ROLE OF \textit{CUZD1} IN MAMMARY DEVELOPMENT AND TUMORIGENESIS

BY

LAVANYA ANANDAN

DISSERTATION
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Doctoral Committee:
Professor Milan Bagchi, Chair
Professor Indrani Bagchi
Professor Benita Katzenellenbogen
Professor Byron Kemper
Assistant Professor Lori Raetzman
ABSTRACT

The mechanisms by which the pathways regulated by the steroid hormones and the epidermal growth factor (EGF) family control mammary development are unclear. We have identified the CUB and zona pellucida-like domain containing protein-1 (CUZD1) as a target of estrogen regulation in the mouse mammary epithelium. Mice lacking Cuzd1 exhibited a striking impairment in steroid-induced proliferation of mammary ductal and alveolar epithelium. Our study revealed that Cuzd1 controls the production of a subset of EGF family growth factors, neuregulin-1, epigen and epiregulin, which act via the ERBB receptors on the mammary epithelial cells. Interestingly, exposure of these cells to the EGF-like growth factors stimulated CUZD1 expression, triggered its nuclear translocation, and promoted CUZD1-dependent mitogenic activity. We postulated that a regulatory loop, involving the EGF-like growth factors and CUZD1, contributes to mammary epithelial proliferation, and that dysregulation of CUZD1 expression may induce tumorigenesis. Indeed, orthotopic injection of mammary epithelial cells overexpressing Cuzd1 into mammary glands of mice led to the development of malignant breast tumors, which metastasized to other tissues. Analysis of Cuzd1 expression in primary human breast tumors revealed a significantly elevated expression of this gene in tumors displaying a high level of estrogen receptor alpha (ESR1) and ERBB signaling. Collectively, these findings suggested that uncontrolled expression of CUZD1, which is a unique integrator of steroid- and EGF family-induced growth pathways in normal mammary epithelium, leads to breast tumorigenesis. Designing therapeutics that target CUZD1 may offer a novel intervention strategy against breast tumor progression, invasion and metastasis.
To my parents, Jayanthi and Anandan
ACKNOWLEDGEMENTS

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CHAPTER 1

BACKGROUND AND SIGNIFICANCE
PART A: SIGNALING NETWORKS IN MAMMARY GLAND DEVELOPMENT AND TUMORIGENESIS

Steroid Hormone Regulation of Mammary Gland Development

The development of the mammary gland is a unique biological process because it is one of the very few organ systems where most of the development takes place after birth. There are two tissue compartments that constitute the mammary gland: the epithelium composed of ductal epithelial cells, myoepithelial cells and milk producing alveolar cells; and the stroma, also known as the mammary fat pad. The development of the epithelium in the mammary gland occurs in well-defined stages. In a newborn animal, only a small rudimentary ductal tree structure, composed of mammary epithelium, is present. At puberty, this ductal tree undergoes accelerated growth and branching induced by pubertal hormones, which eventually lead to the formation of an extended ductal system that fills the entire fat pad in a mature virgin mouse. These ducts branch into smaller ducts that terminate in lobules composed of alveoli. The second phase of development, known as alveologenesis, takes place during pregnancy, leading to the formation of the lobuloalveolar tissue. The alveolar cells later differentiate to become milk producing cells (Fig1.1) [1, 2].

A number of signaling molecules have been implicated in the control of proliferation, differentiation, survival and apoptosis of mammary epithelial cells during development. These include steroid hormones, such as 17β-estradiol (E) and progesterone (P), peptide hormones, such as prolactin, growth factors, such as amphiregulin, cell cycle regulators, such as p27 and cyclinD1, and transcription factors, such as CEBPα or β and LEF1[3]. Specifically, E and the EGF-like growth factors stimulate ductal morphogenesis in the pubertal mammary gland. During
recurrent estrous cycles in the adulthood, E, P and the EGF family members promote ductal side branching. During pregnancy and lactation, these hormones and growth factors act in concert with PRL to induce alveologenesis with lactational differentiation [1, 4, 5].

E acts via estrogen receptor alpha (ESR1) to control mammary epithelial function. The Esr1-null mice exhibit severely compromised ductal morphogenesis during puberty and fails to execute alveologenesis [6-9]. However, global deletion of Esr1 also leads to the failure of E signaling throughout the hypothalamic-pituitary axis, resulting in decreased circulating levels of P and PRL that contribute to the severity of the observed phenotype [6]. Recently, a conditional deletion mouse model in which Esr1 is selectively ablated in the adult mammary epithelium has demonstrated that signaling via this receptor is important for the branching morphogenesis and lobuloalveolar differentiation during pregnancy [8]. However, the identities of the downstream pathways that mediate critical E-regulated functions during mammary gland development remain largely unknown.

**ERBB signaling network during mammary gland development**

Another distinct signaling pathway that is intimately associated with mammary gland development is the ERBB (Erythroblastic Leukemia Viral Oncogene-related) signaling network, also known as the Epidermal Growth Factor Receptor (EGFR) family. This network comprises four homologous receptor tyrosine kinases, named ERBB1-4, that are activated by structurally-related ligands belonging to the EGF family, including heregulins and neuregulins. All members of the ERBB family have in common an extracellular ligand-binding domain, a single transmembrane domain and a cytoplasmic domain containing protein tyrosine kinase activity. The EGF family ligands bind to the extracellular domain of ERBB receptors, triggering the
formation of receptor homo- or heterodimers. This dimerization event stimulates intrinsic tyrosine kinase activity of the receptors and promotes autophosphorylation of specific tyrosine residues within the cytoplasmic domain. The phosphorylated tyrosines then serve as docking sites for cytoplasmic signaling proteins, resulting in the activation of several downstream signaling pathways, such as the Ras-MAP kinase cascade, the AKT/PI3 kinase pathway and the STAT transcription factors. These signaling events regulate cell proliferation, migration, differentiation or survival of mammary epithelial cells [10, 11].

In the mammary gland, all four ERBBs are expressed at various stages of development of this tissue. While only ERBB1/EGFR and ERBB2 proteins are detected in the developing virgin gland, all four ERBB receptors are expressed and activated during pregnancy and lactation [12]. Mouse models have provided important insights regarding the roles of the ERBB-signaling network in mammary gland development. The first indication for a contribution of ERBB1 to mammary gland function came from waved-2 mice, in which the gene encoding ERBB1 is mutated. These mice show impaired alveolar development, leading to reduced milk production [13]. Subsequent mouse models also supported a potential role for ERBB1 in controlling ductal outgrowth [14, 15]. Mice expressing a dominant negative ErbB2 transgene fail to develop a functional alveolar compartment during pregnancy, indicating that ErbB2 plays an essential role in mammary gland development during pregnancy [16]. Although ERBB3 lacks intrinsic tyrosine kinase activity, its heterodimerization with ERBB2 and other ERBB receptors suggests a possible role in alveolar development [17]. Finally, conditional deletion of ErbB4 in the mammary epithelium resulted in alveolar units that failed to undergo complete functional differentiation, indicating a critical role of this receptor in alveolar development [18]. Among the EGF family of ligands, amphiregulin (AREG), which acts primarily via ERBB1, is required for
mammary ductal elongation in the adolescent mouse, whereas the neuregulins (NRGs) and their receptors, ERBB3 and ERBB4, which are predominantly expressed in mammary glands of pregnant mice, are critical for alveologenesis [18-21] Collectively, these studies confirmed the importance of the ERBB-signaling network during mammary gland development.

**Cross-talk between estrogen and ERBB signaling pathways during normal mammary gland development**

There are many reports in the literature indicating a functional link between the ERBB/EGFR signaling network and the ESR1 signaling pathway. It was proposed that E acting via ESR1 in the stroma induces the production of the EGF ligands, which stimulate proliferation of the mammary epithelial cells during puberty. This model was supported by observations that arrest of ductal outgrowth and disappearance of TEBs seen in ovariectomized pubertal mice were rescued when 17β-estradiol was administered locally by means of slow-release pellets [22]. Similarly, pellets releasing EGF, TGF-α, or amphiregulin were also able to induce cell proliferation, TEB formation, and ductal elongation [23, 24]. Studies by Brisken and coworkers showed that AREG is an essential mediator of Esr1 action in ductal morphogenesis during puberty [21]. AREG, however, is not involved in the subsequent development of the mammary gland, raising the possibility that other EGF family ligands may mediate epithelial cell proliferation at later stages of mammary gland development, such as side-branching and alveolar expansion.

**ESR1-mediated signaling in breast cancer**

Many of the signaling networks that control mammary gland growth and differentiation are also involved in breast carcinogenesis. Dysregulation of these molecules results in uncontrolled cell
proliferation and survival, leading to tumor initiation and progression [25]. Signaling by ESR1, the ERBB/EGFR network, and the interaction between these two pathways have long been implicated in breast carcinogenesis. There is strong evidence to suggest that cumulative exposure to E represents a significant determinant of an individual’s lifelong risk of developing breast cancer [26]. In fact, ESR1 is expressed in 40-70% of all breast tumors and these tumors exhibit E responsive growth. ESR1-mediated signaling controls genes that belong to several distinct biological categories, such as cell cycle regulators, modulators of apoptosis, growth factors, cytokines, transcription factors and coregulators that drive initiation, progression and metastases of breast cancer [27, 28]. For example, SRC-3/AIB1, a coactivator of ESR1, is highly amplified in approximately 5% of primary breast cancers, and overexpressed in as many as 60% of primary tumors, suggesting a role of E-dependent gene expression events in tumorigenesis [29, 30]. Recognition that E is important in mammary tumorigenesis has led to the use of tamoxifen, an ESR1 antagonist, and aromatase inhibitors, which block the *in vivo* production of E, in the treatment of ESR1-positive breast cancers [31].

**ERBB/EGF family signaling pathways in breast cancer**

The 30-50% of breast tumors that do not express ESR1 are termed ER-negative and frequently overexpress members of the ERBB family. Several lines of evidences suggest that overexpression or mutation of ERBB network components, including ERBB1/EGFR, ERBB2/Neu, and the EGF family ligands, contributes significantly to initiation of breast cancer in humans and rodents [32]. For example, transgenic mice in which the EGF family ligand TGFα is overexpressed in the mammary gland developed mammary tumors with high efficiency and long latency [33]. Ample evidence has now accumulated, indicating that ERBB2 (HER2/Neu)
plays a primary role in many instances of human breast cancer. Numerous studies have confirmed that the HER2/Neu gene is amplified and overexpressed in 20-30% of human breast tumors [34]. These findings have prompted novel therapeutic strategies targeting HER2/Neu, including the monoclonal antibody Herceptin. A gene knock-in approach, introducing HER2/Neu sequence with a point mutation in mouse genome, resulted in mice that produced mammary tumors with long latency [35]. Molecular analysis also revealed that the HER2/Neu locus was amplified between two-and 22-fold in these tumors.

ERBB3 forms strong heterodimers with ERBB2 and this partnership is important for the proliferation of breast cancer cell lines that depend on ERBB2 signaling pathways [17]. In fact, parallel overexpression of these proteins has been observed in mammary tumors. The significance of ERBB4 in breast cancer remains unclear, given that both oncogenic and tumor suppressive roles have been proposed for it [36]. A role for ERBB4 in mammary epithelial cell proliferation and its frequent overexpression in early stages of breast cancer have been reported previously [37, 38]. Studies proposing its role as a good prognostic marker has also been reported [39, 40]. Although the roles of ERBB1 & ERBB2 in breast cancer are well defined, a deeper understanding of the actions of ERBB3 & ERBB4 during this disease process is needed.

**Cross-talk between ESR1 and ERBB signaling pathways in breast cancer**

There is increasing evidence that the ESR1- and ERBB-mediated signaling pathways interact during tumor proliferation and this may occur via common downstream signaling pathways. It is well known that a number of signaling molecules, including MAPK, PI3K, AKT, NFκB, PKCδ and PLCγ that control cell proliferation, angiogenesis and invasion during carcinogenesis and metastases, also play a role in both ESR1 and ERBB signaling [41]. A number of genes that are
involved in cell proliferation and survival in breast cancer, such as cyclin D1, c-myc and Bcl-XL, lie downstream of both ESR1 and ERBB pathways, providing clear evidence for the presence of nodes of molecular cross talk between the two [42]. The interaction between these two pathways is bidirectional and there are several lines of evidence to make this case.

It is well known that the EGFR/ERBB2-MAPK signaling pathway, in addition to directly driving cell growth, can also target and phosphorylate key serine residues within the AF-1 domain of ESR1 in breast cancer cells. Phosphorylation of these residues, in particular serine-118, which is a target for extracellular signal-regulated kinases 1/2 (ERK1/2), promotes co-activator recruitment and activation of ESR1 transcriptional activity [43]. It was also shown that E induces the expression of EGF family ligands, such as EGF, amphiregulin and TGF-α and the expression of ERBBs, such as EGFR, during proliferation of breast cancer cells [44, 45]. Both genomic (nuclear) and non-genomic (membrane and cytoplasmic) ESR1 activities are likely to be involved in the cross-talk with the growth factor pathways[46].

The interaction between ESR1 and the EGF-like growth factor pathways has been highlighted as a possible mechanism underlying endocrine resistance in breast cancer. In the breast tumors that overexpress ESR1, antiestrogen therapy is effective in inhibiting the tumors. However, the breast tumors that overexpress members of the ERBB signaling pathway are described as more aggressive and are resistant to endocrine therapies [47, 48]. Strategies to disrupt these pathways by using specific inhibitors are clearly necessary to develop effective therapeutics to combat endocrine resistance.
PART B: CUZD1, A CRITICAL REGULATOR OF NORMAL MAMMARY GLAND DEVELOPMENT

Identification of Cuzd1 as a novel E-regulated gene in the mammary epithelium

Recent studies in our laboratory identified CUZD1, Cub and Zona pellucida-like Domain-containing Protein-1 (also known as ERG1 or UO-44) as a critical integrator of the E and ERBB signaling pathways during mammary gland development. E regulates the gene encoding CUZD1 in the mammary gland. Cuzd1 was originally cloned by Kasik and also by our laboratory from rodent reproductive tract [49, 50]. This protein was subsequently found to be present in the oviduct, ovary, pancreas and epididymis [49, 51-53]. The human CUZD1 gene shares strong sequence identity (~80%) with the mouse gene [54]. CUZD1 is a unique protein containing one zona-pellucida (ZP) and two tandem CUB motifs both of which have possible roles in cell adhesion and cell surface binding [49, 50, 54]. Not much is known about the functional significance of these motifs, although their presence is often noted in molecules involved in developmental processes [55].

