Loss of cadherin-based cell adhesion and the progression of Invasive Lobular Breast Cancer

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Lobular Breast Cancer owes its name to the lobular (grape-like) architecture of the milk-producing gland within the breast, called the mammary gland, which is depicted as a flower/sea anemone in this illustration with every petal resembling a lobule. Some of the petals let go and this release exemplifies the loss of cell-cell adhesion, which is characteristic for lobular breast cancer and is one of the main subjects in this thesis. These petals can wander through the body and give rise to a new flower at a distant side. This refers to the process of tumor progression, where tumor cells invade the body and metastasize and colonize other organs. Overall, the human body is presented as an underwater world, because science, experiments and microscopical images reveal the wonders of an entirely different, miraculous and unknown world.

Loss of cadherin-based cell adhesion and the progression of Invasive Lobular Breast Cancer

Verlies van E-cadherin afhankelijke cel adhesie en de progressie van Invasief Lobulair Borstkanker

(met een samenvatting in het Nederlands)

Proefschrift

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door

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geboren op 3 november 1983 te Amsterdam

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Breast Cancer Epidemiology

As 1 out of 8 women develops breast cancer during her life, breast cancer is the most prevalent type of cancer among women in the Netherlands. In 2011, 14 thousand women and 83 men were diagnosed with invasive breast cancer, while over 3 thousand women and 22 men died from the disease [1]. The average age of women diagnosed with breast cancer is approximately 60 years, while this is 67 for men. Risk factors for breast cancer are: heredity, age, no or low number of kids, no breastfeeding or for a short time, first child birth at high age (over 35), early menarche (first menstruation), late menopause, hormonal substitution, contraceptive pill (during, but not after, using it), being overweight, sedentary lifestyle, dense breasts and alcohol [2,3]. About 5% of the breast cancer cases can be attributed to hereditary mutations in breast cancer gene 1 and 2 (BRCA 1 and 2). Women carrying an inactivating BRCA1 or BRCA2 mutation have a 40-85% higher chance of getting breast cancer [2]. In 2011, the total health care expenditure in the Netherlands was 87 billion euros. In that year 696 million euro was spend on breast cancer patients, 79% of which was used for hospital care, while less than 9% can be attributed to prevention costs [2].

Breast Cancer Treatment

Since 1989 women ranging from 50-70 years of age are invited to be screened for breast cancer by X-ray mammography in the Netherlands. In 1998 the program was expanded and now women between 50 up until 75 are screened for breast cancer. Patients who are diagnosed with breast cancer around 1990, have a 10-year survival of approximately 65%. This incidence increased up to 77% in 2002 [1], which was due to both earlier diagnosis and better treatment regimes. Although breast cancer treatment has clearly progressed, many women still die from this disease and further insight in improved treatment possibilities is needed. Current treatment protocols are based on tumor size, histological grade, hormone receptor status and HER2 (also known as ERBB2 or Neu) gene expression. Over time we have learned that breast tumors are heterogeneous in nature, both on a pathological level and on a molecular level. The current view is that personalized medicine, where targeted therapy focuses on specific signaling cascades, can greatly improve breast cancer treatment in the future. To develop targeted therapies, we have to be able to appoint important, driving signaling cascades within every tumor. Therefore, in depth knowledge about the signaling cascades themselves and about signaling crosstalk is vital for future therapy development.

Breast Structure

The mammary gland is an apocrine gland composed of milk producing lobules which drain into ducts and eventually lead to the nipple. The ducts and lobules are formed by an inner luminal cell layer and an outer myoepithelial (basal) cell layer aligned by a basement membrane (see **Figure 3** later in this introduction). The myoepithelial layer can contract to force secretion of milk that is produced by the alveolar luminal cells. It is currently unknown which of the epithelial cell types within the breast, the luminal or the myoepithelial cells, give rise to breast cancer or certain breast cancer types.

Many if not all organs within the human body harbor stem cells or progenitor cells, which are able to give rise to all or a restricted number of cell types found within that organ. As the female mammary gland continuously cycles between proliferation, differentiation and regression cycles due to the menstrual estrous cycle and lactation after pregnancy, stem cells may have a more pronounced role in the mammary gland during life, compared to stem cells in other tissues. Mouse transplantation studies have shown that the transplantation of a small piece of an adult mammary gland can be transplanted into a recipient mammary gland (cleared fat pad of a 3 week old mouse) and result in a full, normal outgrowth [4]. These findings indicated stem cells resided throughout the mammary gland. As stem cells have a high regenerative capacity, tumors may originate from these stem cells, or differentiated mammary cells may dedifferentiate into a cancerous version of these stem cells. Therefore, identifying and studying the biological nature of the mammary stem cell is of high interest. However, the search for stem cell markers and identifying the cell layer, the luminal or the myoepithelial layer, containing the stem cells proved to be very difficult. Recent, progress in the search for stem cell markers indicates that the mammary gland harbors various kinds of stem and progenitor cells, which reside both in the luminal and in the myoepithelial cell layer (reviewed in [5]).

Breast Cancer Subtypes

It is intriguing that while the mammary gland consists of only two cell types, luminal and myoepithelial cells, breast cancer is a heterogeneous disease leading to many kinds of breast cancer subtypes in different patients. With 80%, invasive ductal carcinoma (IDC) (or not otherwise specified (NOS) is the largest collection of breast cancer subtypes based on histology, while invasive lobular carcinoma (ILC) is the second most common type with 10-15% (**Figure 1**). The final 5-10% of breast carcinomas exists of many rare breast cancer subtypes. Subsequently, IDC and ILC can be further divided into subtypes (ILC subtypes are reviewed in Chapter 2). Interestingly, ILC was proven to be different from IDC not only by histology but on a protein level as well, as genome-wide gene expression profiling showed a distinct ILC-subtype based on hierarchical clustering [6]. In contrast to IDC, ILC often does not form a palpable lump [7], early diagnosis is therefore often difficult. Up until 6 years after diagnosis ILC has been shown to have an advantage over IDC based on their disease-free survival [8]. However, after 10 years the ILC patients had a disadvantage over the IDC patients [8]. While the microscopic study of tissues (histology) is more than a hundred years old, stratifying breast cancer tumors based on gene expression profiles is possible only since 20 years. The scientific field hoped this new technique would enable a breast cancer treatment revolution, where we could design treatments directed against real signaling pathways. While, stratification of breast tumors based on gene expression profiles was achieved [9,10], this method turned out to perform just as well as the histology-based method. Although this was a disappointment, the idea to match targeted treatment with specific breast carcinomas has evolved and matured. Currently, the breast cancer research field hopes to identify active signaling pathways in individual breast carcinomas in order to match them with targeted therapy. This so called personalized medicine will hopefully further improve breast cancer treatment in the future.

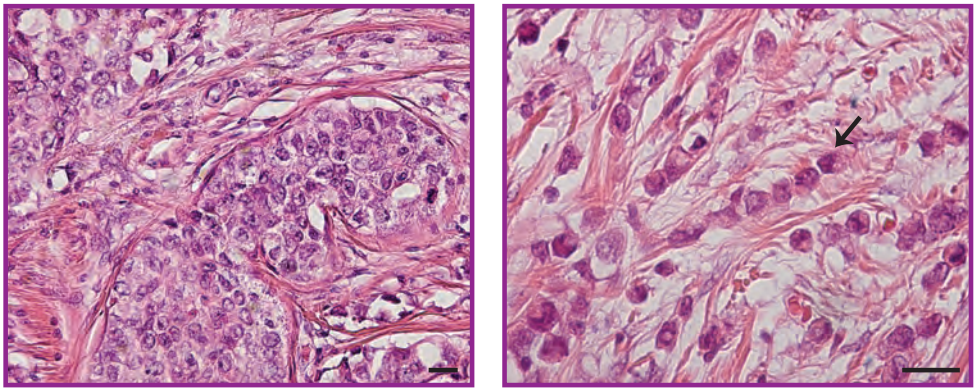


Figure 1. Representative H&E images of human ILC and IDC.
ILC is characterized by a dyscohesive growth pattern of small regular cells, which infiltrate as single cells or as one-layered strands of cells (so called "trabeculae" or "Indian files", see arrow), often in a targetoid pattern around uninvolved ducts. Aside from a more cohesive growth pattern compared to ILC, IDC has no common histological characteristics. Size bar indicates 20 µm.

Invasive Lobular Carcinoma genetics

Genetics are at the basis of signaling cascades within both healthy tissues and tumors. Therefore, genetic studies are important and shed light on the processes present within a tumor. Genetically the majority of breast cancer tumors is characterized by a loss of chromosome arm 16q [11]. However, only in ILC the loss of heterozygosity (LOH) of 16q22.1, which contains the E-cadherin gene *CDH1*, is combined with mutations or methylation of the other *CDH1* allele [12,13]. As a result E-cadherin expression is lost in > 80% of the ILC samples [14], compared to approximately 7% of the IDC patients [15]. ILC is also characterized by a gain at chromosome arm 1q, but it is unknown what gene(s) at this site cause ILC development [16]. Besides frequent somatic mutations, germline E-cadherin mutations have been identified in a minority of lobular breast cancer patients and are associated with bilaterality and familial breast cancer [17-19]. Another strong link between E-cadherin and ILC development was demonstrated by the generation of a mammary specific E-cadherin and p53 knock-out mice, which develop tumors that resembled human ILC [20,21]. Furthermore, somatic *PIK3CA* mutations are found in 33-46% of the ILC patients, compared to approximately 22% of the IDC patients [22]. *PIK3CA* is a gene involved in PI3K signaling and therefore influences survival and proliferation. (A more elaborate review on ILC characteristics can be found in Chapter 2.)

Cell-cell adhesion

Types of cell-cell adhesion and apical-basal polarity

Epithelial cells line the invaginations and surfaces of tissues, thereby forming a protective barrier from the outside world. For this reason the epithelium forms a tight sheet that is supported by different types of cell-cell adhesion complexes; tight junctions (TJ), adherensjunctions (AJ), desmosomes and gap junctions (**Figure 2**). Each junctions has its own specialized

function. The most apical junction, the TJ, functions as a diffusion barrier. AJs are calcium dependent adhesion complexes that connect the cell membrane to the actin cytoskeleton. Desmosomes protect cells from mechanical forces by connecting intermediate filaments to the cell membrane. In epithelial sheets desmosomes and AJs often alternate [23]. Like adherens junctions desmosomes are calcium dependent, but as they mature they become calcium independent and hyper-adhesive, protecting the cell from shearing stresses [24]. Finally, gap junctions, at the most basal side, are needed for intercellular communication by the exchange of ions, metabolites and other small molecules.