To examine whether E regulates Cuzd1 expression in the mammary gland, ovariectomized mice were treated with or without E. As shown in Fig. 1.2A, administration of E to ovariectomized mice stimulated Cuzd1 mRNA expression in both uterine and mammary tissues. In contrast, administration of P had no effects on Cuzd1 mRNA levels (data not shown). Immunohistochemical (IHC) analysis, using an antibody against mouse Cuzd1, indicated a marked increase in the level of CUZD1 protein in the ductal epithelia of the mammary gland upon E treatment (Fig. 1.2B, panel b). The expression of CUZD1 in the mammary epithelium partially overlapped with that of ESR1 (Fig. 1.2B, panel d).
To further investigate whether the E regulation of Cuzd1 expression is mediated by ESR1, we utilized HC11 cells, a non-transformed mammary epithelial cell line containing low levels of endogenous ESR1. These cells were transduced with adenoviral vectors expressing ESR1 or green fluorescence protein (GFP). As shown in Fig. 1.2C, overexpression of ESR1 in these cells induced a robust time-dependent increase in Cuzd1 mRNA. This induction was further enhanced in response to E treatment and strongly inhibited in the presence of ICI 182, 780, an antagonist of ESR1. Collectively, these in vitro studies supported our in vivo findings that E acting via ESR1 stimulates Cuzd1 expression in mammary epithelial cells.

**Cuzd1 controls mammary ductal development and alveologenesis**

To investigate the functional roles of CUZD1 in mammary gland development, we created Cuzd1-null mice in which this gene is deleted from mouse germ line by homologous recombination using mouse embryonic stem cells. A 6-month breeding study revealed that the Cuzd1-null females are fertile and deliver normal size litters. However, ~75% of the delivered pups died within 72 h after birth (data not shown) and it was observed that they had insufficient milk in their stomachs. Almost all pups survived and grew normally when they were transferred to a foster mother immediately after birth. These results indicated that the Cuzd1-null mothers fail to produce an adequate amount of milk to nurse their pups.

To further examine the phenotypic defects, morphological analyses of whole mounts of mammary glands were performed at different stages of development. In comparison to their heterozygous littermates, the mammary ductal outgrowth was significantly delayed in the pubertal glands (6-weeks postnatal) of Cuzd1-null mice (Fig. 1.3, a and b). When examined during pregnancy, mammary glands of mutant mice exhibited a severe deficiency in tertiary
branching during and impaired alveolar development during pregnancy (Day 18, Fig 1.3, c and d) and lactation (Day 2, Fig. 1.3, e and f).

**Cuzd1 controls steroid-dependent mammary epithelial proliferation**

The delay in ductal elongation and impaired alveolar development in *Cuzd1*-null mammary glands raised the possibility that CUZD1 is involved in the control of steroid-induced epithelial proliferation. To test this possibility, we examined mammary epithelial proliferation in response to E and P stimulation. *Cuzd1*-null mice and their heterozygous littermates were treated with E and P for 5 days to mimic the hormonal effects on alveolar development during early pregnancy. Mammary glands were subjected to whole mount and IHC analysis using Ki67 antibody. As shown in Fig. 1.4, administration of E and P led to extensive side branching and robust alveolar budding in the heterozygous mice (panel a). The majority of mammary epithelial cells of these mice showed positive staining for Ki67 (c and e). In contrast, E and P induced only limited side branching, poor alveolar development (panel b) and reduced proliferation rate in mammary glands of *Cuzd1*-null mice (d and f). By our estimation of ki67-positive cells using image J, there was a ~70% reduction in cell proliferation activity in the mutant epithelium (panel g). We also monitored PGR expression in these tissues by IHC. No significant alteration in the percentage of PGR-positive cells was observed in the mammary epithelial cells of these mice, indicating that PGR-mediated signaling is unlikely to be affected in the absence of *Cuzd1* (Fig. 1.4, panels h and i).
**Loss of Cuzd1 impairs the production of a subset of EGF family growth factors in the mammary epithelium**

To identify the signaling pathways that operate downstream of Cuzd1, a microarray analysis was performed to compare the gene expression profiles of mammary epithelial cells isolated from Cuzd1-null mice and their heterozygous littermates on day 18 of pregnancy. Our results revealed that the expression of mRNAs encoding three key members of the EGF family, neuregulin-1 (Nrg1), epiregulin (Ereg) and epigen (Epgn), are significantly down-regulated in the Cuzd1 (-/-) epithelium compared to the Cuzd1 (+/-) epithelium (Fig 1.5A). Interestingly, no significant alteration was seen in the expression levels of mRNAs of several other EGF-family growth factors, such as amphiregulin (Areg), epidermal growth factor (Egf), heparin binding epidermal growth factor (Hbegf), neuregulin-2 (Nrg2), neuregulin-3 (Nrg3) and neuregulin-4 (Nrg4).

The IHC analyses of NRG1 and EPGN further confirmed that Cuzd1 regulates the expression of these proteins in mammary epithelium. Abundant NRG1 or EPGN was detected in mammary epithlia of control mice during lactation (Fig. 1.5B, a and b). The expression levels of these factors were dramatically diminished in the mammary glands of Cuzd1-null mice (Fig. 1.5B, c and d). Taken together, these results indicated that Cuzd1 is necessary for the production of a specific subset of EGF family ligands, NRG1, EREG and EPGN, which are critical mediators of mammary epithelial proliferation and alveologenesis during pregnancy and lactation.

**Loss of Cuzd1 impairs ERBB signaling pathway**

The EGF family ligands signal via ERBB receptors to control mammary gland development [15, 18-20, 56]. Binding of these ligands to the receptors leads to their activation via auto-
phosphorylation of critical tyrosine residues, which subsequently serve as docking sites for further signaling cascades [11]. We, therefore, examined whether the observed alterations in the expression levels of NRG1, EREG and EPGN in the mammary tissue affected the ERBB receptor-mediated signaling. Mammary gland sections obtained from mice during late pregnancy (day 18) were subjected to IHC, using antibodies directed against specific phospho-tyrosine residues critical for activation of ERBB1 (Tyr 1068) ERBB2 (Tyr 877), and ERBB4 (Tyr 1056). As shown in Fig. 6A, abundant activating phosphorylation of ERBB1, ERBB4 and ERBB2 was observed in mammary epithelia of Cuzd1 (+/-) mice (panels a, c, and e), consistent with the considerable proliferative activity seen in this tissue. In contrast, the activating phosphorylation of ERBB1 and ERBB4 was markedly reduced in Cuzd1-null epithelium (Fig 1.6A, panels b and d). Interestingly, the phosphorylation of ERBB2 was not affected in the mutant epithelium (Fig 1.6A, panel f). No alteration was observed in the mRNA and protein levels of ERBB1, ERBB2, and ERBB4 in mammary epithelia of these mice, indicating that Cuzd1-driven production of a specific subset of ligands control corresponding ERBB receptor activation (data not shown).

It is known that signaling downstream of ERBB4 activates the transcription factors STAT5a and STAT5b, which control mammary epithelial cell proliferation and differentiation during alveologenesis [57-59]. No significant change in the levels of Stat5a and Stat5b mRNAs was observed in the mammary glands of Cuzd1 (+/-) or Cuzd1 (-/-) mice (data not shown). STAT5 proteins are activated via phosphorylation of Tyr-694. We, therefore, examined the status of STAT5 phosphorylation (Tyr-694) in the mammary glands of pregnant mice by IHC analysis. We observed a striking loss of STAT5 phosphorylation in the mammary epithelia of Cuzd1-null mice (Fig 1.6B, panel b), whereas abundant phospho-STAT5 was present in the same tissue of Cuzd1 (+/-) littermates (Fig.1.6B, panel a). These results are consistent with the
hypothesis that Cuzd1-mediated production of EGF-like growth factors activates ERBB4 signaling, which is transduced downstream via the activation of STAT5 to control mammary epithelial proliferation and differentiation.

In summary, to study the functional role of Cuzd1 in mammary gland development, we created Cuzd1-null mice by germ line mutagenesis. Phenotypic analysis of adult mutant females revealed a severe impairment in ductal side branching and alveolar development in the mammary gland during pregnancy and lactation. Ablation of Cuzd1 resulted in reduced steroid-induced mammary epithelial proliferation. This study, using in vivo and in vitro approaches, uncovered novel mechanisms linking Cuzd1 to the EGF-like growth factor signaling pathways that guide epithelial proliferation and differentiation during key developmental phases of the mammary gland.

REFERENCES


Fig 1.1. Stages of mammary gland development

Development of mammary gland begins in the fetus (a). A small ductal tree is present in the newborn animal (b). Accelerated growth and branching induced by puberty hormones leads to the formation of extended ductal system that fills the entire fat pad in a mature virgin mouse (c & d). Alveolar development occurs during pregnancy (e). The alveolar epithelial compartment is eliminated during involution (f).

This figure is adapted from Henninghausen and Robinson, *Dev Cell*, 2001
Fig. 1.2

A.

![Bar graph showing fold induction for different conditions](image)

<table>
<thead>
<tr>
<th>Condition</th>
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<tr>
<td>UT/V</td>
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<tr>
<td>UT/E</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>MG/V</td>
<td>4.0 ± 0.4</td>
</tr>
<tr>
<td>MG/E</td>
<td>10.0 ± 1.0</td>
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B.

![Images showing tissue sections](image)

V

Cuzd1

ESR1

C.

![Histogram showing gene expression levels](image)
Fig. 1.2. E regulation of *Cuzd1* expression in the mammary gland.

A. Ovariectomized mice were treated with Vehicle (V) or estradiol (E) at 40 ug/kg body weight (E). After 24 h, total RNAs were isolated from whole uteri (UT) and purified epithelial cells isolated from mammary gland (MG) and subjected to real-time PCR using gene-specific primers for *Cuzd1* or 36B4 (control). Quantitative analysis was performed of induction of *Cuzd1* mRNA expression from three independent experiments after normalization to the level of 36B4 (Mean ± SEM). (Courtesy: Quanxi Li).

B. Localization of CUZD1 and ESR1 in mammary glands in response to E administration. Mammary glands of adult mice were collected following 5 days of treatment with vehicle (V) or estrogen (E) and paraffin-embedded sections were subjected to IHC analysis using an antibody specific for CUZD1 (a and b) or ESR1 (c and d). The reddish brown spots represent immunostaining. Magnification, 20X. Inset magnification: 40X (Courtesy: Quanxi Li and Athi Kannan).

C. HC11 cells were transduced with adenovirus expressing ESR1 or green fluorescence protein (GFP). 24 h post transduction, cells were treated with vehicle (V), E, or E plus ICI for 24h (grey bars) or 48h (black bars). Total RNA was isolated and subjected to real-time PCR using gene specific primers for *Cuzd1* or 36B4. Relative level of *Cuzd1* mRNA at each time point was plotted following normalization with respect to the 36B4 mRNA level (Mean ± SEM).
Fig. 1.3

Reduced ductal branching and impaired alveolar development in Cuzd1-null mammary glands.

Whole mount analysis of no. 4 inguinal mammary glands of virgin heterozygous Cuzd1 (+/-) and homozygous Cuzd1 (-/-) mice at 6 weeks (a and b), pregnancy day 18 (c and d) and lactation day 2 (e and f). Magnification: 4x. (Courtesy: Quanxi Li).
Fig. 1.4

Fig. 1.4. *Cuzd1* controls steroid-induced mammary epithelial proliferation.

No. 4 inguinal mammary glands were collected from *Cuzd1* (+/-) and *Cuzd1* (-/-) mice following 5 days of E+P-treatment and subjected to whole mount analysis (a and b). Magnification, 4X. Mammary tissue sections from the same mice were subjected to IHC analysis using anti-Ki67 antibody (panels c, e, d, f) or PGR antibody (panels h and i). Panels c and d represent a mammary duct. Panels e and f represent an alveolar bud. Magnification, 20x. The estimation of the number of Ki67-positive cells in *Cuzd1* (+/-) and *Cuzd1* (-/-) mammary tissue was performed using image J. Data (panel g) were expressed as average +/- S.D of three separate measurements. (Courtesy: Quanxi Li and Athi Kannan).

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Fig. 1.5

A.

![Gene expression bar chart](chart.png)

- Epgn
- Ereg
- Egf
- Hb-egf
- Areg
- Btc
- Nrg1
- Nrg2
- Nrg3
- Nrg4

B.

![Immunohistochemistry images](images.png)

- Cuzd1^{+/+}
- Cuzd1^{++-}

- L2

- EPGN
- NRG1
Fig. 1.5. *Cuzd1* controls the expression of a subset of EGF family ligands in the mammary epithelium.

A. Total RNA was isolated from purified mammary epithelial cells of *Cuzd1* (-/-) mice and their (+/-) littermates at day 18 of pregnancy. Real-time PCR was performed to analyze relative expression levels of *Epgn, Ereg, Egf, Hbegf, Areg, Btc* (Betacellulin), *Nrg1, Nrg2, Nrg3* and *Nrg4*. Data are represented as average fold induction ± SEM. Statistically significant differences (*P*<0.05) are indicated by *. (Courtesy: Quanxi Li).

B. IHC analysis of *Epgn* (a and c) and *Nrg1* (b and d) protein expression in mammary glands of *Cuzd1* (+/-) and *Cuzd1* (-/-) mice on lactation day 2 (L2). Magnification, 10x.
Fig. 1.6. Loss of *Cuzd1* expression leads to impairment of activation of ERBB1, ERBB4 and STAT5 in mammary epithelium during pregnancy

Mammary tissue sections obtained from *Cuzd1* (+/-) and *Cuzd1* (-/-) mice on Day18 of pregnancy were subjected to IHC using antibodies specific for phospho-ERBB1 (a and b), phospho-ERBB2 (c and d), phospho-ERBB4 (e and f), and p-STAT5 (6B, a and b) to assess the activation of ERBB-mediated signaling pathways. The immunostaining of phospho-ERBB1, phospho-ERBB4, and phospho-STAT5 was sharply diminished in the ductal and alveolar epithelium of *Cuzd1* (-/-) mice. Magnification, 10X. (Courtesy: Quanxi Li and Athi Kannan).
CHAPTER 2

ANALYSIS OF THE ROLE OF CUZD1 IN A MOUSE MODEL OF BREAST TUMORIGENESIS
ABSTRACT

The role of cellular signaling mediated by the epidermal growth factor receptor (ERBB) family in breast tumor growth and progression is well documented. We have recently found that CUZD1, an estrogen-regulated protein, critically controls mouse mammary epithelial cell proliferation by regulating the expression of a specific subset of epidermal growth factor (EGF)-like growth factors in the mammary tissue. Over-expression of Cuzd1 gene in the non-transformed mouse mammary epithelial HC11 cells created HC11-Cuzd1 cells, which proliferated at a faster rate than the control HC11-LacZ cells. In addition, the HC11-Cuzd1 cells exhibited signs of transformation, such as the ability to form colonies on soft agar and increased migration in transwell assays. The tumorigenic property of these cells was further assessed by orthotopic injection into the mammary glands of adult nude or BALB/c mice. No palpable tumor was observed in mice injected with the HC11-LacZ cells. In contrast, animals injected with HC11-Cuzd1 cells invariably developed mammary tumors, which progressed to metastasis. Histological evaluation of these tumors revealed highly proliferative adenocarcinomas in which active signaling by the ERBB receptors, ERBB1 and ERBB4, was observed. Attenuation of expression of ERBB1, ERBB3 and ERBB4 in HC11-Cuzd1 cells effectively inhibited CUZD1-promoted cell proliferation, confirming a critical functional link between CUZD1 and the ERBB pathway. Interestingly, treatment of HC11 cells with the EGF-like growth factors stimulated Cuzd1 expression, triggered the nuclear translocation of CUZD1 protein, and promoted CUZD1-dependent mitogenic activity. We postulated that a regulatory loop, involving Cuzd1 and the EGF-like growth factors, contributes to normal mammary epithelial proliferation. Abnormal elevation in Cuzd1 expression triggers uncontrolled
cell proliferation and tumorigenesis. Collectively, these results indicated that \textit{Cuzd1}, by enhancing the signaling output of the ERBB pathways, plays a critical role in breast tumorigenesis.

\textbf{INTRODUCTION}

Breast tumorigenesis often arises from the overexpression of signaling molecules otherwise involved in normal cellular processes of growth, development, and differentiation \cite{1}. Disruption of signaling pathways regulated by the estrogen receptor alpha (ESR1) and ERBB growth factor receptors, which are essential for normal mammary gland development and lactation, results in uncontrolled epithelial cell proliferation, leading to mammary tumorigenesis \cite{2}.

Overexpression of the ERBB family of ligands and receptor tyrosine kinases is frequently observed in breast cancer and correlates with poor patient prognosis and therapeutic resistance. For example, in a transgenic mouse model in which the EGF family ligand, TGF\(\alpha\), is overexpressed in the mammary gland, the animals developed mammary tumors with high efficiency \cite{3}. Ample evidence has now accumulated, indicating that \textit{ERBB2} (also known as \textit{HER2/Neu}) plays a primary role in many instances of human breast cancer. Numerous studies have confirmed that the \textit{ERBB2} gene is amplified and overexpressed in 20-30\% of human breast tumors \cite{4} These findings have prompted novel therapeutic strategies targeting ERBB2, employing the monoclonal antibody Herceptin. ERBB3 forms strong heterodimers with ERBB2 and this partnership is important for the proliferation of breast cancer cell lines that depend on ERBB2 signaling pathways \cite{5}. In fact, parallel overexpression of these proteins has been observed in mammary tumors. The significance of \textit{ERBB4} in breast cancer remains unclear, given that both oncogenic and tumor suppressive roles have been proposed for it \cite{6}. A role for \textit{ERBB4} in mammary epithelial cell proliferation and its frequent overexpression in early stages
of breast cancer have been reported previously [7, 8]. Studies, proposing that it is a good prognostic marker, have also been reported [9]. Aberrant activation of the ERBBs has often been implicated in resistance to targeted chemotherapeutics in breast cancer. Various strategies have been explored to target the ERBB network [10], including antibodies and gene therapy; however the potential for exploiting this pathway is still in its infancy. A more thorough understanding of the mechanisms that regulate the expression of ERBBs and their ligands is necessary in order to design effective therapeutic strategies that will interfere with their function.

There is increasing evidence that signaling pathways downstream of ESR1 and ERBB interact during tumor proliferation. It is well known that a number of signaling molecules, including MAPK, PI3K, AKT, NfκB, PKCδ and PLCγ, which control cell proliferation, angiogenesis and invasion during carcinogenesis and metastases, are activated downstream of both ESR1 and ERBB signaling [11]. Similarly, a number of genes that are involved in cell proliferation and survival in breast cancer, such as cyclin D1, c-myc and Bcl-XL, lie downstream of both ESR1 and ERBB pathways, providing evidence for the presence of nodes of molecular cross-talk between the two [12]. However, the precise mechanisms that mediate the cross-talk between the ESR1 and ERBB pathways are largely unknown.

Our recent studies revealed that CUZD1, a previously reported estrogen regulated gene (17), is required for mammary epithelial proliferation and differentiation. CUZD1 also regulates the induction of a subset of EGF family mitogenic factors that act via the ERBB signaling system to promote epithelial proliferation and differentiation during mammary gland development and lactation (Chapter 1). It is our hypothesis that CUZD1 acts as an integrator of the steroid hormone and EGF signaling pathways to regulate mammary gland development.
Since many molecules that belong to the proliferative and morphogenic program of mammary gland development are often altered or harnessed to support aberrant growth and tumorigenesis, we considered the possibility that dysregulation of CUZD1 expression may lead to uncontrolled cell proliferation and contribute to cancer initiation or progression. Previously published work has shown that Cuzd1 is overexpressed in many ovarian cell lines and tumors, suggesting that it might have a role in ovarian tumorigenesis [13].

In this study, we demonstrate that overexpression of CUZD1 in untransformed mammary epithelial cells stimulates cell proliferation, cell transformation and eventually leads to development of mammary adenocarcinoma. Using mouse breast tumor tissue, we also establish a critical link between CUZD1 and ERBB signaling pathways during the initiation and progression of breast tumorigenesis.

MATERIALS AND METHODS

Cell line and cell culture
The HC11 cells (generous gift from Dr. Mrinalini Rao of UIC) are a non-transformed mammary epithelial cell line derived from midpregnant BALB/C mice. These cells were grown in RPMI 1640 (Invitrogen) supplemented with 5% (v/v) fetal bovine serum (Hyclone), 10 ng/ml EGF (Sigma) and 5 ug/ml insulin (Sigma).

Creation of Cuzd1 overexpression cell lines
cDNA encoding Cuzd1 was cloned into the lentiviral vector plenti6.3/V5 TOPO (Invitrogen). The plenti6.3-Cuzd1 and a control plasmid containing the LacZ gene were transfected into
293FT along with the lentivirus packaging mixture (according to manufacturer’s instructions) to produce lentiviral particles. HC11 cells were then transduced with Cuzd1 or control lentiviral particles and selected using blasticidin (Invitrogen) to create a stable cell line overexpressing LacZ or CUZD1.

RNA isolation and real-time PCR analysis
Total RNA was extracted from cultured HC11 cells using Trizol RNA purification kit (Invitrogen) according to manufacturer’s instructions. Reverse transcription was performed using the cDNA synthesis kit (Stratagene) following manufacturer’s instructions. cDNA was amplified by quantitative real-time PCR analysis using gene-specific primers and SYBR-Green supermix (Bio-Rad Laboratories). 36B4 was used as the loading control. For a given sample, threshold cycle (Ct) and SD was calculated from individual Ct values from 3-4 replicates of a sample. Normalized mean Ct was computed as ΔCt by substracting mean Ct of 36B4 from Ct of a target gene for control sample. ΔΔCt was then calculated as a difference in ΔCt values between control and experimental groups. The fold change in gene expression was then computed as $2^{-\Delta\Delta Ct}$. Error bars indicates $2^{-\Delta\Delta Ct} \pm SD$.

Cell proliferation using BrdU incorporation
HC11-LacZ or -Cuzd1 cells were plated at a density of $5 \times 10^3$ cells/well in 96-well plates and cultured for 24 h in full growth medium. After 24 h, cells were washed twice with PBS and incubated in serum-starvation medium (RPMI with no FBS) for 48 h. Medium was replaced with RPMI with 10% FBS or with starvation medium (control) and incubated with
Bromodeoxyuridine (BrdU) (100 µM) for 24 h. The uptake of BrdU was quantified using ELISA-based cell proliferation assay according to the manufacturer’s instructions (Roche).

**siRNA-mediated transient transfection**

HC11 cells were transfected with siRNA against Cuzd1 or control siRNA (scrambled), using Lipofectamine-RNAimax reagent following the manufacturer’s protocol (Invitrogen, CA). Briefly, lipofectamine was mixed with 50 nM of siRNA, and allowed to form siRNA-liposome complexes, which were then added to HC11 cells at 60% confluency. After 24 h, the transfection was repeated again. Cells were harvested 48 h after the second transfection and total RNA was isolated and analyzed by quantitative real-time PCR using gene-specific primers.

**Anchorage-independent growth in soft agar**

HC11-LacZ or -Cuzd1 cells (1 x 10⁴) or MCF7 cells (control) were seeded in six-well plates with a bottom layer of 0.48% Bacto agar in DMEM and a top layer of 0.36% Bacto agar in DMEM. Fresh DMEM containing 10% FBS was added to the top layer of the soft agar. The culture medium was changed twice a week. After 16 d, colonies were stained with 0.005% crystal violet. Visible colonies (>0.5 mm in diameter) were counted from representative views from triplicate experiments and the average number of colonies per well was determined.

**Boyden chamber cell migration assay**

Boyden-chambers (Millipore) were placed in 24-well dishes containing chemoattract media (RPMI containing 10% FBS). Serum-starved HC11-LacZ or -Cuzd1 cells (1 x 10⁵ cells/well) were added to the upper compartment and allowed to incubate for 72h at 37°C. Cells that migrated across into the lower chamber of the membrane were quantified by CyQuant.
(Millipore) flurometric assay according to the manufacturer’s instructions. Means were taken from three individual chambers for each experiment.

**Orthotopic injection of cells into mammary gland**

HC11-LacZ or -Cuzd1 cells (1 × 10^6) were suspended in Matrigel and injected into the teat of the fourth abdominal mammary gland of nude (nu/nu) mice as described [14]. Tumor growth was measured by weekly caliper measurement for a period of 18 weeks. All animal experiments were conducted in accordance with a protocol approved by University of Illinois Institutional Animal Care and Use Committee. After 18 weeks, the mice were euthanized by CO₂. The primary mammary tumors, superficial lymph nodes, lungs and livers were harvested from sacrificed animals and fixed in 4% paraformaldehyde for subsequent H&E staining or immunohistochemistry.

**Histology, immunohistochemistry & immunofluorescence**

Organs were fixed in 4% paraformaldehyde overnight, paraffin embedded, and cut into 4- to 5-μm sections. After high-temperature antigen retrieval in citrate or EDTA buffer, sections were stained with H&E. Immunohistochemistry was done on parallel sections overnight at 4°C, with primary antibodies directed against Cuzd1 (in-house), PCNA (Santa Cruz Biotechnology), EPGN (R&D systems), NRG1 (Abcam), phospho-EGFR (Y1068, Cell Signaling), phospho-ERBB2 (Y877, Cell Signaling), ERBB3 (Santa Cruz Biotechnology), phospho-ERBB4 (Y1056, Santa Cruz Biotechnology), phospho-ERK1/2 or phospho-AKT1/2/3 (Santa Cruz Biotechnology). Bound primary antibodies were detected with horseradish peroxidase (HRP)–
conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (Jackson Laboratories). Sections were counterstained with hematoxylin and mounted.

For immunofluorescence-based translocation experiments, HC11 cells were serum starved for 48h and followed by treatment with vehicle (citrate buffer) or Epigen (20ng/ml) or Nrg1 (50ng/ml) (Peprotech, CA). After 24h treatment, the cells were fixed in 3.7% formaldehyde, permeabilized, blocked and incubated overnight with primary antibody against Cuzd1 (in-house). Subsequently, slides were washed in PBS with 0.1% Triton-X and incubated with Alexa Flour 488-conjugated anti-rabbit IgG. Slides were stained for DAPI and mounted.

RESULTS

_Cuzd1 overexpression increases rate of proliferation of HC11 cells_

To assess the functional role of CUZD1 in mammary epithelial proliferation, we stably overexpressed _Cuzd1_ in HC11 cells, a non-transformed mouse mammary epithelial cell line derived from Balb/c mice in mid-pregnancy. Control HC11 cells, overexpressing LacZ, were also generated. We noted that the HC11-Cuzd1 cells expressed markedly higher levels of CUZD1 mRNA and protein compared to the HC11-LacZ cells (Fig 2.1A), confirming that we have successfully created a mammary epithelial cell line overexpressing _Cuzd1_.

We next compared the rates of proliferation of HC11-Cuzd1 and HC11-LacZ cells, using a quantitative BrdU incorporation assay. We observed that the HC11-Cuzd1 cells exhibited significantly higher rates of proliferation relative to the HC11-LacZ cells (Fig 2.1B). Consistent
with this observation, the HC11-Cuzd1 cells exhibited markedly elevated expression of several factors that play critical regulatory functions in the cell cycle progression. For example, the HC11-Cuzd1 cells expressed elevated levels of several factors that promote G1-S phase transition, such as cyclins A1 and B1, Cdk1 and Cdk4, E2F1, and reduced levels of p27, a Cdk-inhibitor (Fig 2.1C). Taken together, these results confirmed that Cuzd1 expression positively regulates proliferation of mammary epithelial cells.

_Cuzd1 promotes expression of a subset of EGF family ligands and their ERBB receptors in HC11 cells_

Previous studies in the mouse (Chapter 1) indicated that a subset of the EGF family of ligands is down regulated and the activation of ERBB1 and ERBB4 is impaired in the mammary epithelia of the Cuzd1-null mice. Consistent with these previous results, assessment of the expression of the EGF family ligands and their receptors in the Cuzd1-overexpressing HC11 cells showed that Cuzd1 specifically stimulates the expression of epigen (EPGN), epiregulin (EREG) and neuregulin-1 (NRG-1) without altering the expression levels of the other EGF family members (Fig 2.2). It is important to note that the expression of the ERBB receptors, ERBB1, ERBB3 and ERBB4, was also elevated in HC11-Cuzd1 cells compared to the control HC11-LacZ cells. Cuzd1, therefore, not only controls the expression of the EGF ligands, but also that of key ERBB receptors, thereby driving the increased rate of proliferation seen in HC11-Cuzd1 cells.

_ERBB signaling is critical for the proliferation of HC11-Cuzd1 cells_

To further evaluate the role of the ERBBs in the control of Cuzd1-mediated cell proliferation, we used siRNA targeted to the mRNAs corresponding to ERBB 1-4. The expression of each of the ERBB mRNAs in HC11-Cuzd1 cells was attenuated and the impact of this intervention on cell
proliferation was measured. Attenuation of mRNA levels of ERBB1 or ERBB3 or ERBB4 led to a marked reduction (50-60%) in cell proliferation, whereas suppression of ERBB2 mRNA did not exert any significant effect (Fig 2.3, A and B). These results confirmed a functional link between ERBB signaling and Cuzd1-mediated cell proliferation.