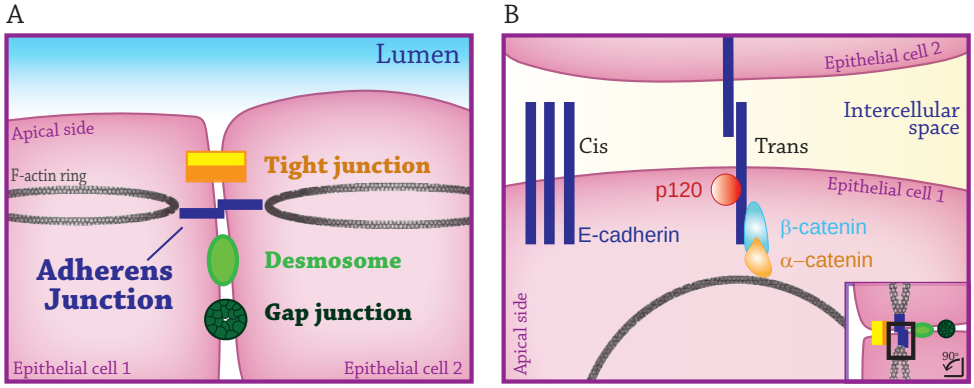


Figure 2. Schematic representation of different cell-cell adhesion structures connecting neighboring breast epithelial cells.

A*. The apical side of the luminal epithelial cells faces the lumen of the duct or lobule. Tight Junctions are the most apical cell-cell adhesion structures on the lateral side of the cell, followed by the Adherens Junctions (AJ), Desmosomes and Gap Junctions. Intracellularly the AJ is connected with the actin cytoskeletal ring made out of actin filaments (F-actin) forming an actin belt or so called zonula adherens.* ***B****. This image zooms in on the AJ, which is comprised of a trans-interaction between two opposing E-cadherin transmembrane molecules (trans-interaction), bound to p120,* β*-catenin and* α*-catenin on the intracellular sides. Subsequently, -catenin is responsible for the connection between the AJ and the actin cytoskeleton, of which the true nature is not clear yet. Both single E-cadherin molecules and full AJ complexes can simultaneously develop cis-interactions between neighboring E-cadherin molecules.*

Assembly of the different types of junctions appears to be interdependent and to follow a certain order. It seems to start with the nectin subfamily, which is part of the AJ and forms Ca^{2+}-independent connections by homo- or heterodimerization either with Nectins or Nectin-like molecules (Necls) on opposing cells (reviewed in [25]). Subsequently, they recruit cadherins, which form an in trans, Ca^{2+}-dependent connection followed by lateral clustering in order to form a mature AJ [26]. Although Nectin trans-dimers are part of the AJ, Nectins and cadherins form independent clusters next to each other rather than mixed clusters [27]. Establishment of the AJ is believed to recruit junctional adhesion molecules (JAMs) on its apical side, which can form in trans homodimers, resulting in a Ca^{2+}-independent junction [28]. Although JAMs function is not fully understood, JAM is known to be essential for proper TJ, but not AJ, formation [29]. The key components of the TJ are occludin and claudin, which are both tetramembrane-spanning proteins. Although occluding has an important role in TJ regulation and function, TJs can also be formed without occludin [27]. TJ formation is Ca^{2+}-dependent and is essential for the establishment of apical-basal polarity andepithelial

barrier function. TJs and desmosomes are dependent on proper AJ formation [30,31]. Premature AJs and TJs are believed to form close to one another and to subsequently separate into mature, discrete junctions [32]. Desmosome and gap junction formation relative to AJ and TJ formation has not been thoroughly investigated yet. It is known that cadherin is needed for desmosome formation, indicating desomosomes depend on AJ formation [33]. However, cell lines mutant for the desmosomal cadherin desmoglein are unable to form desmosomes, but also have a reduced number of AJs showing their interdependence [31,34]. At what exact point of the cell-cell adhesion process gap junctions are formed is unknown.

Cadherin evolution and structure

The cadherin superfamily is comprised of 114 cadherins in humans and based on phylogenetics they can be divided into two major groups, cadherins and related-cadherins [35]. The cadherin family can be further subdivided into eight subfamilies plus the individual members CDH13 (H-cadherin) and CDH26. The type-1 (classical) cadherins comprise one of the eight subfamilies and consists of 5 members, CDH1 (E-cadherin, formerly known as uvomorulin), CDH2 (N-cadherin), CDH3 (P-cadherin), CDH4 (R-cadherin) and CDH15 (M-cadherin) [35]. Myoepithelial breast cells express very minor amounts of E-cadherin and abundant amounts of P-cadherin (**Figure 3**) [36,37]. In contrast, luminal breast epithelial cells only express E-cadherin. R-cadherin is expressed in the breast, but in what cell types has not been determined [38]. N-cadherin expression has been reported in brain, heart and muscle cells and is not expressed in the breast [39]. M-cadherin expression in the breast has not been investigated properly. While mice also harbor these type 1 cadherin members, they are not present in *Drosophila melanogaster* or *Caenorhabditis elegans*, which do have 17 or 12 other cadherin superfamily members respectively [40]. Calcium binding is essential for strong type 1 cadherin based cell-cell adhesion, as it causes a conformational change in the extracellular domain and prevents protease cleavage ([41] and references therein). All type-1 cadherins have five extracellular cadherin (EC) repeats within their N-terminal extracellular tail, a transmembrane domain and a highly conserved p120-catenin (p120) and β-catenin binding site within their intracellular, C-terminal domain (reviewed in [42]). Besides, all type-1 cadherins are able to form calcium dependent cell-cell adhesion structures, but their dynamics and signaling properties can differ. The EC1 domain, at the tip of the N-terminal domain of type-1 cadherins forms strong homotypic interactions in trans, which are stabilized by weak cis interactions with other cadherin molecules [43,44].

Intracellularly type-1 cadherins bind to β-catenin and p120, which are both members of the armadillo protein family. The name 'catenin' is derived from the Latin word 'catena' meaning 'chain', which was chosen as the catenins were anticipated to be involved in the connection between the AJ and the actin cytoskeleton [45]. Indeed β-catenin was found to bind α-catenin, which mediates the link with the actin cytoskeleton [46,47]. β-catenin already associates with E-cadherin within the endoplasmic reticulum, and are transported to the plasma membrane via the Golgi complex [48-50]. p120 and α-catenin join the E-cadherin-β-catenin complex at the cell membrane [48,51]. p120 regulates cadherin recycling (reviewed in [52]) and lateral clustering [53]. Both p120 and β-catenin use their armadillo domain to bind E-cadherin, but are also able to shuttle between the cytosol and the nucleus, where they use their armadillo domain to bind transcription factors. For example, p120 was shown to bind the transcriptional repressor Kaiso, preventing DNA binding and transcriptional repression by Kaiso [54,55]. The nuclear

localization and function of α-catenin has only recently gained some attention. α-catenin has been reported to localize to nuclei of colorectal cells [56], and to influence transcription [57]. Besides, α-catenin was shown to stimulate nuclear actin filament formation also influencing transcription [58].

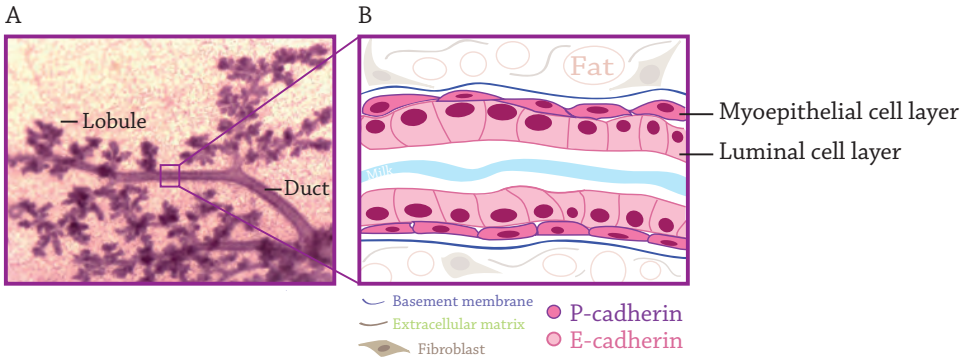


Figure 3. Breast structure.
A*. Ten week old mouse breast stained with carmine (a special dye). Milk is produced in the lobules and transported to the nipple via the ducts.* ***B****. Schematic representation of a mammary duct. The epithelial breast cells are surrounded by a basement membrane and lay within the fat tissue of the breast, where extracellular matrix proteins and fibroblasts can also be found. The outer myoepithelial cell layer can contract and thereby help to transport the milk. The inner luminal cell layer aligns the lumen containing milk or other fluids. While the myoepithelial cells express P-cadherin and maybe very low amounts of E-cadherin, the luminal cells express E-cadherin only.*

Adherens Junction formation and actin dynamics

AJs are so numerous that they form an adhesive belt all around the cell, called the zonula adherens (see **Figure 2**). The AJ is 0.2-0.5 μm in length and at the AJ the cell membranes of the opposing cells are perfectly parallel to each other, containing a space between them of 20 nm [59]. Prior to AJ formation α-catenin monomers bind to E-cadherin-bound β-catenin, while α-catenin homodimers in the cytosol can bind actin filaments [46,60]. Upon cell-cell contact initiation, homotypic trans-interactions are formed between E-cadherin molecules on opposing cells. Meanwhile, α-catenin mediates a connection between the AJ and radial actin fibers sprouting from the cortical actin ring [61]. These punctate focal adherens junctions (FAJ) will subsequently mature into zonula adherens junctions (ZAJ), where perpendicular actin filaments will reorganize to form parallel filaments (relative to the cell membrane) (reviewed in [62]). Several features are indispensable for this process. For example, AJ coupling to the actin cytoskeleton is essential as it reduces E-cadherin mobility within the cell membrane and thereby enables stable lateral clustering [63,64].

Rho, Rac and Cdc42 are the most well studied members of the family of RHO GTPase's, comprising 20 members in total, and are part of the Ras superfamily of small GTPase's. RHO GTPase's are activated at the cell membrane by cell surface receptors including G-protein coupled receptors (GPCRs) and receptor tyrosine kinases. All RHO GTPase's regulate the remodeling of the actin cytoskeleton in their own way. Rho stimulates stress fiber formation and actin contraction by activating Diaphanous-related formins (DRFs) andRho-associated

kinase (Rock). In contrast, Rac inhibits stress fiber and FA formation, and stimulates lamellipodia formation and Arp2/3 mediated branched actin polymerization. The third RHO GTPase Cdc42 stimulates filopodia formation also stimulating both Arp2/3 mediated branched actin polymerization and Dia mediated unbranched actin polymerization (reviewed in [65,66]). RHO GTPase activity is regulated by expression level, GDP or GTP-bound state, phosphorylation state, post-translational modifications and by their localization within the cell. Furthermore, their activity is controlled by different groups of regulatory proteins called guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide exchange inhibitors (GDIs) (reviewed in [65,66]). GEFs enable GTP-loading of a RHO GTPase, while GAPs stimulate the GTPase activity resulting in inactivation. Finally Rho GDIs sequester RHO GTPase's in their GDP-bound form. The high number of regulatory proteins and processes shows the complexity of RHO GTPase regulation.

In case of Rho activation, Rho-GTP will bind and activate its major downstream effectors Rock and mammalian homolog of Drosophila diaphanous (mDia) by releasing their autoinhibition state through a conformational change. mDia is a formin, which is known for actin nucleation properties. By catalyzing actin nucleation and polymerization mDia stimulates unbranched actin filament formation. Rock is a serine/threonine kinase that can phosphorylate various subtrates, including non-muscle myosin II, myosin phosphatase and LIM-kinase (LIMK). Through increased non-muscle myosin II phosphorylation, Rock stimulates actin crosslinking and contraction. Besides, activated LIMK will phosphorylate and inhibit its downstream target cofilin. As cofilin severs actin filaments, cofilin inhibition will promote the stabilization of existing actin filaments. Rock and mDia activity need to be balanced for organized actin bundling and subsequent formation of stress fibers, which are composed of 10-30 actin filaments bundled together by cross-linking proteins like non-muscle myosin II and α-actinin (reviewed in [67]).

The Adherens Junction and the microtubule network

During epithelial polarization not only the actin cytoskeleton but also the microtubules reorganize [68]. Upon polarization, the microtubule organizing centers (the centrosomes) move apically, away from the nucleus and split into separate centrioles. The microtubules that sprouted radially from the centrioles are greatly reduced and new microtubules start sprouting from the zonula adherens in a basal direction. Apart from these minus-end microtubules connected to the AJ, plus-end microtubules ending at the AJ have also been reported [69-71]. Overall, the microtubule network seems to provide an outward-force spreading the cell, while the actin cytoskeleton creates a contracting force going inward (reviewed in [72]). The actin and microtubule network also facilitate vesicles and organelle transport or cell movement. Interestingly, next to the actin cytoskeleton, Rho seems to influence the microtubule network as well. By the activation of mDia, Rho influences microtubule stabilization and stimulates microtubule alignment along actin filaments [73]. On the other hand microtubules can transport Rho and may also affect their activity, which may create a feedback loop (reviewed in [74]). Moreover, microtubule disassembly stimulates Rho signaling through the release of microtubule-bound Rho-GEF, GEF-H1 [75]. Ect2, another Rho GEF, localizes at the AJ in a microtubule-dependent manner where it activates Rho [76]. These findings indicate the actin cytoskeleton and the microtubules can talk to each other through Rho, but also show the current lack of knowledge in this area.How microtubules are connected to the AJ exactly is also not well understood, but both p120 and β-catenin have been shown to form a complex with microtubule binding proteins [69,77-79]. Recently, dynamitin was found to link cytosolic αE-catenin

to a protein called dynactin, which regulates overall microtubule organization and connects cargoes with microtubule motor proteins [79]. Although α-catenin loss does not disrupt cortical microtubule formation [80], α-catenin depletion was found to markedly reduce the presence of microtubules at cell-cell contacts [79,80], and membrane-targeted expression of αE-catenin, but not p120 or β-catenin, stabilizes microtubules [81]. However, experiments using the microtubule stabilizing agent taxol in α-catenin depleted or p120 depleted cells can rescue microtubule reorganization showing that AJ formation stabilizes but is not essential for cortical microtubule organization [82]. Microtubule disassembly by nocodazole treatment has various outcomes, but sometimes causes AJ disruption [70,71,83].

Adherens Junctions and Cancer

The use of various different mouse models over the past years has gained insight in the role of cadherin-based adhesion in epithelial integrity and tumorigenesis. Somatic loss of AJ members turned out to be embryonically lethal and conditional loss is also not tolerated in epithelial cells and will lead to apoptosis. This has been learned from three conditional E-cadherin deletion models, based on the MMTV, K14 or WAP promoter, where E-cadherin loss was found to cause apoptosis leading to a perturbation in alveolar development and a marked reduction in milk production, while none of the mouse models developed mammary tumors [20,21,84]. Besides, mammary specific loss of AJ members α-catenin and p120 also causes apoptosis [85,86], whereas the effect of β-catenin loss has not been investigated *in vivo*. E-cadherin expression is marginal in myoepithelial and abundant in luminal cells in the mammary gland. In contrast, P-cadherin, another classical cadherin, is specifically expressed in myoepithelial (basal) cells (**Figure 3**) [36,37]. The expression pattern of P- and E-cadherin in the skin is slightly different, where both P- and E-cadherin are expressed in the basal cell layer of the skin, while the suprabasal layers only express E-cadherin [36,37,87]. Moreover, E-cadherin loss in the skin evokes an increase in P-cadherin expression in the basal cell layer, securing normally localization of epithelial markers like Par3 and formation of desmosomes and TJs [31,88]. P-cadherin hereby rescues basal cell apoptosis and causes hyperplasia with age. However, silencing of both P- and E-cadherin in the skin does cause apoptosis, hyperthickening (but not hyperproliferation), abnormal Par3 localization and disturbed TJ formation [31]. Together, these mouse studies show that E-cadherin loss is compensated by P-cadherin in the skin, but not in the mammary gland and that loss of cadherin-based adhesion is not tolerated in epithelial tissues. Interestingly, when E-cadherin loss was combined with the loss of a renowned tumor suppressor p53, invasive mammary tumors were induced, which closely resembled human ILC tumors, while loss of p53 alone caused non-invasive mammary tumors [20,21]. The combined loss of E-cadherin and p53 leading to invasive mammary tumors and the loss of E-cadherin in the skin leading to hyperplasia, indicate E-cadherin has an important role in tumorigenesis. Similarly, loss of the AJ member α-catenin in the skin also causes defects in intracellular adhesion and epithelial polarity, resulting in hyperproliferation, suprabasal mitoses, and multinucleated cells [89].The importance of cadherin-based adhesion in human carcinogenesis is outlined by the high number of different carcinoma types associated with E-cadherin loss, including lobular breast cancer (discussed above), pancreatic ductal carcinoma and colorectal cancer (reviewed in [90]). Besides, the loss of E-cadherin expression in these tumors is often correlated with poor prognosis. Aside from the somatic E-cadherin loss, 30% of the hereditary diffuse gastric cancers (HDGC) carry a CDH1 germline mutation [91-93]. Interestingly, a germline mutation in the α-catenin gene, CTNNA1, has also been reported to possibly cause

HDGC recently [94], indicating loss of proper cell-cell adhesion due to α-catenin loss may also be responsible for tumor initiation.

An important cancer related phenomenon associated with proper cell-cell adhesion is 'contact inhibition'. The term "contact inhibition (of cell movement/proliferation)" was originally introduced to denote the phenomenon that when normal cells collide, their locomotion is inhibited [95] and has now been extended to include cell immobilization due to AJ formation. Later the term "contact inhibition of growth/cell division/proliferation" was added to refer to the inhibition of mitosis upon cell-cell adhesion or higher cell density upon cell-cell contact. Both types of contact inhibition are very important during tumorigenesis as both processes seem to become dysfunctional. Unfortunately the mechanisms behind these processes and its relation with cell-cell adhesion is not fully understood yet. Interestingly, the loss of "contact inhibition of proliferation" seems to be a recurrent phenotype upon loss of cell-cell adhesion. E-cadherin is involved in "contact inhibition of proliferation" [96], where the connection of E-cadherin with β-catenin appears to be essential [97]. Depending on the cell type and β-catenin's subcellular localization, β-catenin seems to mediate this phenotype either via the Wnt pathway or the YAP pathway [98,99]. As α-catenin in connected to the AJ via β-catenin, the loss of β-catenin binding also means loss of α-catenin binding. Indeed, α-catenin negativity has also been linked with loss of contact inhibited growth [89]. The mere binding of p120 to E-cadherin was proven not to affect contact inhibition [97-99]. However, complete loss of p120 expression will cause E-cadherin downregulation [100,101], and will therefore definitely have an effect upon contact inhibition [102].

α-catenin

Genetic homologies

α-catenin (formerly known as Cadherin Associated Protein 102 kDa (CAP102) has been discovered in 1989 [45,103]. Although p120 and β-catenin come from the same family, structurally α-catenin is related to vinculin and α-catulin [104]. The α-catenin family consists of three members: αE-catenin, αN-catenin and αT-catenin. Specifics on the different α-catenin homologues can be found in **Table1**. Mouse and human αE-catenin share 99.2% sequence identity [105]. Furthermore, all homologues can give rise to different isoforms. αE-catenin, for example, is known to have 44 splice variants, of which 27 are really protein coding [106]. The homologue expression patterns differ, where αE-catenin is ubiquitously expressed and αT-catenin is mainly expressed in the heart and testis. Interestingly, while αN-catenin expression is confined to the neuronal tissue, αE-catenin and αT-catenin evolved from the αN-catenin gene [104]. Little is known about the functional differences between the α-catenins. αE-catenin's homodimerization affinity is ten times weaker than αN-catenin [107]. Besides, αE-catenin has a higher affinity for actin filaments compared to αN-catenin, and is therefore more effective in the recruitment of vinculin [108]. On the other hand, αN-catenin can translocate to the nucleus, where it is bound and recruited by a transcriptional repressor called ZASC1 [109], αE-catenin was unable to bind this protein. In contrast, αE-catenin can bind Yes-associated protein 1 (YAP1) [110], and whether αN-catenin or αT-catenin can do this too is unknown. Finally, forced expression of αT-catenin in an α-catenin negative colon cell line, showed αT-catenin is able to form AJs together with E-cadherin and β-catenin [111]. However, although cardiomyocytes express both αT-catenin and αE-catenin, in some muscle cell types their localization is not overlapping, which suggests specific functions [112].

α-catenin homologues	Gene	Mol. weight (kDa)	Human		Mouse	
			Sequence identity	Sequence similarity	Sequence identity	Sequence similarity
αE-catenin	CTNNA1	102	100%	100%	100%	100%
αN-catenin	CTNNA2	102	76%	83%	77%	83%
αT-catenin	CTNNA3	100	59%	74%	56%	74%
α-catulin	CTNNAL1	82	21%	34%	25%	34%
Vinculin	VCL	124	18%	31%	23%	31%

Table 1. Specifications of human α-catenin homologues.
Indicated sequence identity and similarity are relative to αE-catenin [113,114].