The EGF family ligands control expression and nuclear translocation of the CUZD1 protein

In the mouse mammary epithelial cells in vivo, the CUZD1 protein is present in both nucleus and cytoplasm (Chapter 1, Fig 1.1B). When the HC11 cells were grown in a medium without serum, CUZD1 showed mostly cytosolic localization. Interestingly, addition of medium containing serum triggered nuclear localization of this protein (data not shown). We next tested whether EGF-like factors in the serum could promote nuclear translocation of CUZD1. As shown in Fig 2.4, panels B and C, upon stimulation with EPGN or NRG1, CUZD1 was localized in the nucleus. Additionally, treatment of HC11 cells with EPGN or NRG1 markedly up-regulated Cuzd1 mRNA levels (Fig 2.4A). Collectively, these results raised the possibility that overexpression of Cuzd1 may create an autocrine positive feedback signaling loop in which CUZD1 up-regulates the expression of EPGN, EREG, and NRG1, and their ERBB receptors; the activated ERBB signaling pathway then promotes Cuzd1 expression, triggers the nuclear translocation of CUZD1 protein, which, via an unknown mechanism contributes to stimulated production of these EGF-like growth factors. We postulate that establishment of such an autoregulatory signaling loop may contribute to uncontrolled Cuzd1-driven mammary epithelial cell proliferation, leading to breast cancer.
**Overexpression of Cuzd1 leads to transformation of HC11 cells**

To test whether the overexpression of Cuzd1 promotes tumorigenic transformation of mammary epithelial cells, we performed the soft agar assay to assess anchorage independent growth, a well-known marker of cell transformation. Whereas the HC11-Cuzd1 cells formed large colonies when cultured in media containing soft agar, the HC11-LacZ cells remained as single cells in the agar suspension. As a control, we used MCF7 breast cancer cells, which are known to form robust colonies on soft agar (Fig 2.5A). When subjected to a cell invasion assay in which cells are tested for their ability to migrate across a barrier, the HC11-Cuzd1 cells exhibited significantly higher motility than the control HC11-LacZ cells. We used MDAMB-231 breast cancer cells, which are highly migratory and metastatic, as a positive control in this experiment (Fig 2.5B). Additionally, HC11-Cuzd1 cells expressed elevated levels of the metalloproteases MMP2 and MMP9 (Fig 2.5C), both of which have been heavily implicated in breast tumor invasion and metastasis [15, 16]. Our findings indicate that the overexpression of Cuzd1 in HC11 cells induces two important hallmarks of tumor-like transformation in these cells, the ability to form colonies and exhibit increased migratory properties.

**HC11-Cuzd1 cells form breast tumors in mice**

We next investigated whether the HC11-Cuzd1 cells form breast tumors in vivo. To test this possibility, we orthotopically injected HC11-lacZ or HC11-Cuzd1 cells into the teats of the mammary glands of nude mice. The injected cells penetrated into the ducts and lodged themselves in the fat pad. In mice injected with HC11-Cuzd1 cells, palpable breast tumors were detected as early as two weeks post-injection. Tumors did not appear in mice injected with HC11-lacZ cells. The tumor growth in mice injected with HC11-Cuzd1 cells was assessed
weekly, using digital calipers, and compared to mice injected with HC11-LacZ cells. The breast tumors continued to grow over the eighteen weeks assessment period, reaching sizes between 200-250 mm$^2$ (Fig 2.6 A and B). This growth pattern was observed in 100% of the mice that were injected with HC11-Cuzd1 cells compared to none in mice injected with HC11-LacZ cells. Mice were sacrificed eighteen weeks following injection and tumors were removed and examined by H&E staining. The tumors were verified as high-grade adenocarcinomas upon pathological examination (Fig 2.7A, bottom panels). Upon immunohistological assessment of the tumor tissue, we observed a robust expression of CUZD1 in both cytoplasmic and nuclear compartments (Fig 2.7B, left panel). These tumors also expressed a high level of PCNA, indicative of proliferative activity (Fig 2.7B, right panel). Therefore, the overexpression of *Cuzd1* in mammary epithelial cells led to the formation of high-grade mammary adenocarcinomas, establishing it as a novel tumor-promoting gene in breast cancer.

We also performed similar experiments, using Balb/c mice, the strain of mice from which HC11 cells are derived. Injection of the HC11-Cuzd1 cells into mammary glands of these mice also led to the formation of breast tumors, although at a relatively slower pace (data not shown).

**Activation of ERBB signaling in Cuzd1-driven tumorigenesis**

We next investigated, using IHC, the expression levels of selected EGF family ligands and the ERBB receptors in CUZD1-overexpressing breast tumors. The expression of high levels of EPGN and NRG1 was evident in the tumor sections (Fig. 2.8, panels a and b). Abundant active forms of ERBB1, ERBB3 and ERBB4 (panels c, d, f) were also present in the tumor tissue. In contrast, activated ERBB2 was undetectable in the tumor (panel e). These results were in
agreement with our previous finding that overexpression of Cuzd1 involves active signaling by ERBBs 1, 3 and 4, but is not accompanied by ERBB2 activation.

Activation of ERBB receptors is often accompanied by activation of downstream ERK1/2 and/or PI3K-AKT pathways. To assess ERK1/2 and PI3K-AKT activation in Cuzd1-overexpressing tumors, IHC was performed, using antibodies against phospho-ERK1/2 or phospho-AKT1/2/3 (panels g and h). Our results showed the presence of abundant phospho-ERK1/2, but not phospho-AKT in the tumor tissue, indicating that the activation of downstream ERK1/2 signaling is the predominant mechanism by which CUZD1 regulates proliferation of breast tumor cells.

DISCUSSION

Creation of the Cuzd1-null mouse model in our laboratory had previously allowed us to defined the critical role of this gene in epithelial cell proliferation during mammary gland development (Chapter 1). Our studies indicated that the ERBB signaling network is activated downstream of Cuzd1. The present study provided direct evidence that elevated expression of Cuzd1 stimulated the production of a subset of EGF family ligands and their ERBB receptors. These included EPGN, EREG and NRG1, which act via ERBBs 1, 3 and 4. Attenuation of these ERBB receptors in Cuzd1-overexpressing cells inhibited cell proliferation, thereby revealing the critical importance of ERBB signaling in Cuzd1-mediated cell proliferation. Interestingly, ERBB2, whose activation is frequently associated with breast cancer, does not appear to mediate the effects of Cuzd1. Collectively, our results suggested that the mitogenic activity downstream of
Cuzd1 is mediated via activation of the ERBB signaling system, presumably via formation of homo- or heterodimers between ERBB1, ERBB 3 and ERBB 4.

Ample evidence in the literature suggests that activation of the ERBB pathway plays a key role in generating and maintaining malignant phenotypes, including cell proliferation, differentiation, invasion and tumor cell survival [17]. This raised the possibility that constitutive Cuzd1-driven activation of the ERBB signaling network may lead to breast tumorigenesis. The present study provides direct evidence for the tumorigenic potential of Cuzd1. Overexpression of this gene in a non-tumorigenic mammary epithelial cell line resulted in rapid cell proliferation and anchorage independent growth, hallmarks of premalignant phenotypes of mammary epithelial cells. The Cuzd1-overexpressing cells formed tumors in vivo. When injected orthotopically into mammary glands of nude mice, these cells progressed with high frequency, to high-grade mammary adenocarcinomas. It is important to note that the same response was also noted in normal Balb/c mice, albeit at a slower rate, indicating that it is not a phenotype that occurs only in immunocompromised mice. These results established the role of Cuzd1 as a critical player in breast tumorigenesis.

The growth of breast cancer cells is controlled by cellular growth factors that stimulate proliferation, migration and differentiation of the tumor cell. A variety of growth factors, such as insulin-like growth factors, transforming growth factor α, fibroblast growth factors and EGF family growth factors, are known to stimulate the proliferation of breast cancer cells [18, 19]. In cell culture studies, we have shown that treatment with EPGN or NRG1 robustly stimulates the expression of Cuzd1 in HC11 cells. In addition, exposure of HC11 cells to these EGF-like growth factors triggers nuclear translocation of CUZD1. Our studies suggested a critical nuclear
role of CUZD1, which promotes the synthesis of EPGN, EREG and NRG1. Interestingly, Cuzd1 itself does not possess a nuclear localization signal. So, its nuclear translocation and mechanism of action are likely dependent on interactions with as yet unknown nuclear proteins. Perhaps its translocation to the nucleus occurs in association with a transcription factor, which then participates in the regulation of the growth factor genes. Future biochemical experiments will explore the mechanisms by which Cuzd1 achieves this regulation.

In our proposed model (Fig 2.9), E induces the expression of Cuzd1 in normal mammary epithelial cells. The EGF family growth factors, such as EPGN and NRG1, trigger the translocation of the CUZD1 protein to the nucleus. CUZD1 then act in the nucleus to control the production of EPGN, EREG and NRG1 and their ERBB receptors via an unknown mechanism. Secretion of these growth factors leads to enhanced activation of the ERBB and ERK1/2 signaling pathway, promoting mammary epithelial cell proliferation. These results suggest that constitutive overexpression of Cuzd1 may result in the establishment of an autoregulatory loop that drives uncontrolled epithelial cell proliferation and tumorigenesis. Our findings, therefore, provide a plausible mechanism by which elevated levels of Cuzd1 give rise to high-grade mammary adenocarcinomas.

Invasion by malignant cells requires degradation of the extracellular matrix (ECM) and the basement membrane. Cuzd1-overexpressing cells showed invasive properties as assessed by the transwell migration assays. Indeed, in mice bearing tumors larger than 150mm², the primary tumors metastasized to distant sites. Metastatic lesions were observed in the liver and lung (data not shown). It is plausible that Cuzd1 may be involved in reprogramming cancer cells toward a
potentially more invasive state, leading to invasion and metastases. The potential role of Cuzd1 role in metastasis is currently under investigation in our laboratory.

In summary, we have identified Cuzd1 as a critical regulator of breast tumorigenesis. Our finding supports the concept that Cuzd1, which controls the proliferative program of mammary gland development, when dysregulated, promote the genesis of breast cancer. Designing therapeutics that target Cuzd1 may offer a novel intervention strategy for ERBB-dependent breast cancers.

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REFERENCES


FIGURES

Fig. 2.1

A.

![Graph A]

B.

![Graph B]

C.

![Graph C]
**Fig 2.1. Overexpression of Cuzd1 leads to an enhanced rate of HC11 cell proliferation.**

A. HC11 cells were transduced with lentivirus harboring Cuzd1 or LacZ cDNA to create stable cells overexpressing Cuzd1 (HC11-Cuzd1) or LacZ (HC11-LacZ), respectively. Total RNA was isolated from these cells and subjected to real-time PCR using Cuzd1-specific primers to validate Cuzd1 mRNA expression.

B. HC11-Cuzd1 and HC11-LacZ cells were cultured under serum-free condition for 48 h and then medium containing 10% FBS was added. BrdU was administered 24 h prior to cell harvest, and BrdU incorporation was measured using an ELISA-based assay. The relative levels of BrdU incorporation from three independent measurements are shown (Mean ± SEM).

C. HC11-LacZ and HC11-Cuzd1 were serum-starved for 48 h and stimulated with FBS for 24 h. RNA was prepared from these cells and subjected to real-time PCR analysis using gene-specific primers to assess the expression of E2F Transcription-1 (E2F1), p27, Cyclin dependent kinase 1& 2 (Cdk1 &2), Cyclin A1 and Cyclin B1
Fig. 2.2. *Cuzd1* regulates the expression of a subset of EGF family ligands and ERBB receptors in HC11 cells

HC11-*Cuzd1* and HC11-LacZ cells were cultured for 48 h in serum-free media and then exposed to 10% FBS for 24 h. RNA was isolated and real-time PCR was performed to analyze relative expression levels of *Epgn, Ereg, Egf, Hbegf, Areg, Btc, Nrg1, Nrg2, Nrg3* and *Nrg4*. Data are represented as average fold induction ± SEM.
Fig. 2.3

A.

B.
Fig. 2.3. Regulation of proliferation of HC11-Cuzd1 cells by ERBB receptors

A. HC11-Cuzd1 cells were transfected with siRNA (50nM each) targeted against ERBB1-4 or scrambled siRNA (control) as described in the Materials and Methods. Total RNA was prepared from these cells 48 h post-transfection and subjected to real-time PCR analysis, using gene-specific primers, to assess the expression of ERBB 1-4 mRNAs.

B. HC11-Cuzd1 cells were transfected with siRNA to attenuate the expression of ERBB1-4. 48 h post transfection, the siRNA transfection mixture was removed and replaced with fresh growth medium. BrdU was added at this point and its incorporation was measured after 24 h, using an ELISA-based BrdU assay, according to the manufacturer’s protocol.
Fig. 2.4

A.

![Bar chart showing Relative Fold Induction for Cuzd1](chart.png)

B.

![Immunofluorescence images showing Vehicle and 20ng/ml Epigen](image.png)

C.

![Immunofluorescence images showing Vehicle and 50ng/ml Nrg1](image2.png)

24h Treatment
Fig. 2.4. Regulation of Cuzd1 expression and translocation of CUZD1 protein in response to the EGF family ligands

A. HC11 cells were serum starved for 48 h followed by treatment with epigen (20 ng/ml) or neuregulin-1 (50 ng/ml) for 24 h. Control cells were treated with vehicle. RNA was isolated from all treatment groups and subjected to quantitative real-time PCR, using gene-specific primers for Cuzd1.

B. HC11 cells were serum starved for 48 h and stimulated with epigen (20 ng/ml) for 24 h. Cells were fixed and subjected to immunofluorescence, using an antibody specific for CUZD1. Positive staining for CUZD1 is indicated in green and DAPI stained nuclei are shown in blue.

C. HC11 cells were serum starved for 48 h and stimulated with neuregulin-1 (50ng/ml) for 24 h. Cells were fixed and subjected to immunofluorescence, using an antibody specific for CUZD1. Positive staining for CUZD1 is indicated in green and DAPI stained nuclei are shown in blue.
Fig. 2.5

A.

B.
Fig. 2.5 (cont.)

C.

![Bar graph showing relative fold induction of MMP2 and MMP9 for HC11-LacZ and HC11-Cuzd1](image-url)
Fig 2.5. Overexpression of Cuzd1 leads to transformation of HC11 cells

A. MCF7 cells (control) or HC11-Lacz or HC11-Cuzd1 cells were plated in media containing soft agar. Colonies were allowed to form for 16 days and stained with crystal violet overnight. Visible colonies (>0.5mm) were counted, using a dissecting microscope. Data are presented as mean+/- standard error from three independent experiments. Figures below show a representative colony from each treatment group.