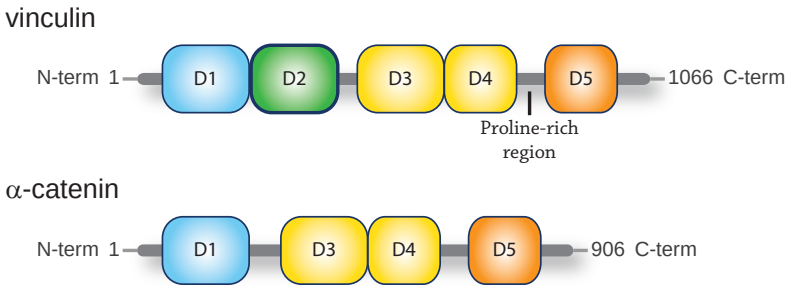
Structure, binding partners and mechanosensing

Human and mouse αE-catenin is 906 amino acids long and as it is a vinculin homologue it harbors three regions with strong vinculin homology, VH1-3 [115]. Since the crystallization of vinculin in 2004, which showed that vinculin is comprised of eight four-αhelix bundles [116], vinculin has been divided in so called D-domains, D1-D5. The overall structure of αE-catenin is very similar albeit it lacks the D2 domain and the proline-rich region between D4 and D5 (**Figure 4**) [117,118]. While the D2 domain has no known obvious function, vinculin binds several proteins like vinexin, ponsin, VASP and Arp2/3 via this proline-rich region (reviewed in [119]). Many proteins involved in adhesion have been shown to have an 'open' and 'closed' conformation, which includes αE-catenin, vinculin, Rock, diaphanous (Dia) proteins and some integrins ([108,120-122] and reviewed in [123]). Besides, vinculin and αE-catenin are involved in mechanosensing [120,124]. Mechanotransduction is a process in which a mechanic stimulus is converted into a chemical, cellular response. The closed conformation of αE-catenin for example, is unfurled by tension changing its conformation and revealing protein binding domains that affect intracellular signaling [122,124]. Vinculin is comprised of a large, N-terminal head domain, while domain D5 at the C-terminus forms the tail [116]. In the closed conformation vinculin has a very tight head-to-tail binding, which (partly) occludes binding to αE-catenin with D1-D3, and F-actin or phosphatidylinositol-4,5-bisphosphate (PIP_2) with D5 [116,122]. Although αE-catenin also has an open and closed conformation, it does not have a head-to-tail construction. Instead, binding of N-terminal D1 to β-catenin and C-terminal D5 to F-actin are never constricted by loss of tension, while the middle part, D3 and D4, is unfurled by tension enabling binding to vinculin [108,125]. Surprisingly, direct and simultaneous binding of αE-catenin to β-catenin and F-actin, in an *in vitro* reaction mixture, proved to be difficult and could only be demonstrated upon the addition of the actin binding protein Eplin [46,47,126]. A recent publication by Buckley et al., has shown that the AJ dissociates quickly from the actin cytoskeleton in the absence of tension, while forces between 5-10 pN most optimally increased the lifetime of the bondage between the AJ and the actin cytoskeleton [127]. These insights lead to the following model for AJ formation and concurrent actin cytoskeletal dynamics: Before AJ formation αE-catenin forms homodimers within the cytosol. However, upon AJ assembly αE-catenin heterodimerizes with E-cadherin-bound β-catenin [107], which cannot occur simultaneously with homodimerization as the homo- and heterodomerization

domains overlap. Unlike vinculin, αE-catenin will be able to bind filamentous actin (F-actin) in its closed conformation [108]. Only upon myosin II induced tension will αE-catenin unfurl into its open, activated state and will the dissociation constant decrease enabling a direct connection between the AJ and the actin cytoskeleton. The open, unfurled state of αE-catenin recruits and activates vinculin at the AJ and enables vinculin homodimerization, F-actin crosslinking and strengthening of the AJ [122,128-131].

Actin remodeling during AJ formation and the role of αE-catenin

Once AJs form a connection with the actin cytoskeleton they are called primal, punctate AJs, or focal adhesion complexes (FAJs). These AJs have unidirectional actin bundles, where the barbed-end is facing the cell membrane. Subsequently, they can mature by the development of mixed-polarity actin bundles allowing lateral AJ clustering [140]. Next, radial F-actin bundles are reorganized into parallel oriented F-actin bundles (relative to the cell membrane) forming a circumferential actin belt. Together these zonula AJs (ZAJs) form an apical belt, called the zonula adherens, and will lead to epithelial polarization [62,141]. Tension created by the motor



Domain (aa):	α-catenin binding partners:	Function:
1-290	MgcRacGAP	Part of the centralspindlin complex
57-264	β-catenin	AJ member
82-279	α-catenin (homodimerization)	AJ member, F-actin binding protein
117-161	DLC1	RhoGAP
300-500	Formin	Nucleates unbranched actin polymerization
325-394	α-actinin	F-actin binding protein
326-509	Vinculin	F-actin binding protein
509-906	Afadin	F-actin binding protein
668-906	F-actin	Cytoskeleton of the cell
691-848	ZO-1	F-actin binding protein
unknown	Eplin	F-actin binding protein
unknown	ARHGAP10	RhoA and CDC42 GAP
unknown	Merlin	Binds TJ-associated polarity protein Par3
unknown	Ajuba	F-actin binding protein

Figure 4. Schematic representation of the structural composition of α-catenin and vinculin.
The vinculin protein can be divided into 5 domains (D1-D5) and it contains a proline-rich region between domain 4 and 5. The α-catenin protein resembles vinculin, but lacks domain 2 and the proline-rich region. The currently known α-catenin binding partners are depicted below, with the responsible α-catenin binding domain on the left side and their main function on the right side [32,62,76,107,132-139].

protein non-muscle myosin II is essential for this AJ maturation process [82,142,143]. Experiments using an E-cadherin tension sensor showed the E-cadherin complex can already be stretched by actin cytoskeletal contraction before a homotypic interaction is initiated [144]. αE-catenin is essential for this relay of tension by the actin cytoskeleton onto the E-cadherin molecule, as αE-catenin depletion caused E-cadherin intramolecular tension to abrogate [144].

How the actin cytoskeleton can become multi-directional and reorient their direction to establish a connection between the AJ and the cortical actin ring is not well understood ([23,145] and reviewed in [62]). Interestingly, fibroblasts can form punctate AJ structures as well, but they lack cortical actin filaments and are unable to form an adhesion belt with mature AJs [142]. Instead their actin cables sprouting from the AJ are all directed towards the cell nucleus [145]. As Eplin is highly expressed in epithelial cells and is required for the transformation of radial into circumferential F-actin as well as the associated AJ maturation [126,146,147], Eplin could play an important role in the actin cytoskeletal reorganization needed for epithelial polarization.

As the connector between the actin cytoskeleton and the AJ, αE-catenin has an important role in AJ maturation. Next to this connector role αE-catenin also bundles actin filaments and recruits formin-1, which nucleates the polymerization of unbranched actin cables [114]. In the absence of αE-catenin, cortical actin filaments and E-cadherin homotypic interactions are still formed, but the actin cytoskeleton can no longer be attached to the AJ [23]. As this attachment reinforces E-cadherin cis-interactions and is needed for stable cluster formation [64], loss of αE-catenin decreases E-cadherin clustering and stability at cell-cell contact sites decreasing the lifetime and the tensile strength of the junction [143,148].

The role of cytosolic αE-catenin

Next to being part of the AJ, αE-catenin also resides within the cytosol as a monomer, homodimer or heterodimer with β-catenin. The role of cytosolic αE-catenin is still largely unknown. Deletion of the first 57 amino acids from αE-catenin enhances dimerization and inhibits AJ formation [149]. Sequestering of αE-catenin dimers to different locations within the cell showed that it can influence actin dynamics without affecting cell-cell adhesion, proving that αE-catenin dimers can regulate actin dynamics independently of cell-cell adhesion [150,151]. The αE-catenin dimer has a higher affinity for F-actin than the monomer. Furthermore, the dimer inhibits Arp2/3 mediated branched actin filament nucleation and actin depolymerization by cofilin [46,60,150,152]. While, αE-catenin bound to the AJ does not inhibit Arp2/3 very well, αE-catenin does recruit Eplin which can also inhibit Arp2/3 [153]. Interestingly, the ratio of αE-catenin monomers, homodimers or heterodimers is actin polymerization independent [150].

αE-catenin and Cancer

Loss of αE-catenin expression is a prognostic factor for poor survival of cancer patients (reviewed in [154]). Furthermore, various human αE-catenin negative cell lines have been discovered; HCT-8, MDA-MB-330, MDA-MB-468, OV2008, PC3 and PC9 [155-160]. However, the presence of αE-catenin mutations in primary tumors has not been analyzed and there is only one study reporting an αE-catenin germline mutation in a Dutch family, where it may be responsible for hereditary diffuse gastric cancer [94]. Relative to the studies concerning the

role of αE-catenin in humans, the experiments in mice are more numerous. For instance, conditional ablation of αE-catenin in the skin causes a thick, unorganized epidermis with frequent and significantly higher percentages of aberrant mitotic figures, a condition reported to resemble squamous cell carcinoma *in situ* in humans [89]. αE-catenin$^{-/-}$ keratinocytes derived from this K14-Cre; CTNNA1$^{fl/fl}$ mouse are not contact inhibited *in vitro*, while their wild-type counterparts do show contact inhibition of proliferation [89,110]. In line with these results, αE-catenin depletion in the cerebral cortex (Nestin-Cre; CTNNA1$^{fl/fl}$) caused severe dysplasia and hyperplasia, forming invasive tumor-like masses, causing these mice only to survive for 2 or 3 weeks [161]. Furthermore, inducing the expression of wild-type αE-catenin in a couple of the human αE-catenin negative cell lines reduced tumorigenic potential *in vivo,* when subcutaneously injected into mice [157,162]. These findings show αE-catenin is clearly involved in tumorigenesis. In contrast, its role in the breast is less well studied. Remarkably, conditional ablation of αE-catenin in the mammary induces cell death instead of tumorigenesis [85]. This is not surprising as it is in line with studies showing that the conditional ablation of E-cadherin in the mammary also causes cell death [84]. However, in the skin single E-cadherin ablation causes increased P-cadherin levels rescuing cell-cell adhesion [88], and double E- and P-cadherin ablation causes epidermal hyperthickening. This phenotype was similar to the epidermal phenotype seen in αE-catenin ablated mice, although proliferation defects and an inflammatory influx were absent in the double cadherin ablated skin [31]. In contrast to single deletion of E-cadherin in the mammary, combined loss of E-cadherin and the tumor suppressor p53 in the mammary gland induces invasive mammary tumors and shows E-cadherin is a recessive tumor suppressor in the mammary gland [20,21]. These results indicate that although single ablation of αE-catenin in the mammary gland causes cell death, αE-catenin may function as a recessive tumor suppressor in the mammary gland just like E-cadherin.

Yes-associated protein

YAP structure and isoforms

Yes-associated protein (YAP) is a transcriptional coactivator, which was first identified in 1994 [163]. It contains a WW domain (a sequence motif containing two conserved and consistently-positioned tryptophan (W) residues), which it uses to bind PPXY motifs. As there are many PPXY motif containing transcription factors, there are many putative YAP binding partners (reviewed in [164]). Interestingly, the most renowned binding partner of YAP is the TEAD family of transcription factors, which does not contain a PPXY motif. Recently many Yap isoforms have been identified. All mammals, including mice and humans, seem to express a YAP paralog called transcriptional coactivator with PDZ-binding motif (TAZ) [165]. Moreover, in humans two YAP isoforms can be detected, named YAP1 and YAP2, whichcontain one or two WW-domains respectively, while in mice only one YAP isoform has been described (YAP65) containing two WW-domains [166]. Furthermore, analyzing the YAP cDNA yielded the description of 4 putative YAP1 and 4 putative YAP2 isoforms [167]. Currently, little is known about the functional differences between these different YAP isoforms.

YAP regulation

Since YAP and its orthologue Yorkie in Drosophila, were found to be under the control of the Hippo kinase pathway regulating organ size, the interest in the functions of YAP has grown [168]. The Hippo kinase cascade inhibits YAP by phosphorylation, which enables binding to the scaffolding protein 14-3-3 and sequesters YAP within the cytosol [169]. Succeeding studies showed temporal overexpression of YAP within mouse liver caused a significant increase in organ size due to increased proliferation, without affecting the overall organ structure [169,170]. Furthermore, cessation of YAP expression caused apoptosis and showed the YAP-induced increase in organ size to be reversible. However, continued overexpression of YAP eventually lead to tumorigenesis disrupting the natural organization of the organ. In contrast to YAP overexpression in the liver, YAP overexpression in the intestine lead to severe hyperplasia and dysplasia within a few days, showing that the effects of YAP signaling to be organ specific [170]. YAP can be regulated by different mechanisms, including phosphorylation, degradation and sequestration by protein binding. YAP contains at least five HXRXXS motifs that can be phosphorylated by various kinases, including Lats, Mst, Jnk, CK1δ and Akt [166,171,172]. The scaffolding proteins Angiomotin, angiomotin-like (amotl) protein and 14-3-3 can directly bind and inhibit YAP by sequestering YAP within the cytosol [110,173-176]. Lats phosphorylation of YAP will promote phosphorylation by CK1δ and subsequent degradation by an E3 ubiquitin ligase [172]. The fact that Lats can phosphorylate Angiomotin as well, and stimulate Angiomotin - 14-3-3 binding and cytosolic sequestration of YAP, adds to the complexity of YAP regulation [177].

Link between YAP and αE-catenin

αE-catenin, YAP and the actin cytoskeleton seem to be interrelated. For instance, both αE-catenin and YAP are associated with mechanosensing/actin remodeling. As role of αE-catenin in mechanosensing has been discussed before and relates to the fact that αE-catenin unfurls upon tension. Besides, YAP signaling is influenced by tension as culturing cells on stiff matrices or on large surfaces causes nuclear translocation of YAP and can be prevented by Rho, Rock or actin polymerization inhibitor treatment [178-181]. Interestingly, F-actin and YAP compete for binding to Angiomotin/amotl, creating a link between actin dynamics and YAP regulation [182]. Furthermore, YAP overexpression in mouse skin caused hyperproliferation and thickening, which resembles the phenotype of the αE-catenin knockout mouse model and suggests a common downstream responsible signaling pathway [89,176]. Remarkably, epidermal or cerebellar specific, αE-catenin depleted conditional mouse models show prominent nuclear expression of YAP [110]. αE-catenin binds phosphorylated YAP via the scaffolding protein called 14-3-3 and sequesters it within the cytosol [89,176]. Interestingly, YAP mutation experiments in αE-catenin depleted keratinocytes, showed the interaction between YAP and the TEAD transcription factor family is responsible for the loss of contact inhibition of proliferation. These findings indicate that αE-catenin acts as a tumor suppressor in the skin and cerebellum by suppressing YAP signaling via the TEAD family of transcription factors. Whether αE-catenin is a tumor suppressor in the mammary as well and effectuates this by suppression of YAP signaling remains to be addressed.

Thesis outline

This thesis focuses on different aspects of the lobular breast cancer subtype. As the group of lobular patients is smaller compared to the ductal group of breast cancer patients, the number of lobular studies is relatively small. Despite the fact that lobular tumors are recognized as a different subtype with different genetic and molecular characteristics, lobular breast cancer patients are not treated differently compared to ductal breast cancer patients, as specific lobular treatment regimens have not been developed yet. Therefore, more knowledge on this subject could enable their development. Finally, the genetic and molecular status of lobular tumors seems to be more uniform within the lobular compared to the ductal breast cancer group, which might make the new therapies beneficial to a relatively large part of the lobular patients.

In **Chapter 2** we expatiate on the pathological characteristics like morphological subtypes, molecular markers, incidence and prognosis of lobular carcinoma. We also describe the genetic background, the defining biological signaling cascades and current and possible future treatment options for lobular carcinoma.

In **Chapter 3** we identify methylation-based biomarkers specific for pleomorphic invasive lobular carcinoma (ILC). We also compare global methylation profiles between invasive ductal carcinoma (IDC), classic and pleomorphic ILC.

In **Chapter 4** we address the role of α-catenin in breast tumorigenesis. We describe the effect of α-catenin loss on AJ formation and cell morphology and address its function on actin cytoskeletal contraction and anoikis resistance.

In **Chapter 5** we assess the expression of Yes-associated protein (YAP) in ILC patient samples and human and mouse breast cancer cell lines.

In **Chapter 6** we compare YAP expression patterns between lobular carcinoma *in situ* (LCIS), ductal carcinoma *in situ* (DCIS), ILC and IDC. We also investigate the expression patterns in matched LCIS and ILC samples, and also for DCIS and IDC samples taken from the same patient.

In **Chapter 7** we highlight the general conclusions and we discuss how Rho/Rock signaling can be activated in ILC and if this can cause YAP activation. Finally, we will also discuss Rho/Rock inhibitor-based therapeutic intervention opportunities for the treatment of ILC.

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Chapter 2

Lobular breast bancer: pathology, biology, and options for clinical intervention

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Abstract

Lobular carcinoma is a breast cancer subtype comprising approximately 15% of all breast cancers cases. Clinical diagnosis of this subtype is difficult due to a characteristic growth pattern that inhibits detection using palpation or standard X-ray mammography. While clinical intervention based on hormone antagonists has proven an effective strategy, hormone receptor negative or nonresponsive disease cannot be treated successfully, indicating the need for alternative curative approaches. In contrast to its well-defined histopathological characteristics that were first recognized a century ago, the surface of the underlying biology has only recently been scratched. Progress was made in understanding the biology of the disease, which will hopefully have its impact on future treatment modalities and initiate development of novel intervention strategies. Here, we review the pathological and molecular features of lobular breast cancer and report on the currently known mechanisms that control disease development and progression. Finally we will reflect on past, present and future treatment options.

Keywords: lobular breast cancer, pathological features, clinical features, molecular features

Pathology and Molecular Features of Lobular Breast Cancer

Terminology and History

World-wide, breast cancer causes approximately half a million deaths annually [1]. Lobular carcinoma accounts for approximately 10–15% of all breast cancer cases, thereby representing the second most common type of breast cancer [2,3]. At the beginning of the 19th century, the general opinion was that all breast tumors were ductal carcinomas [4]. In 1932, Broders was the first to define a non-invasive variant termed lobular "carcinoma *in situ*" (LCIS) [5]. The term lobular neoplasia (LN) was subsequently introduced by Haagensen et al. [6], who used this terminology to cover the whole non-invasive spectrum of the disease; from atypical lobular hyperplasia (ALH) to LCIS. LN was described not only to be associated with invasive lobular breast cancer, but also as a risk factor for subsequent development of invasive cancer [7-9]. This hypothesis was subsequently supported by molecular evidence indicating that LCIS is a precursor of invasive lobular carcinoma (ILC), which will be discussed later in this review. Also columnar cell lesions can be precursor lesions of ILC [10], but they fall beyond the scope of the current review (for review see [11]).

Lobular Breast Cancer Subtypes

Morphological Subtypes

Lobular breast cancer is characterized by a noncohesive growth pattern and can be grouped in non-invasive and invasive subtypes (**Fig. 1A and B**, respectively). In 1941, Foote and Stewart defined LCIS as a population of small monomorphic cells filling and distending from the terminal ducto-lobular unit spreading in a Pagetoid manner through the ductal system [12]. ALH resembles LCIS, but ALH lesions do not completely solidify the acini within the lobular structures of the mammary gland [13]. In view of the problems when differentiating between ALH and LCIS and the wide overlap in molecular features, the noninvasive forms of lobular cancer are currently grouped as LN. Although usually located in the lobule, cells may spread to the ductal system, denoted "Pagetoid spread" [7,14,15]. Very often, LN is diffusely dispersed in the affected breast. High grade variants are pleomorphic LCIS defined by atypical nuclei and occasional apocrine differentiation, and the macroacinar (sometimes denoted "florid") type with hugely distended acini that tend to become necrotic in the center because of severe hypoxia, and may contain micro calcifications (**Fig. 1A**) [16]. These high grade forms of LCIS are more difficult to discern from ductal carcinoma *in situ* (DCIS) and tend to behave more as DCIS in the sense that they usually form localized disease, which can be treated surgically. Alternatively, LN has been divided into three progressive groups based on grading; LIN1, LIN2 and LIN3 [17]. LIN1 is comparable to ALH, LIN2 to classic (low grade) LCIS, and LIN3 to pleomorphic, macroacinar or pure signet ring cell LCIS. While LN lesions are always surrounded by a myoepithelial cell layer and an intact basement membrane, invasive tumors penetrate the basement membrane and invade into the surrounding stroma. Moreover a rare type called microinvasive lobular carcinoma has been reported. In these tumors LN lesions are present with an intact basement membrane combined with microivasive foci <1mm that have penetrated the basement membrane [18,19]. Classic ILC is characterized by small regular cells, frequently containing cytoplasmic vacuoles (which may induce "signet cell" formation), with small rounded nuclei with no or small nucleoli. The tumor cells grow dysco-

hesively and infiltrate as single cells or as one-layered strands of cells (so called "trabeculae" or "Indian files"), often in a targetoid pattern around uninvolved ducts (**Fig. 1B**). The mitotic rate is usually low and there are few apoptotic cells.

Different ILC variants have been described. First, the alveolar and solid subtypes, which show a different architecture, but both harbor a classic lobular cytonuclear appearance. The alveolar variant has an architectural pattern of round, balloon-like aggregates of 20 cells or more, surrounded by thin sheets of intervening stroma [20]. In contrast, solid ILC is characterized by large solid masses of lobular carcinoma cells with little intervening stroma (**Fig. 1B**, top row) [21]. Second, the pleomorphic (**Fig. 1B,** top row). The pleomorphic variant represents approximately 10% of the lobular lesions and displays polygonal cells with highly atypical nuclei and harbors more frequent mitoses [16]. Some ILC variants show histiocytic, myoid or apocrine differentiation, the latter marked by eosinophilic cytoplasm (**Fig. 1B**, bottom row).

Immunophenotype

Historically, microscopical examination of fixed and embedded tissue was one of the first tools of classifying breast tumors. The main purpose of classifying breast tumors was to be able to predict patient prognosis. Today markers like hormone receptors and Her2/Neu (ERBB2) are used to predict patient outcome and treatment response. The vast majority of ILC shows estrogen and/or progesterone receptor expression, while lacking expression of ERBB2 [2,22]. In contrast, 35–81% of pleomorphic ILC expresses ERBB2 [23,24]. Compared to classical ILC, pleomorphic ILC is significantly more often p53-positive [23-25], and 46–100% shows aprocrine differentiation based on expression of the apocrine differentiation marker gross cystic disease fluid protein 15 [23,24,26]. There is very frequent loss of E-cadherin expression and abnormal subcellular localization of p120-catenin (p120) in all ILC subtypes [27,28]. (Expression and loss of function of the cadherin-catenin complex will be discussed in part two of this review.) ILC lacks expression of basal markers like cytokeratin (CK)5 and CK14, but expresses the luminal epithelial markers CK8 and CK18 [29,30]. ILC in general does not show overexpression of the epidermal growth factor receptor (EGFR) [2,16].