B. Serum-starved MDAMB-231 cells (positive-control) or HC11-LacZ or HC11-Cuzd1 cells were placed in Boyden chambers and allowed to migrate toward 10% FBS for 72 h. The number of invading cells was quantitated, using CyQuant fluorescence labeling (grey bars), and compared to corresponding cells without exposure to the serum (black bars). Data are presented as mean+/-standard error from three independent experiments.

C. HC11-LacZ and HC11-Cuzd1 were serum-starved for 48 h and stimulated with FBS for 24 h. RNA was prepared from these cells and subjected to real-time PCR analysis using gene-specific primers to assess the expression of MMP2 and MMP9 (Matrix-degrading metalloprotease).
Fig. 2.6

A.

HC11-LacZ

HC11-Cuzd1

Exterior

Mammary Gland

B.

Tumor Volume (mm2)

Weeks

H-LacZ

H-Cuzd1

Weeks

Tumor Volume (mm2)
Fig. 2.6. HC11 cells overexpressing Cuzd1 form tumors in vivo

A. HC11-LacZ or HC11-Cuzd1 cells were injected orthotopically into the teat of the 4th mammary gland of immunocompromised female nude mice. Mice were sacrificed 18 weeks post injection and examined for tumor growth. Top panels represent an exterior view of the animal and bottom panels show the mammary gland after dissection.

B. Tumor volume was quantified weekly using digital calipers from time of injection (week 0) to time of sacrifice (week 18). Tumor volume=1/2(length × width²)
Fig. 2.7

A.

HC11-LacZ
(Normal Mammary Gland)

HC11-Cuzd1
(Mammary Tumor)

B.

Cuzd1

PCNA
Fig 2.7. HC11 cells overexpressing Cuzd1 form high-grade mammary adenocarcinomas

A. Mammary glands (HC11-LacZ, top panel) or tumors (HC11-Cuzd1, bottom panel) were collected 18 weeks post-injection, fixed, embedded in paraffin, sectioned and subjected to H&E staining. Left panels are 5x images and right panels are 40x images.

B. Mammary tumor tissue of mice injected with HC11-Cuzd1 cells were collected, fixed, and embedded in paraffin. Serial sections of these tissues were subjected to IHC analysis, using antibodies against CUZD1, pan-cytokeratin and PCNA. Figures are 40x images.
Fig. 2.8. Tumors resulting from HC11-*Cuzd1* cells express high levels of EGF-like ligands and ERBB receptors

Mammary tumor tissue from mice injected with HC11-Cuzd1 cells were collected, fixed, and embedded in paraffin. Serial sections of these tissues were subjected to IHC analysis, using antibodies against epigen (a), neuregulin-1 (b), phospho-ERBB1/EGFR (c), ERBB3 (d), phospho-ERBB2 (e), phospho-ERBB4 (f), phospho-ERK1/2 (g) or phospho-AKT (h). Magnification: 40X. Inset in (e) show ductal cells within the sample that stain positive for p-ErbB2
Fig. 2.9

E induces the expression of Cuzd1 in normal mammary epithelial cells. The EGF family growth factors, such as EPGN and NRG1, trigger the translocation of the CUZD1 protein to the nucleus. CUZD1 then act in the nucleus to control the production of EPGN, EREG and NRG1 and their ERBB receptors via an unknown mechanism. Secretion of these growth factors leads to enhanced activation of the ERBB and ERK1/2 signaling pathway, promoting mammary epithelial cell proliferation. These results suggest that constitutive overexpression of Cuzd1 may result in the establishment of an autoregulatory loop that drives uncontrolled epithelial cell proliferation and tumorigenesis.
CHAPTER 3

THE ROLE IN CUZD1 IN HUMAN BREAST CANCER
ABSTRACT

Our laboratory has recently identified Cuzd1 as a novel estrogen (E)-regulated gene in the mouse mammary gland. Deletion of the Cuzd1 gene from the mouse genome resulted in greatly reduced proliferation of the mammary epithelium in response to E and progesterone (P), indicating a central role of this gene in steroid-induced growth of the mammary gland. The Cuzd1 gene is highly conserved between the mouse and the humans. The linkage between Cuzd1 and mammary epithelial proliferation raised the possibility that it might be involved in the uncontrolled proliferation of human breast cancer cells. Screening of several breast cancer cell lines for Cuzd1 expression revealed that Cuzd1 is present in low or undetectable levels in ESR1-negative tumor cells lines, but is expressed at high levels in ESR1-positive cell lines. Small interfering RNA (siRNA)-mediated attenuation of Cuzd1 expression in human breast carcinoma MCF7 cells led to a dramatic loss of E-induced proliferation of these cells. This observation hinted at an important connection between Cuzd1 and E-dependent breast cancer. The siRNA-mediated downregulation of Cuzd1 also significantly reduced the expression levels of epigen (EPGN) and epiregulin (EREG), growth factors belonging to the EGF family, indicating that Cuzd1 regulates the ERBB signaling in human breast cancer cells. Analysis of Cuzd1 expression in primary human breast tumors revealed a significantly elevated expression of this gene in tumors displaying a high level of ESR1 and ERBB signaling, compared to normal (control) breast tissues. Collectively, these studies indicated that Cuzd1 regulates uncontrolled proliferation of human breast cancer cells by acting as a critical integrator of the ESR1 and ERBB signaling pathways.
INTRODUCTION

An extensive body of basic and clinical research established that the steroid hormone estrogen (E), acting via its receptor ESR1, controls epithelial proliferation during normal mammary gland development as well as uncontrolled cell division in hormone-dependent breast malignancies. Although ESR1 is expressed in 40-70% of breast cancers, the exact mechanism by which E controls tumorigenesis is unknown. To understand this, it is necessary to identify E-regulated molecular pathways that mediate the proliferative actions of E in the breast tissue [1-5]. Although ample evidence has accumulated, implicating the epidermal growth factor (EGF) signaling pathway as a downstream mediator of E-induced growth of human breast tumors, the precise molecular mechanisms that connect these signaling pathways during mammary gland development or carcinogenesis remain unclear [6-10]. Understanding the key players which integrate the E & ERBB signaling pathways to regulate the proliferation of breast cancer cells is critical to the development of novel targeted therapies for cancer prevention and treatment.

We have identified Cuzd1 as a novel E-regulated gene in the mouse mammary gland. The mammary tissue of Cuzd1-null mice exhibits significantly delayed ductal outgrowth during puberty. A severe impairment in ductal side branching and alveolar development was also evident in these mutant mice during pregnancy and lactation. Strikingly, loss of Cuzd1 resulted in greatly reduced proliferation of the mammary epithelium in response to E and P, indicating a central role of this factor in steroid-induced growth of this tissue. Gene expression profiling studies, using wild type and mutant mammary epithelia, further revealed that Cuzd1 critically regulates the ERBB signaling pathway during mammary gland development. Collectively, these
studies uncovered Cuzd1 gene product as a potential integrator of the growth promoting effects of steroid hormone and ERBB receptor pathways during normal mammary epithelial proliferation.

The identification of Cuzd1 as a regulator of E-dependent epithelial cell proliferation during normal mammary gland development and its potential significance in breast tumorigenesis led us to investigate its role in human breast cancer cells. Like mouse Cuzd1, its human ortholog (CUZD1) contains two ZP domains and one CUB domain. The CUB-domain containing proteins are developmentally regulated and have known roles in protein adhesion or interaction with the extracellular matrices. ZP domains function as conserved modules for polymerization of extracellular proteins and are also responsible for sperm-adhesion of the ZP. The human CUZD1 shares ~80% identity with the mouse Cuzd1 at the nucleotide level. Cuzd1 has been implicated in diverse physiological functions, including E-induced proliferation of uterine epithelium during early pregnancy [11, 12], protection against severity of pancreatitis [13] and cell adhesion [14]. A recent study [14] reported a high level of expression of Cuzd1 in human ovarian tumor cells, suggesting a potential role for Cuzd1 in the development of ovarian cancers. These findings raised the possibility that Cuzd1 may also play an important role in human breast tumorigenesis.

To study the role of CUZD1 in E-mediated breast tumor cell proliferation, we chose the human breast carcinoma cell line, MCF7. These cells express endogenous CUZD1 mRNA and CUZD1 protein, and high levels of ESR1. Addition of E to these cells led to a marked enhancement in CUZD1 expression. Also, ICI 182,780, a well-known ESR1 antagonist, dramatically reduced the expression of CUZD1 in MCF7 cells, indicating that this factor is
regulated by ESR1 and a potential downstream mediator of ESR1 function in human breast cancer cells. Silencing of $CUZD1$, using small interfering RNAs (siRNAs), resulted in the inhibition of cell growth and proliferation, suggesting a vital role of this gene in E-dependent breast cancer cell proliferation. Most importantly, analysis of $CUZD1$ expression in human primary breast tumors revealed elevated expression of its mRNA in ~60% of tumors compared to their normal counterparts. There was also a tight correlation between the expression of $CUZD1$, ESR1 and active ERBB1 proteins in primary breast tumors. Our recent breast tumor tissue microarray analysis revealed strong correlation between the expression of $CUZD1$, $ESR1$ and later stages of breast cancer. Taken together, these findings support a potential role for $CUZD1$ in human breast tumorigenesis.

MATERIALS AND METHODS

Cell line and cell culture

The human non-transformed mammary epithelial cell line, MCF10A and human breast cancer cell lines MDAMB468, MDAMB231, BT20, SKBR3, T47D and MCF7 were obtained from American Type Culture Collection ($ATCC$) and cultured according to the manufacturer's specifications.

RNA isolation and real-time PCR analysis

Total RNA was extracted from cultured MCF7 cells using the Trizol RNA purification kit (Invitrogen) according to the manufacturer’s instructions. Reverse transcription was performed using the cDNA synthesis kit (Stratagene) following manufacturer’s instructions. cDNA was amplified by quantitative real-time PCR analysis using gene-specific primers and SYBR-Green
supermix (Bio-Rad Laboratories). 36B4 was used as the loading control. For a given sample, threshold cycle (Ct) and SD was calculated from individual Ct values from 3-4 replicates of a sample. Normalized mean Ct was computed as ΔCt by subtracting mean Ct of 36B4 from Ct of a target gene for control sample. ΔΔCt was then calculated as a difference in ΔCt values between control and experimental groups. Fold change in gene expression was then computed as $2^{-\Delta\Delta C_t}$. Error bars indicates $2^{-\Delta\Delta C_t} \pm$ SD.

For scanning expression levels in human primary tumors, a real-time PCR–based TissueScan Breast Cancer Panel containing 48 tissues covering four disease stages and normal tissues (Origene) was used to evaluate CUZD1 expression levels in human breast cancers. All data were normalized to the endogenous β-actin.

**siRNA- mediated transient transfection**

MCF7 cells were transfected with siRNA against CUZD1 or control siRNA (luciferase) using Silentfect lipid reagent following the manufacturer’s protocol (Bio-Rad Laboratories). Briefly, silentfect was mixed with 150 nM of siRNA, allowed to form siRNA-liposome complexes and added to MCF7 cells at 60% confluency. After 24 h, the transfection was repeated again. Cells were harvested 48h after the second transfection and total RNA was isolated and examined by quantitative real-time PCR using gene-specific primers.

**Cell proliferation using BrdU incorporation**

MCF7 cells were plated at a density of 5 x 10^3 cells/well in 96-well plates and cultured for 24 h in full growth medium. Following siRNA transfection, siRNA was replaced with medium containing E (10^{-7} M) and cells were allowed to grow for 72 h. BrdU was added at this point and
its incorporation was measured after 24 h using an ELISA-based BrdU assay and the resulting color reaction was measured using a plate reader at 370nm. To assess the mitogenic effects of epigen, MCF7 cells were grown in media containing low (0.5%) serum and were treated with recombinant epigen (20ng/ml). In parallel experiments, positive controls were performed by adding EGF (100ng/ml) or E (10^{-7} M) to these cells. BrdU incorporation was measured over a period of 24 h.

**Immunofluorescence and immunohistochemistry**

MCF10A or MCF7 cells were cultured in media containing 10% FBS for 24 h. Cells were fixed in 3.7% formaldehyde, permeabilized, blocked and incubated overnight with primary antibody against CUZD1 (in-house). Subsequently, slides were washed in PBS with 0.1% Triton-X and incubated with Cy3-conjugated anti-rabbit IgG. Slides were stained for DAPI and mounted.

Paraffin-embedded tumor tissue sections from Normal/Stage 0, stage II or IV, that were pathologist verified were obtained from Origene. Immunohistochemistry was performed on adjacent sections overnight at 4^0C, with primary antibodies directed against CUZD1 (in-house), ESR1 (Novacastra), phospho-EGFR (Y1068, Cell Signaling) or phospho-ERBB4/HER4 (Y1056, Santa Cruz Biotechnology). Bound primary antibodies were detected with horseradish peroxidase (HRP)–conjugated goat anti-rabbit or anti-mouse secondary antibody (Jackson Laboratories). Sections were counterstained with hematoxylin and mounted.
Tissue microarray analysis

A tissue microarray (TMA), assembled from paraffin-embedded breast cancer tissue samples, was provided by the Breast Center of Baylor College of Medicine, Houston, TX. The TMA consisted of six slides, each containing 45 breast cancer samples and one internal control sample. Information regarding age, disease stage, receptor status for ESR1, PGR and HER2, were provided with each slide of the TMA. To perform the CUZD1 expression analysis, slides were subjected to IHC using an antibody specific for CUZD1. The images were captured with a brightfield microscope within 72 h of staining and were obtained at the following magnifications: 5x, 10x, and 20x. The images were scored for positive or negative immunostaining by two independent observers.

We assessed the localization and intensity of CUZD1 immunostaining. Cytoplasmic staining of CUZD1 was evaluated qualitatively (negative, weak, medium, medium-high and strong), while nuclear staining was evaluated quantitatively. Nuclear staining was assessed using ImageJ software and the positive cells ranged between negative, weak (<5%), medium (5-10%), and high intensities (>10%). Samples with score of medium or higher were considered for the analysis. Finally, the CUZD1 expression data were combined with other information regarding the tumors to assess correlations with tumor stage and receptor status.
RESULTS

E acting via ESR1 regulates \textit{CUZD1} expression in MCF7 cells

To investigate the regulation of \textit{CUZD1} by E in human breast cancer cells, we added E to cultures of MCF7 cells grown in the absence of hormone and monitored the expression of \textit{CUZD1} and pS2, a well known E-regulated gene, at different time points after ligand addition. Upon E treatment, we observed a marked up-regulation of \textit{CUZD1} and pS2 (positive control) mRNAs at 2 h. The level of pS2 mRNA increased progressively at 10 h and 24 h. On the other hand, \textit{CUZD1} mRNA level increased robustly at 10 h and then declined at 24 h (Fig. 3.1A). When ICI 182,780, an ESR1-antagonist, was added together with E, a drastic reduction in the expression of both genes was seen (Fig. 3.1B). These results provided clear evidence that E regulates \textit{CUZD1} expression in mammary cells in an ESR1-dependent manner.