Molecular subtypes

More than a century later, clinicians are still trying to improve patient outcome and treatment response predictions by subgrouping the breast cancer patients. Approximately ten years ago, Perou and coworkers [31] proposed a classification system based on RNA expression profiling. Unsupervised cluster analysis pointed to 5 novel sub-types of breast cancer: Luminal A, Luminal B, Basal-like, Her2/Neu and Normal [32]. In this context, the majority of ILC tumors were considered Luminal A-type lesions, based on the absence of basal expression RNA markers (e.g. ERBB2) and hormone receptor presence [33]. Because pleomorphic and apocrine lobular carcinomas tend to be ERBB2-positive, they were grouped in the "HER2" or "Luminal B" subgroups, depending on their hormone receptor status [20,33]. Pleomorphic tumors with a basal classification are rare, but have also been reported [24]. Recently, the RNA expression profiling was combined with copy number aberration analysis to define DNA-RNA paired profiles, which revealed ten new breast cancer subgroups [34]. The subgroups were correlated with differences in survival, but it is still unclear whether these new subgroups will enable better prognosis or treatment strategies. In order to improve treatment efficiency, we will have to extend our knowledge beyond the past profiling studies and reach through to the underlying biology.

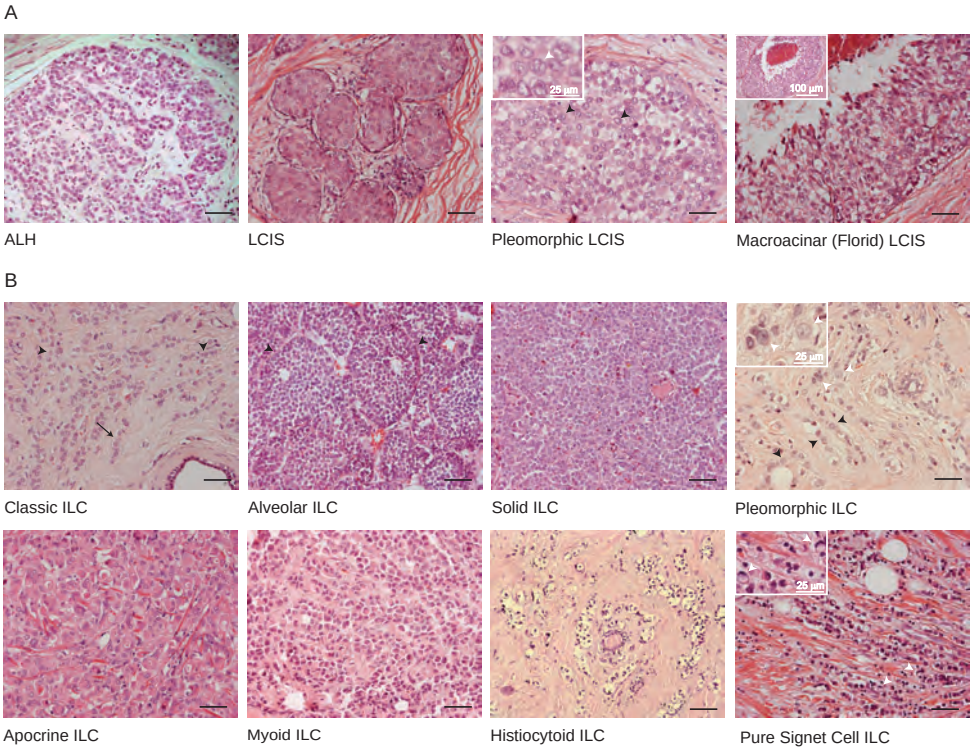


Figure 1. Lobular breast cancer subtypes based on histology.

A. Lobular neoplasia (LN).

H&E stainings showing different types of LN. Atypical lobular hyperplasia (ALH), presenting incomplete filling of the acini characterizes ALH (left panel). LCIS characterized by complete filling of the acini (middle panel). A case of pleomorphic LCIS, which is characterized by nuclear atypia (arrowheads, right panel). Inset shows an enlargement of the area indicated by the white arrow.

B. Invasive lobular carcinoma (ILC).

Shown are H&E stainings of representative examples of classic ILC (top left panel), which is characterized by tumor cells growing individually or in trabecular growth patterns, 'Indian files' (arrowheads) distributed around unaffected ducts in a targetoid arrangement (arrow). In alveolar ILC the growth pattern resembles the preceding lobular architecture (arrowheads, top middle panel). Solid ILC, a fairly compact mass of cells with little intervening stroma (top right panel). Pleomorphic ILC is also characterized by nuclear atypia (arrowheads, middle left panel). Inset shows an enlargement of the area indicated by the white arrows. Also shown are ILC variants with apocrine or myoid differentiation (middle, middle and right panel). Histiocytoid ILC is characterized by abundant, vacuolated cytoplasms (bottom, left panel). Central necrosis present in structures with a lobular growth pattern are called macroacinar (florid) ILC (bottom, middle panel). Inset shows an overview of the area. Signet cells resemble signet rings as the nucleus is pushed to the side by a large cytoplasmic vacuole (bottom, right panel). Inset shows an enlarged area with typical examples of signet cells indicated by the white arrows.

Size bars on the lower right indicate 50 µm.

Clinical Aspects

Diagnosis

LN is usually too small and scattered to be detected as a palpable lesion, and presents a challenge for detection by radiologists. Detection limitations and symptomless disease progression renders LN difficult to diagnose and estimate disease incidence. Only occasionally, LCIS is accompanied by micro calcifications [35-38], especially the macroacinar type. Micro calcifications are mostly observed around areas of sclerosing adenosis and apocrine metaplasia juxtaposed to tumor areas and they facilitate diagnosis of LN by radiology. Research indicated that core-needle biopsies can underestimate breast cancer risk, as surgical excision biopsies from women diagnosed with LN by core-needle biopsies, showed invasive disease in 14% of the cases [2]. As a consequence it was concluded that surgical biopsy should be considered to determine the presence of ILC. As for LN, the diagnosis of ILC using physical examination, mammography, sonography, MRI and PET scanning is also often difficult due to the fact that also ILC often does not form a palpable lump [39-43]. While MRI is regarded as the most sensitive technique in the detection of ILC, a recent study concluded that there was no statistically significant difference between the sensitivity of mammography, sonography, MRI, and breast-specific gamma imaging [44].

Incidence

LCIS is found in about 5% of all breast cancer excision specimens, whereas ILC comprises approximately 15% of all breast cancers [2,45,46]. The incidence of LCIS and ILC appear to have increased in the last two decades [45,47], an increase likely due to the improvement of screening methods, together with the increased awareness of the pathologists about this lesion [48,49]. Furthermore, it was suggested that the increase in ILC may be linked to usage of post-menopausal hormone replacement therapy [50]. Incidence of the ILC subtypes is as follows: 57% classic, 19% alveolar, 11% solid, and 13% pleomorphic, signet ring cell, histiocytoid, or apocrine ILC tumors [51].

Prognosis

Lobular neoplasia. The relative risk to develop subsequent invasive cancer was originally estimated to be 4–5-fold for ALH and 8–10-fold for LCIS. The relative risks of the ductal variants, ADH and DCIS are in the same order [52]. More than 50% of women diagnosed with LCIS present multifocal tumor development in the ipsilateral breast. Surprisingly and specific for lobular breast cancer, 30% of patients with ipsilateral involvement also develop LCIS in the contralateral breast [2,53]. The latter case is still a point of debate since there are conflicting reports on the risk of contralateral breast cancer associated with LCIS [54,55]. Despite these differences, both studies showed that a large proportion of women diagnosed with LCIS developed ILC later in their lifetime. In view of the above described multifocality and high rate of ipsilateral invasive recurrences and contralateral disease, the proper surgical treatment for LN would be bilateral mastectomy, but this is generally considered over treatment as only few patients develop ILC. Unfortunately, there are relatively few validated prognostic factors for LN. Bodian et al. [56] stressed the importance of age at diagnosis of LCIS and family history as reliable prognostic factors. These factors appeared to reflect poor prognosis for this

malignancy [56]. Both of these factors were previously reported by others as well [6]. Furthermore, the number of lobules with LCIS within a specimen and nuclear size significantly relate with recurrence of the disease [57].

Invasive lobular cancer. Reported comparisons between the prognoses of ILC versus Invasive ductal carcinoma (IDC) are inconsistent; while some studies report that ILC has a better prognosis [58], others conclude that there is no difference [59] or even that ILC has a worse prognosis [60,61]. A common drawback of these studies however is the fact that small groups of patients were included. A study on a large cohort of breast cancers (4140 ILC cases versus 45169 IDC cases) showed no prognostic difference between these two biologically different subtypes (five year survival of 86% and 84%, respectively), while the metastatic spectrum did differ in ILC compared to IDC [2]. Interestingly, another study on a large cohort of patients (767 ILC cases versus 8607 IDC cases) showed that up until 6 years of disease free survival the ILC cohort had an advantage over the IDC cohort, however after ten years the ILC cohort had a disadvantage [62]. This indicates that the long-term prognosis of ILC patients is worse compared to IDC patients. This is illustrated by a case report of a ILC patient diagnosed at the age of 41, who presented with recurrence 40 years later [63]. Pleomorphic ILC has been reported to be significantly larger then classic ILC tumors[64]. Besides the lymph nodes of pleomorphic ILC patients are more often positive and significantly more patients presented with metastatic disease[64]. Moreover, recurrence in pleomorphic ILC patients is significantly worse compared with classical ILC patients [65]. Overall this indicates that pleomorphic ILC is a more aggressive form compared to classical ILC. Nothing is known about prognosis of the rare type of lobular carcinoma called microinvasive carcinoma, but in general no lymph node metastases are found in these patients indicating this rare subtype is probably not very aggressive[18,19].

Lobular Breast Cancer Biology

Genetic Features

Genome wide gene expression profiling indicated that ILC forms a distinct subtype based on hierarchical clustering [33]. ILC displayed downregulation of genes involved in actin cytoskeleton remodeling, ubiquitin conjugation, DNA repair, cell adhesion and transforming growth factor (TGF)-β signaling. Among the up-regulated genes were those implicated in transcription regulation of immediate early genes, lipid/prostaglandin biosynthesis, and cell migration. Furthermore, comparative genomic hybridization (CGH) showed that IDC carries a higher frequency of copy number alterations compared to ILC [66,67].

Most lobular carcinomas are E-cadherin negative, which is often due to inactivating mutations and subsequent loss of heterozygosity (LOH) of the unaffected *CDH1* allele, which is located on 16q22.1[68]. As a consequence, expression of E-cadherin can be used to efficiently differentially diagnose ILC versus IDC, since IDC mostly retain expression [69]. Apart from genetic alterations in *CDH1* at chromosome arm 16q, ILC is characterized by a gain at chromosome arm 1q. Unfortunately it is unknown which genes at 1q are causal to ILC pathology [70]. Another hallmark of ILC is the presence of somatic *PIK3CA* mutations, which are found in 33–46% of ILC. In contrast IDC shows *PIK3CA* mutations in approximately 22%. *PIK3CA* encodes for a catalytic subunit called p110α, which together with the p85 subunit forms a lipid kinase called phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K).

PIK3CA mutations are generally positioned in the interface of p110α and p85 (Huang et al. 2008). PI3K converts phosphatidylinositol (4,5)-diphosphate (PIP-2) into phosphatidylinositol (3,4,5)-triphosphate (PIP-3), which is a prominent substrate for the protein kinase B/AKT1 pathway [71]. The *PIK3CA* mutation spectrum seems to vary between breast cancer subtypes [72], the majority of mutations in ILC are positioned in exon 9 (helical domain) and exon 20 (the kinase domain) [73]. Besides mutations in *PIK3CA,* many other genes in the PI3K signaling pathway were found mutated in breast cancer, like phosphatase and tensin homolog tumor suppressor gene (*PTEN*), nuclear factor (*NF-κB*), v-akt murine thymoma viral oncogene homolog 1 (*AKT1)*, insulin receptor substrates and PIK3 regulatory subunits (Kalinsky et al. 2011; Wood et al. 2007). *PTEN* truncating mutations are found in around 2% of the ILC tumors [74]. Another gene often affected in breast cancer is *ERBB2,* which encodes for a protein-tyrosine kinase receptor and has high homology with the EGFR. V-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (*ERBB2)* amplification can be found in 13% of the lobular and in 46% of the ductal tumors, and is associated with poor prognosis [75]. Activating somatic *ERBB2* mutations occur more often in ILC compared to IDC [72]. Finally, tumor protein p53 (*TP53)* is mutated in about 19% of the ductal breast cancer samples [76,77], while somatic *TP53* mutations are a rare event in classical ILC (approximately 6%) (Oliveira et al. 2002). However, the incidence in pleomorphic ILC may be as high as 46% [78,79].

Reports on hereditary genetic predispositions in lobular carcinoma patients indicated there might be a slightly higher family incidence of lobular cancer [80,81]. As the frequency of somatic E-cadherin mutations in lobular carcinoma is so high, *CDH1* would be a plausible candidate for this hereditary risk. Indeed, E-cadherin germline mutations were observed in lobular breast cancer patients albeit with a low frequency [82-84]. E-cadherin germline mutations were also found in about 30% of the hereditary diffuse gastric cancers (HDGC) [82,85,86]. Moreover, patients carrying an inactivating E-cadherin germline mutation have a high cumulative risk to develop gastric cancer (67% for men and 83% for women) if they would reach an age of 80 years [87]. Compared to the cumulative risk to develop breast cancer this risk is 39%, which makes it five times more likely for a female mutation carrier to develop gastric cancer than breast cancer [87]. Interestingly, E-cadherin mutation carrier families are often associated with either breast or gastric cancer, and only a small overrepresentation of lobular breast cancer has been found in HDGC families [83,88,89]. Many different E-cadherin mutations have been described in lobular and gastric cancer [90]. Interestingly, almost all of these mutations were found in the extracellular part of the E-cadherin protein. Interestingly, most of the mutations found in lobular breast cancer cause premature stop codons resulting in a complete loss of the E-cadherin protein, while diffuse gastric cancer often contain mutations resulting in exon-skipping and in-frame deletions, rendering expression of a mutant E-cadherin protein [90]. It is currently unknown whether these differences in mutation patterns differentially affect signaling and how these are connected to tissue-specific cancer types.

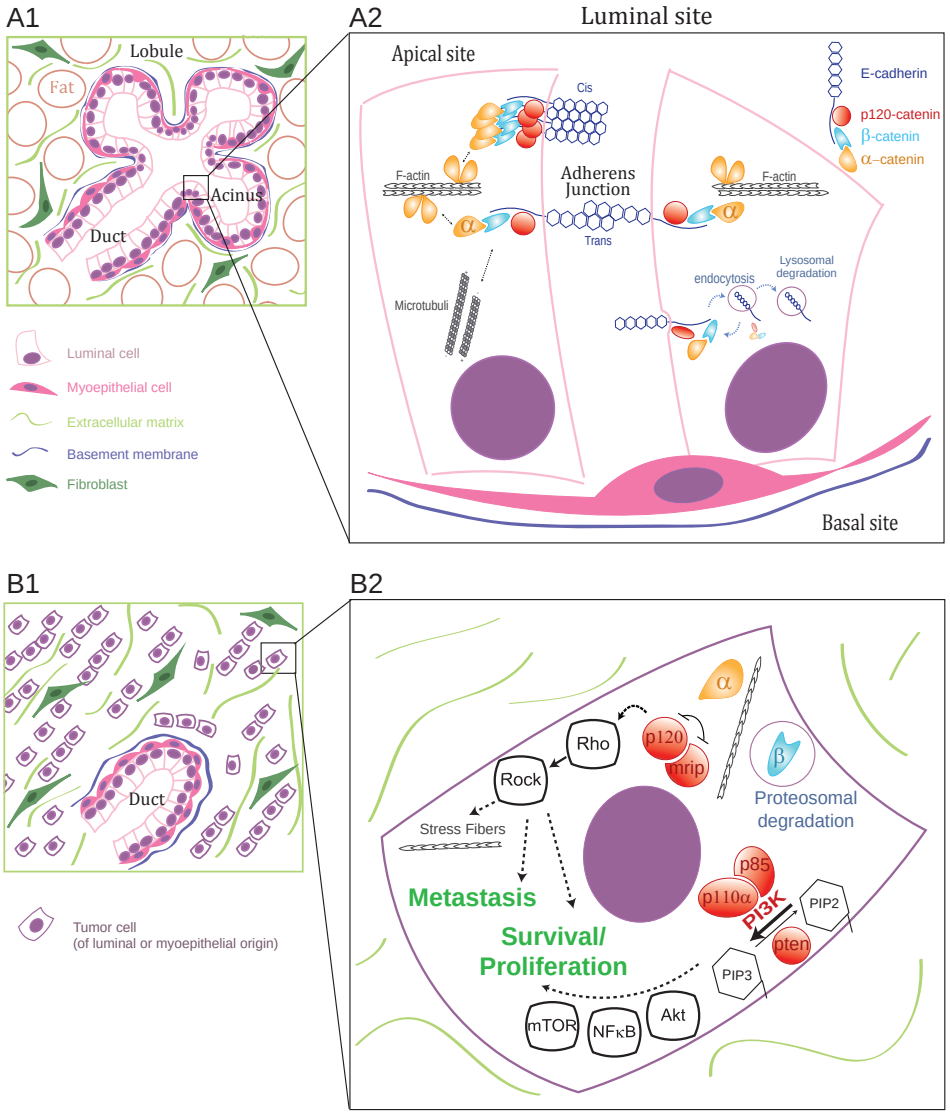
Besides mutational inactivation of E-cadherin, expression of E-cadherin can also be lost due to LOH upon promoter methylation or epigenetic silencing. Methylation of the E-cadherin promoter has been found in a substantial percentage of ILC tumors and LN lesions [91,92]. Although more rare, E-cadherin can also be epigenetically silenced via the TGF-β/SMAD2 pathway [93,94]. While expression of wild-type E-cadherin is retained in approximately 15% of the ILC cases [91], functionality of the adhesion complex seems to be impaired. In these cases, α-catenin expression is lost and p120 is translocated to the cytosol [94,95]. Moreover, biallelic in-

activating mutations were identified in α-catenin (*CTNNA1*), another E-cadherin associated molecule [96]. While the authors of this study did not observe somatic inactivating mutations in *CTNNA1* in patient material, immunohistochemistry revealed loss of protein expression, which correlated with the lobular phenotype. These findings indicate that loss of E-cadherin expression or function can be caused by different genetic alterations. Interestingly, somatic mutations in *CTNNB1* or *CTNND1* have not been described to date, indicating that mutations in α-catenin or p120 are rare.

Recent genomic advances may also aid to delineate tumor etiology and clonal relationships in lobular cancer. It has long been discussed whether LN is a precursor lesion of ILC. In 1974, a patient follow-up study reported that LN only in some cases leads to invasive carcinomas, but if they do they are often of the lobular kind [7]. A few decades later, genomic analyses showed a clonal relationship between LN and ILC, as patients samples containing LN adjacent to ILC showed identical mutations or genomic alteration patterns in both lesions [8,9,97-100]. Besides loss of chromosome arm 16q, the majority of the lobular carcinomas harbor a gain of 1q based on whole-arm CGH analysis [8,100,101]. As these genetic alterations can already be found in LN, they are both thought to be early onset chromosomal abnormalities. In short, genetic analysis by CGH and mutation analyses show that loss of chromosome arm 16q and gain of 1q are early onset alterations in lobular breast cancer, confirming that LN is indeed an ILC precursor.

Pleomorphic lobular carcinoma is an interesting lobular carcinoma variant from a cancer progression point of view. Phenotypically it has both lobular and ductal features while displaying apocrine differentiation. It has been under debate whether pleomorphic ILC is an E-cadherin mutated high-grade ductal carcinoma, or if it represents a lobular carcinoma with some ductal features. Genetic studies have indicated that both the *in situ* and the invasive component can be best classified as a lobular carcinoma, as they also harbor 16q losses and 1q gains [33,100,102,103]. Our unpublished data (Ercan, Moelans et al. submitted) indicate that based on multiplex ligation-dependent probe analysis studies on 21 established breast oncogene and tumor suppressor genes, pleomorphic ILC more frequently harbors copy number changes than classic ILC. Cluster analysis demonstrated classic and pleomorphic ILC to be rather separate entities and pleomorphic ILC as an entity molecularly in between classic ILC and infiltrating ductal cancer (Ercan, Moelans et al. submitted).

Next generation DNA sequencing indicated that mutations in *PIK3CA* and *TP53* may reflect driver mutations [104]. Interestingly, a recent study reported that *PIK3CA* mutations appear to be more common in the primary tumor than in tumor cells at a metastatic site, suggesting that *PIK3CA* mutations may not be required for metastases formation [105]. Also, a breast cancer case study showed that a specific *TP53* mutation was only present in the recurrent tumor and the distant metastases, but not in the original primary tumor, indicating that the p53 mutation had evolved as a secondary event [106]. Other research showed that breast cancer can become genetically heterogenic by forming different subclones at early stages of tumor development [104]. During ILC development significant tumor evolution may take place; next-generation sequencing on a single lobular carcinoma sample revealed that 5 of the 32 somatic coding mutations in the metastases could be traced back to the primary tumor that arose 9 years earlier [107]. Surprisingly, next to genetic alterations in the epithelial tumor cells, genetic alterations in the tumor-associated stroma have also been found. *PTEN* and *TP53*



mutations were observed in carcninoma cells as well as in tumor associated stroma. In most cases mutations were found either in the stromal or in the epithelial compartment and only rarely in both compartments [108]. From an evolutionary point of view it would be very interesting to know in which cellular compartment somatic mutations arise first. Little is known about genetic alterations within the tumor microenvironment of lobular breast tumors.