High levels of \textit{CUZD1} mRNA is present in ESR1-positive human breast cancer cell lines

To further investigate \textit{CUZD1} expression in breast cancer, we examined 6 human breast cancer cell lines (three ESR1-negative and three ESR1-positive) to profile the expression of \textit{CUZD1} mRNA. We observed low or undetectable levels of \textit{CUZD1} in ESR1-negative tumor cells lines, but significantly higher, from 5-20 fold in ESR1-positive cell lines when compared to non-transformed MCF10A cells. The expression was highest in MCF7 cells where ESR1 is abundantly expressed (Fig 3.1C). Furthermore, localization of \textit{CUZD1} protein via immunofluorescence revealed a high level of expression in nuclei of MCF7 cells, while it was
undetectable in non-transformed MCF10A cells (Fig 3.1D). Taken together, these results
demonstrated that overexpression of \textit{CUZD1} mRNA is correlated with ESR1 expression in
breast cancer cells.

**Attenuation of \textit{CUZD1} expression suppresses E-dependent proliferation of MCF7 cells**

To confirm the functional role of \textit{CUZD1} in E-mediated proliferation of breast cancer cells, we
employed siRNA strategy to suppress \textit{CUZD1} mRNA expression in MCF7 cells. As shown in
Fig 3.2A, we observed that 48 h following transfection, there was ~80% reduction in \textit{CUZD1}
mRNA levels. Transfection of luciferase siRNA had no appreciable effect on \textit{CUZD1} mRNA
levels. In order to determine the role of \textit{CUZD1} in E-mediated proliferation of MCF7 cells, an
ELISA-based colorimetric assay was employed to quantitate the amount of BrdU incorporation
in cells in which \textit{CUZD1} expression was attenuated by siRNAs. In this experiment, 48 h
following \textit{CUZD1} or luciferase (control) siRNA transfection, cells were treated with medium
containing E for 72 h. The BrdU was then added and its incorporation was assessed after 24 h.
This experiment showed that E-induced BrdU incorporation was reduced approximately 60% in
cells treated with \textit{CUZD1} siRNA compared to those treated with control siRNA (Fig 3.2B).
These results provided convincing evidence that \textit{CUZD1} is an important regulator of E-mediated
proliferation of breast cancer cells.

**\textit{CUZD1} mediates the expression and mitogenic effects of EPGN & EREG during E-induced
proliferation of MCF7 cells**

Loss of epigen and epiregulin expression in the mammary epithelia of \textit{Cuzd1}-null mice indicated
that these growth factors are regulated downstream of \textit{Cuzd1}. To determine whether E regulates
the expression of these growth factors in MCF7 cells, E was added to cells grown in the absence of hormone and the expression of various EGF family of growth factors, such as epigen, epieregulin, EGF and amphiregulin, was monitored at different times after E addition. Upon E treatment, a marked up-regulation of expression of epigen was observed. The expression of epieregulin and EGF was also up regulated by E, although to a lesser extent (Fig 3.3A). The E regulation of amphiregulin is well established, [16] hence it was used as a positive control. Interestingly, attenuation of CUZD1 mRNA expression led to the downregulation of epigen and epieregulin, but not EGF, amphiregulin and other EGF-like ligands (Fig 3.3B). These results indicated that although E stimulates the production of various EGF family growth factors, the induction of only a subset of these factors is mediated by CUZD1.

It is possible that the EGF family growth factors produced by MCF-7 cells in response to E treatment contribute to cell proliferation. To test this possibility, we examined whether epigen exerts mitogenic effects on MCF7 cells. For this purpose, an ELISA-based BrdU incorporation assay was performed. Treatment with epigen resulted in a three-fold increase in BrdU incorporation compared to vehicle-treated control cells. Interestingly, the extent of epigen-induced cell proliferation was comparable to that induced by EGF or E (Fig 3.3C). Collectively, these results supported the hypothesis that E-induced production of a subset of the EGF family growth factors, such as epigen, is mediated by CUZD1 and these growth factors play a key role in E-dependent proliferation of human breast cancer cells.
Elevated expression of \textit{CUZD1} in human primary breast tumors

To further explore the functional link between CUZD1, ESR1 and the EGFR signaling pathway during breast cancer, we analyzed the expression patterns of \textit{CUZD1}, \textit{ESR1}, \textit{ERBB1} and \textit{ERBB4} in primary breast tumors, using real-time PCR based analysis (Fig 3.4A-D). We analyzed the expression of these genes in 5 normal breast tissues as well as 43 primary breast tumors obtained from patients at different stages of breast cancer ranging from stages I to IV. We observed low levels of expression of \textit{CUZD1} mRNA in several normal breast tissues that we analyzed. Of the primary tumors analyzed, in \textasciitilde 60\% of the primary tumors, \textit{CUZD1} mRNA expression was significantly elevated (at least 6-fold or higher) compared to the normal breast tissue (Fig 3.4A). The expression pattern of \textit{ESR1} mRNA revealed that \textasciitilde 70\% of \textit{CUZD1}-positive tumors are also \textit{ESR1}-positive (6-fold or higher) (Fig 3.4B). These tumors were also verified as ESR1-positive based on the immunohistochemical data provided with the samples. A significant population of these tumors was positive for ERBB1 or ERBB4 (2.5-fold or higher) (Fig 3.4 C and D). Statistical analysis (Chi-squared test) reveals significant correlation between the expression of \textit{CUZD1}, \textit{ESR1} and HER1/HER4 in these cancer samples ($P < 0.005$) (Fig 4E). Strikingly, highest levels of mRNA expression was observed in breast tumors classified as stages III & IV in which \textit{CUZD1} expression was increased ranging from 10- to 60-fold compared to normal breast tissue (Fig 3.4A). This result hints at a link between the expression level of \textit{CUZD1} and progression of human breast tumorigenesis.

We next employed IHC to analyze the expression of CUZD1, ESR1, ERBB1 and ERBB4 proteins in nonmalignant and malignant mammary tissues. We detected low levels of these proteins in normal tissues. Consistent with the RNA data presented in Fig. 3.4, significantly higher and mostly nuclear expression of CUZD1 protein was observed in Stage III tumor tissue.
The nuclear expression of CUZD1 was further increased in the Stage IV/metastatic tissue (Fig 3.4F). The Stage III tumor tissue sections also exhibited high levels of ESR1, ERBB1 and ERBB4 compared to normal/stage 0. Additional Stage IV samples could not be obtained to perform the ErbB staining profile. These results again supported a strong correlation between CUZD1, ESR1 and the EGFR signaling system in a specific subset of tumors.

**CUZD1 expression analysis using human primary breast tissue microarray**

In order to assess the relationship between CUZD1 and tumor stage or receptor status, archival breast cancer tissue microarrays (TMA) consisting of 276 individual tumor samples, provided by Baylor College of Medicine (BCM), were screened for CUZD1 expression by IHC staining. The majority of the tumor samples were ductal carcinomas and information regarding the stage and receptor status was available for each individual sample. For cytoplasmic staining of CUZD1, the staining intensity was graded as negative, weak, medium, medium-high and strong. For nuclear staining, the percentage of CUZD1-positive cells were determined by image-J software and was graded as negative, weak, medium and high. Samples that scored medium or higher were considered for the analysis. Representative images for each of these categories are provided in Fig 3.5A. Samples in negative or low expression categories were not included in our analysis.

In this study, our first aim was to investigate whether there is any correlation between the overall level of CUZD1 expression, the stage of cancer and its nuclear/cytoplasmic localization. Our study revealed that 63% of all samples were CUZD1-positive. CUZD1 was predominantly present in the late stages of cancer (IIB, III & IV) as compared to the early stages (0, I and IIA), i.e., 61% of all CUZD1-positive tumors were late stage and this observation was statistically
significant as determined by the chi-squared test (<0.05) (Fig 5B (i)). There was an approximately even split between the cytoplasmic and nuclear localization of CUZD1 in tumor samples and no clear correlation emerged between the cellular localization of CUZD1 and tumor stage.

The second goal of this study was to determine whether CUZD1 levels were linked to a specific steroid receptor profile of the tumor tissue. Our analysis revealed that 55% of CUZD1-positive tumors were also categorized as ESR1-positive, PGR-positive and ERBB2-negative (++) and this observation was statistically significant as determined by the chi-squared test (<0.05) (Fig 3.5B (ii)). Interestingly, a small but significant fraction of CUZD1-positive tumors were ESR1-negative, PGR-negative and ERBB2-negative (---). In conclusion, CUZD1 expression is predominantly associated with late stage breast cancers that are positive for ESR1 and PGR but negative for ERBB2.

**DISCUSSION**

Abnormal expression of ESR1 is found in many human breast cancers, and about 50% of ESR1-positive breast cancer patients respond to anti-estrogen therapy [5, 10]. However, the exact signaling mechanism by which E controls mammary tumorigenesis remains elusive. To understand this, it is necessary to identify E-regulated molecular pathways that mediate the proliferative actions of E in the breast tissue. In this study, we have identified *CUZDI* as a novel E-regulated gene in a human breast cancer MCF7 cells. Addition of ICI 182,780, a pure antagonist of ESR1, completely abolished the expression of *CUZDI*, indicating that its
expression is mediated by ESR1. Furthermore, we demonstrated that the endogenous levels of 
CUZD1 mRNA in a variety of breast cancer cell lines were correlated with their ESR1 status. 
Taken together, these results provide evidence consistent with the ESR1 regulation of CUZD1. The induction of CUZD1 as early as 2 h in response to E indicates that it might be a direct, primary target gene of ESR1. Recent in silico analysis revealed several potential ESR1 binding sites within the 10 kilobase 5’-flanking sequence of the CUZD1 gene (J. Mapes, unpublished data). Chromatin immunoprecipitation experiments are underway to determine whether one or more of these sites interact with ESR1.

The proliferative response of MCF7 cells to E is well documented. It was previously reported that E induces the expression of several EGF family ligands, such as EGF, AREG and TGFα. E also regulates the expression of ERBBs, such as ERBB1, which are known mediators of proliferation of breast cancer cells [6, 9]. Recent studies in our laboratory, using the Cuzd1-null mouse model and mouse model of tumorigenesis (described in Chapters I and II), showed that CUZD1 acts by modulating the ERBB signaling pathway, a critical regulator of mammary epithelial cell proliferation during normal development and tumorigenesis. Here, we investigated whether a similar molecular link exists between CUZD1 and the ERBB signaling pathway in human breast cancer cells. Indeed, attenuation of CUZD1 by administration of siRNA targeting its mRNA led to a marked reduction in the production of the EGF family growth factors, EPGN and EREG, which act via the ERBB signaling pathway. We have demonstrated that Epgn mRNA expression is robustly induced by E in MCF7 cells. EPGN also stimulates the proliferation of MCF7 cells when added to cell cultures maintained in the absence of serum. In one study, Finak et al have reported significant correlation between the expression patterns of CUZD1 and Epgn in certain breast tumors (18). Taken together, these results raised the possibility that the
mitogenic effect of E in human breast cancer cells is mediated at least in part by \textit{CUZD1} via production of growth factors, such as EPGN.

Analysis of human primary breast tumors show that \textasciitilde60\% of these tumors displayed significantly up-regulated levels of \textit{CUZD1} compared to normal breast tissue. The \textit{CUZD1} mRNA overexpression is also tightly linked to the expression of mRNAs corresponding to \textit{ESR1}, \textit{ERBB1} and \textit{ERBB4}. Since \textit{CUZD1} acts as functional link between the \textit{ESR1} and \textit{ERBB} signaling pathways, it emerges as a target of therapeutic drugs in the context of a subset of breast tumors.

Our study also suggested a link between \textit{CUZD1} overexpression and advanced stages of breast cancer. Tissue microarray analysis of over 250 samples revealed significant correlation between \textit{CUZD1}-positive samples and advanced stages of breast cancer. This analysis also showed a tight correlation between \textit{CUZD1} and tumors that belong to the subtype \textit{ESR1}-positive, PGR-positive and \textit{ERBB2}-negative. Analysis of the Oncomine database, a publicly available database summarizing gene expression profiling across tumor tissue types, provided additional evidence for a possible relationship between \textit{CUZD1} and \textit{ESR1} in the context of \textit{ERBB2}-negative breast tumors (15). In one study, statistically significant (p=2.90E-4) up-regulation of \textit{CUZD1} expression was observed in invasive ductal breast carcinoma compared to their normal counterparts (17). This and other studies show significant correlation between the expression patterns of \textit{CUZD1} and \textit{ESR1} and inverse correlation between \textit{CUZD1} and \textit{ERBB2} expression. Recent studies have shown that the classical adjuvant therapies are not effective for \textit{ESR1}-positive, \textit{ERBB2}-negative tumors because they often develop endocrine resistance [19]. Endocrine resistance is often attributed to the cross-talk between \textit{ESR1} and the \textit{ERBB} pathways
Our study raises the possibility that specific therapeutic drugs targeting CUZD1 may prevent endocrine resistance in the ESR1-plus/ERBB2-minus subtype of breast tumors.

REFERENCES


20. Arpino, G., et al., *Crosstalk between the estrogen receptor and the HER tyrosine kinase receptor*
FIGURES
Fig. 3.1

A.

B.

C.

D.
Fig 3.1. Estrogen regulation of *CUZD1* in MCF7 breast cancer cells

A. MCF7 cells grown in E-free culture medium were treated with E (10^{-8}M) for indicated times. Cells were harvested at various time points, total RNA was isolated from these cells, and subjected to real-time PCR analysis, using gene-specific primers, to assess the expression the expression of pS2 (control) and *CUZD1*.

B. MCF-7 cells were treated with E in the absence or presence of ICI 182,780 (10^{-6} M) for 24 h. Total RNA was isolated and subjected to real-time PCR analysis, using gene-specific primers, to assess the expression the expression of pS2 (control) and *CUZD1*.

C. Total RNA was isolated from a non-tumorigenic human mammary epithelial cell line, MCF10A (control) or breast cancer cell lines MDAMB 468, MDAMB 231, SKBR3 (ESR1-negative), BT20, T47D, MCF7 (ESR1-positive) and subjected to real-time PCR analysis using gene-specific primers to assess the expression of *CUZD1*. MCF10A was used as the control cell line.

D. MCF10A or MCF7 cells were cultured in 10% FBS for 24 h. Cells were fixed and subjected to immunofluorescence using an antibody specific for CUZD1 (orange) and the nuclei were stained with DAPI (blue)
Fig. 3.2

A. MCF7 cells were transfected with siRNA (150 nm) targeted against CUZD1 or Luciferase siRNA (control) as described in the Materials and Methods. Total RNA was prepared from MCF7 cells at 48 h after transfection and subjected to real-time PCR, using gene-specific primers, to assess the expression of CUZD1.

B. MCF7 cells grown in E-free culture medium were transfected with 150 nM siRNA targeted to CUZD1 or control luciferase mRNA for 48 h. The siRNA was replaced with medium containing E (10^{-7} M) and cells were allowed to grow for 72 h. BrdU was added at this point and its incorporation was measured after 24 h using an ELISA-based BrdU assay, according to the manufacturer’s protocol.
Fig. 3.3

A.

![Gene Expression Bar Graph]

B.

![Fold Induction Bar Graph]

C.

![Absorbance Graph at 370nm]
Fig 3.3. **CUZD1 mediates the expression and mitogenic effects of EPGN and EREG during E-induced proliferation of MCF7 cells**

A. MCF-7 cells grown in E-free culture medium were treated with E (10^{-8}M) for indicated times. Cells were harvested at various time points, total RNA was isolated from these cells, and subjected to real-time PCR analysis, using gene-specific primers, to assess the expression of Epgn, Ereg, Egf and Areg mRNA.

B. MCF7 cells grown in E-free culture medium were transfected with 150 nM CUZD1 or control luciferase siRNA for 48 h. Total RNA was prepared from HC11 cells 48 h after transfection and subjected to real-time PCR, using gene-specific primers, to analyze relative expression level of CUZD1 and Epgn, Ereg, Egf, Hbegf, Areg, Btc, and Nrg1-4.

C. MCF7 cells grown in E-free culture medium were treated with E (10^{-7} M) or recombinant EPGN (20 nM) or EGF (20 nM) for 72 h. BrdU was then added and its incorporation was measured after 24 h, using an ELISA-based BrdU assay, according to the manufacturer’s protocol.
Fig. 3.4

A.

B.
Fig. 3.4 (cont.)

C.

D.
E.

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<th>H&amp;E</th>
<th>Cuzd1</th>
<th>ESR1</th>
<th>p-HER1</th>
<th>p-HER4</th>
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<td>Stage 0</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
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<tr>
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<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
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<tr>
<td>Stage IV</td>
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<td><img src="image14.png" alt="Image" /></td>
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Fig. 3.4. Correlation between Cuzd1, ESR1, ERBB1 and ERBB4 in human primary breast tumors

A-D. A breast cancer TissueScan Real-Time qPCR array, containing five normal/Stage 0 cDNAs and forty-three human breast cancer cDNAs, was analyzed for expression of Cuzd1 (6A), ESR1 (6B), ERBB1/HER1 (6C) or ERBB4/HER4 (6D) by real-time PCR. Averages of relative Cuzd1 mRNA expression from two independent plates are plotted with clinical status indicated.

E. The breast cancer tissue samples were categorized based on their Cuzd1 status. Relative mRNA fold induction of 6 or higher was considered positive. The Cuzd1-positive samples were then further categorized based on the status of ESR1 and ERBB/HER (ERBB1/HER1 or ERBB4/HER4). Relative mRNA fold induction of 6 or higher was considered positive for ESR1 and a fold induction of 2.5 fold or higher was considered positive for ERBB1/HER1 and ERBB4/HER4.

F. Tissue sections from normal/Stage 0 and Stage III breast cancer were subjected IHC analysis using antibodies against Cuzd1, ESR1, ERBB1/HER1 and ERBB4/HER4. Stage IV sample was subjected IHC analysis using antibodies against Cuzd1 and ESR1.
Fig. 3.5

A.

**Nuclear**

<table>
<thead>
<tr>
<th></th>
<th>Negative</th>
<th>Weak</th>
<th>Medium</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cytoplasm</strong></td>
<td>Weak</td>
<td>Medium</td>
<td>Medium-high</td>
<td>Strong</td>
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</tbody>
</table>

B.

(i) | Cuzd1 status | Cuzd1+ | Cuzd1- |
<table>
<thead>
<tr>
<th></th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td><strong>Stage</strong></td>
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<td>Early Stage</td>
</tr>
<tr>
<td></td>
<td>61%</td>
<td>39%</td>
</tr>
<tr>
<td>*p-value=.0397</td>
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</tbody>
</table>

(ii) | Cuzd1 status | Cuzd1+ | Cuzd1- |
<table>
<thead>
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<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Receptor Status</strong></td>
<td>++-</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>55%</td>
<td>30%</td>
</tr>
<tr>
<td>*p-value=0.0434</td>
<td></td>
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</tr>
</tbody>
</table>
Fig 3.5. Correlation between CUZD1 expression, steroid receptor status and stages of breast cancer in human breast tumor tissue microarrays

A. Representative immunohistochemical images of the nuclear and cytoplasmic scoring of CUZD1 staining are shown.

B. The breast cancer tissue samples were categorized based on their CUZD1 status. For cytoplasmic staining of CUZD1, the staining intensity was graded as negative, weak, medium, medium-high and strong. For nuclear staining, the percentage of CUZD1-positive cells were determined by image-J software and was graded with a score of negative, weak (<5%), medium (5-10%), and high intensities (>10%). Samples that were scored medium or higher were considered for the analysis. The correlation between CUZD1 and stage of breast cancer is shown in upper panel (i). Stages O, I and IA are early stage breast cancers, IIB, III and IV are late stages. The correlation between Cuzd1 and receptor status is shown in lower panel (ii). Receptor status is represented as ESR1 status, PGR status, ERBB2/HER2 status as determined by IHC or FISH by pathologists at Baylor College of Medicine.
CHAPTER 4: ON-GOING STUDIES

ROLE OF CUZD1 IN EPITHELIAL MESENCHYMAL TRANSITION (EMT) AND METASTASES
ABSTRACT

Our recent studies indicated that overexpression of Cuzd1, a unique integrator of steroid hormone- and EGF family-induced growth pathways, in untransformed HC11 mammary epithelial cells leads to breast tumorigenesis. Interestingly, immunohistochemical analysis of primary mammary tumors derived from HC11-Cuzd1 revealed that 20-30% of the tumor cell mass is not epithelial in nature. This cell population resembles mesenchymal cells and may represent cells that have undergone epithelial-mesenchymal transition (EMT), which is a critical event in the progression toward cancer metastasis. Indeed, in mice bearing tumors larger than 150 mm², metastatic lesions were observed in the liver and lung. Analysis of expression of well-known EMT markers in the primary tumor tissue showed a strong up-regulation of the mesenchymal-marker vimentin and the transcriptional regulator Snail1. We also observed a marked downregulation of the epithelial marker E-cadherin in the primary tumors. To further analyze the role of Cuzd1 in EMT and metastasis, we subjected the primary tumors to enzymatic digestion and derived cell lines from HC11-Cuzd1 tumors. These cell lines are important tools to understand how the gene expression pattern progressively changes from untransformed HC11 cells to transformed HC11-Cuzd1 cells isolated from the mammary tumor. A preliminary microarray analysis was performed comparing the mRNA expression profiles in these cells. This analysis revealed a progressive up-regulation of several factors that influence cellular movement, invasion, and metastases, particularly the ILK, Rho, TGFβ, and Wnt signaling pathways, and downregulation of factors that mediate cell-cell tight junctions. These results are providing an initial glimpse of the molecular pathways that are regulated by Cuzd1 during tumorigenesis and metastasis.
INTRODUCTION

During breast cancer progression, epithelial cells detach from the primary tumor, and adhere to and invade the surrounding stroma. They intravasate into blood vessels and disseminate to distant tissues and organs where they extravasate and initiate secondary tumors or metastasis. Metastasis underlies the majority of cancer-related deaths [1]. Therefore, understanding the molecular mechanisms that enable tumor cell dissemination is a vital health issue. The transdifferentiation of epithelial cells to mesenchymal cells, called epithelial-mesenchymal transition (EMT), endows tumor cells with enhanced motility and survival attributes that facilitate dissemination to permissive niches [2]. Epithelial cells establish close contacts with their neighbors through the rearrangement of adherens junctions, desmosomes, and tight junctions. Conversely, mesenchymal or stromal cells are loosely organized in a three-dimensional extracellular matrix and comprise connective tissues adjacent to epithelia. The EMT is fundamental for embryonic development and tumor invasion. It involves profound phenotypic changes that include the loss of cell-cell adhesion, the loss of cell polarity, breakage of tight junctions, degradation of the basement membrane ECM components and the acquisition of migratory and invasive properties [3].

One of the earliest steps in EMT is the loss of E-cadherin function, and in fact it is generally accepted that EMT-inducing factors initiate epithelial reorganization by suppressing the expression or impairing the function of E-cadherin [4]. Many different extracellular cues have been shown to trigger epithelial dedifferentiation and EMT, such as those involving transforming growth factor-β (TGF-β), Notch, fibroblast growth factors (FGFs) and Wnt signalling pathways [5]. Most of these EMT-inducing signals exert their action through the
modulation of transcription factors that (i) repress epithelial genes, such as those encoding E-cadherin and cytokeratins, and (ii) activate transcription programs that specify fibroblast-like motility and an invasive phenotype. Several transcription factors are known to drive EMT, including the basic helix loop helix (bHLH) families of transcription factors, and other factors such as Twist, Zeb2 (ZEB2/SIP1), and the Snail/Slug family transcription factors [6]. This multistep process results in the loss of apical-basal cell polarity and the acquisition of spindle-shaped morphology, and the gain in expression of mesenchymal-specific genes, such as vimentin and N-cadherin [7]. During tumor progression, these changes are thought to promote tumor cell migration across the basement membrane and invasion into the surrounding microenvironment, ultimately leading to metastasis.

Although CUZD1 is expressed in the epithelial cells of mammary glands, uterus and ovary, its expression in mesenchymal or stromal cells has not been reported. We have shown that overexpression of CUZD1 in mouse mammary epithelial cells bestows distinct tumorigenic properties on these cells. Most notably, CUZD1-overexpressing cells show invasive properties as assessed by the transwell migration assays and also express robustly elevated levels of the matrix-degrading metalloproteases, that are known to promote tumor cell motility, invasiveness, and EMT. Indeed, in mice bearing tumors larger than 150 mm², metastatic lesions were observed in the liver and lung. Since EMT has been implicated as an essential process for metastases, it is plausible that CUZD1 is involved in reprogramming cancer cells toward a mesenchymal and potentially more invasive state, leading to invasion and metastases. Therefore, we investigated whether CUZD1 plays a role in EMT and if so, what the EMT-specific genes and signaling pathways it regulates. Here we provide evidence that overexpression of CUZD1 promotes activation of key signaling pathways that are critical for EMT.
MATERIALS AND METHODS

Orthotopic intraductal injection of cells into mammary gland

HC11-LacZ or HC11-Cuzd1 cells (1 × 10^6) were suspended in Matrigel and injected into the teat of the fourth abdominal mammary gland of nude (nu/nu) mice as described [8] Tumor growth was measured by weekly caliper measurement for a period of 18 weeks. All animal experiments were conducted in accordance with protocols approved by University of Illinois Institutional Animal Care and Use Committee. After 18 weeks, the mice were euthanized by CO₂. The primary mammary tumors, superficial lymph nodes, lung and liver were harvested from sacrificed animals and fixed in 4% paraformaldehyde for subsequent H&E staining or immunohistochemistry.

Isolation of primary breast tumor cell lines

Isolated tissue from late stage tumors (>20-weeks post-injection) was first placed into a cell culture dish containing PBS and the necrotic tumor tissue was removed. Tumor tissue was cut into small pieces, immediately placed in a digestion mixture of dispase (6 g/L) and collagenase (0.5 g/L) and incubated in 37°C incubator for 1 hour with agitation. The resulting mixture was passed through a 100 mm cell strainer and the flow-through was centrifuged at 1000 rpm for 5 minutes. The cell pellet was washed twice in HBSS and resuspended in DMEM-F12 media with 10% FBS, penicillin and streptavidin for 24 h. After culturing the cells for 3-4 days, they were replenished with RPMI-1640 along with 2.5 ug/ml of blasticidin to select for a pure population. This cell line was named HC11-Cuzd1Tumor
RNA isolation and real-time PCR analysis

Total RNA was extracted from cultured HC11 cells using the Trizol RNA purification kit (Invitrogen) according to the manufacturer’s instructions. Reverse transcription was performed using the cDNA synthesis kit (Stratagene) following the manufacturer’s instructions. cDNA was amplified by quantitative real-time PCR analysis using gene-specific primers and SYBR-Green supermix (Bio-Rad Laboratories). 36B4 was used as the loading control. For a given sample, threshold cycle (Ct) and SD was calculated from individual Ct values from 3-4 replicates of a sample. Normalized mean Ct was computed as ΔCt by substracting mean Ct of 36B4 from Ct of a target gene for control sample. ΔΔCt was then calculated as a difference in ΔCt values between control and experimental groups. Fold change in gene expression was then computed as $2^{-\Delta\Delta C_t}$. Error bars indicates $2^{-\Delta\Delta C_t} \pm SD$.

Histology and immunohistochemistry

Organs were fixed by incubation in 4% paraformaldehyde overnight, paraffin embedded, and cut into 4- to 5-μm sections. After high-temperature antigen retrieval in citrate or EDTA buffer, immunohistochemistry was performed on parallel sections for overnight at 4°C, with primary antibodies directed against Cuzd1 (in-house), pan-Cytokeratin (Cell Signaling), vimentin (Sigma), twist (abcam), E-cad (Cell signaling) and slug (Cell signaling). Bound primary antibodies were detected with horseradish peroxidase (HRP)–conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (Jackson Laboratories). Sections were counterstained with hematoxylin and mounted.
Microarray and ingenuity pathway analysis

Cells were harvested from three cell lines HC11-LacZ, HC11-Cuzd1 or HC11-Cuzd1Tumor and the total RNA was isolated by Trizol reagent (Invitrogen, CA). RNA samples were processed at the Biotechnology Center of the University of Illinois at Urbana-Champaign. RNA integrity was verified using the Agilent 2100 bioanalyser. Each RNA sample was processed for microarray hybridization using Genechip Mouse Genome 430A 2.0 arrays (Affymetrix) following established protocols. The resulting data files were analyzed by Affymetrix GeneChip Expression Console software.

Canonical pathways analysis identified the pathways that were most significantly enriched in the data set from the Ingenuity Pathways Analysis (Ingenuity Systems) (http://www.ingenuity.com/) a library of canonical pathways. Subsets of the related genes that were associated with a canonical pathway in the Ingenuity Pathways Knowledge Base were considered for further analysis.
RESULTS

_Cuzd1-driven tumors metastasize to the liver and lung_

Mice harboring CUZD1-overexpressing tumors were examined 20 weeks following tumor initiation for the presence of metastasis in the liver, lung and the peritoneum. Indeed, in mice bearing tumors larger than 150 mm$^2$, surface abnormalities and visible lesions were found in the liver and occasionally in the lung, but not in other visceral organs. Histological examination of the liver and lung tissue sections confirmed invasion of mesenchymal-like breast tumor cells in hepatic tissue of the liver (Fig 4.1A, top panel) and alveolar tissue of the lungs (Fig 4.1B, top panel). These lesions were verified by pathologists as adenocarcinomas and histologically consistent with the primary tumor. Furthermore, these cells stained positively for CUZD1, confirming that CUZD1-overexpressing cells have indeed metastasized to the liver and the lung (Fig 4.1A and B, bottom panels).

_Cuzd1-overexpressing tumors undergo EMT_

EMT is considered as a prerequisite to metastasis that allows epithelial tumor cells to become more fibroblastic and migratory, resulting in tumor cell dissemination and formation of secondary tumors at distant sites. One of the earliest steps in EMT is the loss of epithelial markers and appearance of mesenchymal markers. To confirm whether EMT occurs in CUZD1-overexpressing tumors, we analyzed the expression and localization of epithelial markers, such as cytokeratin and E-cadherin, and mesenchymal markers, such as vimentin and Slug in serial sections of these primary breast tumors. Although cytokeratins are abundantly expressed in CUZD1-overexpressing primary mammary tumors, 20-30% of the tumor cells did not stain
positively for pan-cytokeratin and histological analysis revealed that these cells are not epithelial in nature (Fig. 4.2A). These cells were also marked by downregulation of E-cadherin. We inferred that these cells most likely represented mesenchymal cells since they exhibited fibroblast-like morphology and showed strong expression of mesenchymal markers, such as vimentin, and Twist (Fig 4.2A).

In order to further understand the role of CUZD1 in EMT and metastasis, we have recently created a cell line, HC11-Cuzd1Tumor, by enzymatically dissociating tumors (>20-weeks post-injection) that formed in mice injected orthotopically with cells stably overexpressing CUZD1. A population of primary tumor cells was isolated following proper selection of the antibiotic resistance markers. To monitor the CUZD1-driven gene expression profiles that drive tumorigenesis, we utilized three cell lines: the control cell line HC11-LacZ, Cuzd1-overexpressing cells HC11-Cuzd1 and the tumor-derived cell line HC11-Cuzd1Tumor. Real-time PCR analysis of EMT markers was performed on total RNA prepared from these three cell lines. This analysis revealed a down-regulation of the epithelial marker E-cadherin and up-regulation of the mesenchymal marker vimentin in HC11-Cuzd1Tumor cells compared to HC11-LacZ cells. There was also an increase in the expression of mRNAs encoding EMT-related transcription factors, such as Snail and Zeb2, in the HC11-Cuzd1Tumor cells, confirming our immunohistochemical data. The expression of other transcription factors, such as Slug and Zeb1, did not change in the tumor cells, indicating that CUZD1 may regulate specific signaling pathways acting via Snail and Zeb2 (Fig 4.2B). These data suggested that CUZD1 may drive the reprogramming of cancer cells toward a mesenchymal and potentially more invasive state, leading to metastasis.
Identification of *Cuzd1*-regulated gene networks associated with EMT

To further examine the hypothesis that CUZD1 controls EMT during breast tumorigenesis, we sought to explore downstream signaling pathways associated with this transition. Our approach involved comparing the gene expression profiles of three HC11-based cell lines namely HC11-LacZ, HC11-Cuzd1 and HC11-Cuzd1Tumor. The HC11-LacZ cells overexpress lacZ and serve as a control non-tumorigenic cell line. The HC11-Cuzd1 cells overexpress Cuzd1 and exhibit some tumorigenic properties, such as colony formation on soft agar and increased migration (described in Chapter 2). The HC11-Cuzd1Tumor cells are derived from late-stage Cuzd1-overexpressing tumors that develop metastatic adenocarcinoma in liver and lung. We believe that the HC11-Cuzd1Tumor cells have undergone biological reprogramming and transformation from epithelial to mesenchymal-like cells in the microenvironment of the primary tumor. Total RNA was isolated from these three cell lines and subjected to microarray analysis. When we compared gene expression profiles of HC11-LacZ and HC11-Cuzd1 cells, we observed alteration in expression levels of many genes, which likely contributed to the tumorigenesis pathway. Similarly, the expression of many genes was significantly altered when gene expression profile of HC11-Cuzd1Tumor cells was compared to that of HC11-LacZ. These genes are likely involved in EMT and metastasis.

When the results of these two microarray analyses were compared, we found that the expression of 250 genes was up-regulated (>1.5-fold, p-value <0.1) and that of 168 genes was down-regulated (<1.5-fold, p-value <0.1) in both microarray analyses. The candidate up-regulated (Fig 4.3, A and B) and down-regulated genes (Fig 4.3, C and D) were classified according to their
known biological functions and the canonical pathways by ingenuity pathway analysis (IPA). Among the genes whose expression was altered, several with known roles in tumorigenesis and metastasis were prominent. These genes control cellular growth and proliferation, cellular development, cell-cell interaction, cellular movement and invasion. Among the up-regulated genes were the ILK (integrin-linked kinase), Rho, TGF-β, and Wnt signaling pathways. We also observed downregulation of the tight junction pathway. These pathways are implicated in EMT.

Genes identified by microarray analysis were further validated using real-time PCR analysis. As shown in Fig 3.4E observed a marked up-regulation of (i) molecules that belong to the TGFβ and Wnt signaling pathways, such as TGF-β1 and β-catenin coactivator BCL9; (ii) molecules that belong to the ILK pathway, such as the actin, ACTG2, and the integrin, IGTB4; and (iii) molecules that belong to the Rho signaling pathway, such as the guanine nucleotide exchange factor, NGEF. Additionally, the expression of molecules that belong to the tight junction pathway, such as the ECM component EFEMP2, the cadherins, desmocollin, and protocadherin18, and the cell adhesion molecule contactin, were significantly down-regulated. Up-regulation of molecules involved in invasion and downregulation of molecules involved in adhesion clearly showed that CUZD1 helps establish the molecular signature for EMT. Further analysis is required to define the gene regulatory networks and precise mechanisms that operate during CUZD1-induced EMT and metastasis.
DISCUSSION

We have shown previously that CUZD1-overexpressing cells exhibit invasive properties as assessed by the *in vitro* migration assays. These cells also expressed elevated levels of the matrix-degrading metalloproteases, MMP2 and MMP9 ((Chapter 2), which are known to promote tumor cell motility, invasiveness, and EMT. Several lines of evidence suggest that EMT is involved in cancer progression, particularly during invasion and intravasation when tumor cells migrate to distant organs to form metastases [6, 9]. In this study, we show that CUZD1-overexpressing tumors metastasize to distant secondary sites, such as the lung and the liver. Our results showed that overexpression of CUZD1 leads to an elevated expression of the mesenchymal marker, vimentin, and downregulation of the epithelial cell adhesion marker, E-cadherin. These changes are well-established hallmarks of EMT and support a role for CUZD1 in the regulation of EMT.

It is important to note that Snail, the transcriptional repressor of E-cadherin, is up-regulated in Cuzd1-overexpressing tumors, as assessed by real-time PCR and immunohistochemical studies, indicating that Cuzd1-induced down-regulation of E-cadherin is likely mediated by Snail. In future experiments, we will assess the expression of EMT markers in tissues obtained from lung and liver adenocarcinomas to understand if similar pathways are being activated.

The microarray analysis links CUZD1 to many critical inducers of EMT, such as TGF-β, ILK, Rho and Wnt signaling pathways. TGF-β1, a well-known mediator of EMT, is highly induced in CUZD1-overexpressing cells. TGF-β1 functions both as a tumor suppressor and a tumor promoter [10]. In fact, the TGF-β pathway is known to prevent epithelial cell transformation by inhibiting proliferation and inducing senescence or apoptosis. On the other hand, during advanced tumorigenesis, enhanced TGF-β signaling is associated with cancer progression via
increased tumor cell motility, invasiveness and ultimately metastasis. TGF-β cooperates with the Ras and canonical Wnt/β-catenin pathway to induce EMT. It was also reported that a Snail-SMAD3/4 transcriptional repressor complex promotes TGF-β-mediated EMT [11]. ILK is a key component of focal adhesions that binds to integrins β1-3 and is indispensable for integrin function during development [12]. During EMT and metastasis, overexpression of ILK leads to nuclear translocation of β-catenin, increased invasiveness and repression of E-cadherin [13] via upregulation of Snail expression [14, 15]. ILK is also involved in TGF-β-mediated EMT of human keratinocytes [16]. Rho family GTPases and their regulatory molecules, GEFs and GAPs, exert important roles in epithelial plasticity and are essential effectors of EMT induced by TGF-β and other stimuli [17]. There is plenty of evidence from the literature, suggesting an interplay between the Wnt, Rho and ILK pathways and indicating TGF-β as an important regulator of these pathways.

CUZD1 dramatically induces the expression of TGF-β1 and its downstream signaling molecules, such as the MMPs, ACTG2 and NGEF. Taken together, these data raise the possibility that CUZD1 regulates EMT via the TGF-β pathway. Classic TGF-β signaling requires that it binds to the type II TGF-β receptor followed by transphosphorylation of a type I receptor, and subsequent phosphorylation of Smad2 and Smad3. Phosphorylated Smad2/3 forms a trimer with Smad4, which then translocates to the nucleus and interacts with transcription machinery to suppress epithelial cell-specific genes and promote the expression of mesenchyme-specific genes. Many groups have shown that TGF-β can induce EMT, but the precise signaling cascades involved are not completely understood. A future goal of this project is to use cell-based assays
and siRNA approaches to functionally address how CUZD1 regulates TGF-β and its downstream pathways to direct EMT.

It is also important to understand the role of EMT in later stages of the invasion-metastasis cascade, including intravasation, tumor-cell survival in the circulation, extravasation, and finally establishment/expansion of the micro-metastatic lesion in a distant target site. For this, we have created HC11-LacZ and HC11-Cuzd1 cells that are tagged with ZsGreen, a brighter and more stable alternative to GFP for fluorescent tracking of these cells (J.Mapes and MKB, unpublished). Using these cells, it should be possible to determine the time course and pattern of tumor growth and metastasis in vivo by tracking fluorescence through intravital microscopy.

Finally, it is also important to analyze human breast cancer tissues and assess possible correlation between CUZD1 expression, tumor recurrence and metastasis. Understanding the mechanism of action of CUZD1 and other molecules in EMT and metastasis may help us formulate new intervention strategies to prevent tumor metastasis. Both EMT and tumor-host microenvironment cross-talk contribute to the progression and severity of metastasis. In the future, it will be important to understand the molecular signature of EMT and aberrant host-microenvironment interactions to develop effective therapies against invasion and metastasis. Development of specific drugs that target and control these two broad mechanisms would provide powerful treatment strategies for cancer.
REFERENCES


FIGURES

Fig 4.1

A. Ctrl Liver  Lesion Liver

H&E  (10x)

Cuzd1  (20x)

B. Ctrl Lung  Lesion Lung

H&E  (10x)

Cuzd1  (10x)
Fig 4.1. Cuzd1-overexpressing breast tumors metastasize to distant secondary sites

A and B. The mammary glands of mice were injected with HC11-LacZ or HC11-Cuzd1 cells as described previously (Chapter 2). Liver (Fig A) and lung (Fig B) tissues were collected 20 weeks post injection, fixed, embedded in paraffin, and sectioned. Sections were subjected to H&E staining (top panels) or immunofluorescence or immunohistochemical analysis using an antibody against CUZD1 (bottom panels).
Fig 4.2

A.

B.
Fig 4.2. Cuzd1-overexpressing tumors undergo EMT

A. Mammary glands of mice were injected with HC11-Cuzd1 cells as described previously. Mammary tumor tissues were collected 20 weeks post injection, fixed, and embedded in paraffin. Serial sections from these tissues were subjected to IHC analysis using antibodies specific for CUZD1, pan-cytokeratin, vimentin, E-cadherin and Snail.

B. Total RNA was prepared from HC11-LacZ, HC11-Cuzd1 or HC11-Cuzd1Tumor cells and subjected to real-time PCR to analyze relative expression levels of mRNAs corresponding to E-Cadherin, N-Cadherin, Vimentin, Snail, Slug and Cuzd1. Data are represented as average fold induction ± SEM.
Fig 4.3

A.

B.
Fig 4.3 (cont.)

C.

D.
Fig 4.3 (cont.)

E.

![Bar chart showing TGFβ, ILK, Rho, and Tight Junction signaling in HC11-L, HC11-C, and HC11-CT.](chart)
Fig 4.3. Identification of EMT and metastasis associated pathways

A. List of common genes whose expression is up-regulated (>1.5 fold change) in HC11-Cuzd1 cells compared to HC11-LacZ (genes implicated in tumorigenesis) and in HC11-Cuzd1Tumor cells compared to HC11-Cuzd1 (genes implicated in EMT/Metastases). These pathways were categorized by biological function using Ingenuity pathway analysis software (IPA).

B. List of common genes whose expression is up-regulated (>1.5 fold change) in HC11-Cuzd1 compared to HC11-LacZ (genes implicated in tumorigenesis) and in HC11-Cuzd1Tumor cells compared to HC11-Cuzd1 (genes implicated in EMT/Metastases). These pathways were categorized by canonical pathways using Ingenuity pathway analysis software (IPA).

C. List of common genes whose expression is down-regulated (>0.5 fold change) in HC11-Cuzd1 cells compared to HC11-LacZ (genes implicated in tumorigenesis) and in HC11-Cuzd1Tumor cells compared to HC11-Cuzd1 (genes implicated in EMT/Metastases). These pathways were categorized by biological function using Ingenuity pathway analysis software (IPA).

D. List of common genes whose expression is down-regulated (>1.5 fold change) in HC11-Cuzd1 compared to HC11-LacZ (genes implicated in tumorigenesis) and in HC11-Cuzd1Tumor cells compared to HC11-Cuzd1 (genes implicated in EMT/Metastases).
These pathways were categorized by canonical pathways using Ingenuity pathway analysis software (IPA).

E. Selected genes from the microarray analysis were verified by real-time PCR. Total RNA was isolated from HC11-LacZ, HC11-Cuzd1 and HC11-Cuzd1Tumor cells and subjected to real-time PCR to analyze relative expression levels of *TGFB1, TGFB2, BCL6, TGFB2, FOXC1, ACTG2, ITGB4, MYLK, NGEF, GNA13, PCDH18, CNTN*. Data are represented as average fold induction ± SEM.