Figure 2. Schematic overview of cellular process in normal and invasive lobular tumor cells.
A. Normal epithelial cells within the breast.
A1*, within the fat tissue of the breast epithelial cells form ducts and lobules. There are two types of breast epithelial cells; luminal and myoepithelial cells. Luminal cells are able to produce milk, while myoepithelial cells can contract and hereby eject the milk out of the ductal system. The myoepithelial cells are enwrapped by basement membrane separating the epithelial cells from the stroma.* ***A2****, only luminal cells express E-cadherin molecules, which form adherens junctions (AJ) by homotypic interactions with molecules on neighboring cells (trans). E-cadherin molecules expressed by the same cell can also interact with each other (cis). On the intracellular site E-cadherin binds to p120-,* β*- and* α*-catenin. The AJ may be connected to microtubuli via the catenin molecules. Besides,* α*-catenin is needed for the connection between the AJ and the actin filaments. E-cadherin molecules can be recycled via endocytosis or degraded by lysosomal degradation.*
Invasive lobular tumor cells within the breast.
B1*, invasive tumor cells burst through the basement membrane and make direct contact with the tumor stroma of the breast. Compared to ductal tumors the desmoplastic reaction in lobular tumors is limited.* **B2***, E-cadherin negative lobular tumor cells do not form AJ. p120- and* α*-catenin are therefore no longer localized at the cell membrane, but translocate into the cytosol, while* β*-catenin is degraded by the proteasome. p120-catenin (p120) binds MRIP, which alleviates the inhibitory effect of p120 and MRIP on the Rho-Rock signaling pathway. This leads to stress fiber formation, anoikis resistance and metastasis formation. Many lobular tumors also harbor mutations that overactivate the PI3K pathway; activating PIK3CA mutations and inactivating PTEN mutations. The PI3K pathway effects Akt, NF*κ*B and mTOR signaling and it is unknown, which pathways are responsible for induction of proliferation and survival.*

Biological Features

Although loss of E-cadherin expression characterizes lobular tumors, until recently surprisingly little was known about the intracellular signaling pathways that are affected upon E-cadherin loss and how they promote tumor development and progression. Conditional E-cadherin knock-out mouse models were generated that have provided the tools to understand the underlying cellular biochemistry. Using these models it has become evident that aberrant activation of downstream catenins and the relationship with the actin cytoskeleton are crucial in lobular carcinoma biology and the metastatic phenotype.

The Epithelial Adherens Junction and Its Functional Role in Lobular Breast Cancer

E-cadherin is a calcium-dependent transmembrane protein that controls epithelial integrity by homotypic (*cis* and *trans*) interactions. Together with the associated catenins it forms the adherence junction (AJ) on the apical side of the basolateral membrane of the cell [109] (**Fig. 2 A2**). E-cadherin regulates multiple epithelial processes through linkage to the actin cytoskeleton through β-catenin and α-catenin [110]. The AJ can also connect to microtubuli via α-catenin, β-catenin or p120 [111-114], although these ties remain controversial. The AJ is linked to the actin cytoskeleton via α-catenin, but the exact mechanism still remains unclear. It seems that α-catenin cannot bind β-catenin and actin filaments simultaneously due to its transition between "active" and "inactive" conformations [115,116], suggesting highly dynamic and mutually exclusive interactions. The AJ is not a rigid structure; even when cells are grown in confluency and cell-cell junctions are stable, a small pool of E-cadherin is recycled [117] (**Fig. 2A2**). Stability and recycling of the AJ complex is mainly dependent on p120-regulated endocytosis [118]. The p120-binding domain of E-cadherin overlaps with the region responsible for endocytosis [119-121]. Recent studies indicated that HAKAI-mediated ubiquitination of the E-cadherin jux-

tamembrane domain inhibits p120-binding, and targets E-cadherin for degradation [122]. The link between p120 and E-cadherin internalization was further illustrated by the observation that loss of p120 leads to E-cadherin, β-catenin and α-catenin downregulation [118]. Moreover, recently data indicated that α-catenin is required to strengthen the association between p120 and E-cadherin, and as such may influence E-cadherin internalization [123].

Given that E-cadherin is mostly absent in lobular breast cancer as a result of somatic mutations, conditional knockout mice targeting E-cadherin were generated to model lobular breast cancer. Due to the fact that E-cadherin loss in the mammary gland is not tolerated [124,125], E-cadherin was ablated in the context of p53 loss. Concomitant inactivation of E-cadherin and p53 in the mouse mammary gland resulted in a shift from noninvasive carcinoma to invasive and metastatic tumors resembling human ILC [125,126]. Mouse ILC (mILC) tumors grew in a noncohesive manner typical for invasive lobular tumors. Furthermore, different lobular subtypes were observed including LN, classical ILC and solid ILC (**Fig. 3**). mILC tumors were relatively pleomorphic in appearance and diagnosed as high grade. Similar to human ILC, mILC showed trabecular invasive growth (Indian files), targetoid periductal distribution and stellate "scar"-type lesions. Interestingly, next to common metastatic sites such as bone, lungs and lymph nodes, mILC showed dissemination to the peritoneum and the gastro-intestinal tract, which are typical metastatic sites for human ILC as well. Occasionally, signet ring cells (an occasional characteristic trait of human ILC) were detected (**Fig. 3B**). While human ILC tumors expressed only cytokeratin 8, mILC cells showed a mixed but mutually exclusive expression of epithelial cytokeratins 8 and 14. Some estrogen receptor (ER) expressing cells were detected in low grade elements of mILC lesions, but most mILCs were ER-negative suggesting that ER expression is inversely correlated with tumor grade in these mouse models.

Using the mILC model as starting point, Schackmann et al. (2011) confirmed that p120 translocated to the cytoplasm upon E-cadherin loss. The authors went on to show that p120 played a key oncogenic role through regulation of anchorage independent survival. In contrast, β-catenin was subject to proteasomal degradation, thereby silencing canonical Wnt signaling [127]. These findings were supported by observations in human breast cancer samples showing: (i) that missense (NH2)-terminal domain β-catenin mutations were exclusively found in metaplastic breast cancer [128,129], and (ii) that β-catenin expression is very low or absent in most ILC cases [28]. In ILC, p120's Rho GDP-dissociation inhibitor functions are inhibited by binding to the Rho antagonist myosin phosphatase Rho interacting protein. Net result of this interaction is activity of the Rho-Rock signaling axis, which positively controls anchorage independent tumor growth and subsequent metastasis of ILC cells [127] (**Fig. 2B2**). It however remains unclear how signals downstream of p120 prevent induction of anoikis. Nonetheless, since Rock is active and susceptible for pharmacological inhibitions, this research opened new avenues for the treatment of metastatic lobular breast cancer. Finally, our unpublished results suggest that conditional homozygous ablation of p120 in the mILC model severely inhibits ILC formation, while tumor incidence, onset and metastatic potential remained unaffected (Tenhagen, manuscript in preparation).

The contribution of α-catenin to ILC development and progression is less well defined. Mutational analyses in human breast cancer cell lines revealed biallelic inactivating mutations in *CTNNA1* resulting in functional inactivation of the protein [96]. These four mutated cell lines (MDA-MB-468, MDA-MB-330, MDA-MB-157 and HCC1187) retained E-cadherin expression at the membrane and three out four showed a rounded cell morphology suggesting impaired cell-cell adhesion. Interestingly, E-cadherin still seems to form a complex with p120, actin, α-actinin and high levels of vinculin in MDA-MB-468 [130,131]. Others showed

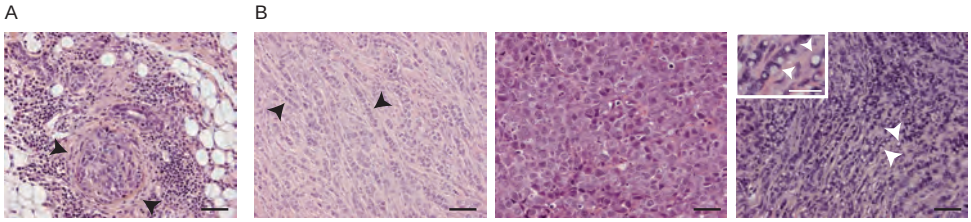


Figure 3. Subtypes of mouse Lobular carcinoma.
A*. LN lesion with complete filling of alveoli encapsulated by inflammatory stroma (arrowheads).* ***B****. First panel, classical mouse ILC. Tumor cells showing a trabecular infiltration pattern ('indian files') typical for ILC (arrowheads). Second panel, a solid variant of mouse ILC, characterized by compact growth of tumor cells. Right panel, pure signet cell ILC characterized by cells resembling signet rings. Inset shows an enlarged area with typical signet cells (white arrowheads), white ize bars indicates 25 μm. Lower right size bars indicate 50 μm.*

by immunofluorescence that also the tight junctions and desmosomes were affected in this cell line [123]. Together, these data suggest that the disturbance of the connection between the AJ and the actin cytoskeleton is crucial for lobular tumor development and that this can be caused by either mutational inactivation of *CDH1* or *CTNNA1*. A lot is still to be learned, as causality between α-catenin loss and lobular development has not been established. Furthermore, it is still unclear how α-catenin mediates the link between the AJ and the actin cytoskeleton. α-catenin contains different domains, each able to bind to different proteins of which many are actin binding proteins themselves [132], suggesting the formation of a bigger complex enabling indirect binding of α-catenin to the actin cytoskeleton.

Other Signaling Pathways Involved in ILC

The PI3K/Akt pathway is disturbed in at least one third of the ILC tumors [73,74,105]. *PIK3CA* mutations increase PI3K activity, which stimulates AKT-induced cell survival, cell cycle progression, cell motility and metabolism (Fig. 2B2). PTEN is a protein/lipid phosphatase that also affects the PI3K/Akt pathway, as it reverses the synthesis of PIP3 [133,134]. Although AKT signaling seems to play a major role in breast cancer, mice carrying activated AKT under the control of the mouse mammary tumor virus promoter crossed with heterozygous p53 mice did not have a different median survival compared to the heterozygous p53 mice [135]. This suggested that PI3K and PTEN might promote cancer progression not via the AKT pathway, but via another, like the NF-κB pathway. Supporting this hypothesis is the finding that mutations in NF-κB or in other proteins involved in the NF-κB pathway have been observed in breast cancer [136]. Mouse models with mammary-specific activation of the PI3K pathway showed excessive ductal branching and tumor development, but give little insight into the role of this pathway in lobular breast cancer development and progression [137,138]. In conclusion, the high frequency of *PIK3CA* mutations observed in lobular breast cancer suggests an important role for the PI3K pathway in tumor progression. Although cancer evolution studies advocated PI3K as a key player in tumor initiation [104], its role in tumor progression remains controversial [105,139,140].

Surgical Management

Lobular Neoplasia

In the past, mastectomy was a frequently applied management strategy for the case of LCIS [141,142]). Bilateral mastectomy was also suggested as a possible management strategy with the claim that LCIS gives rise to invasive cancer in both breasts with an equal risk [143,144]. However, LN is usually an incidental finding and recent investigations show only a small percentage of LCIS patients treated with local excision present with recurrence (Fisher et al. 2004). Besides, recurrence developed relatively late as more than 50% occurred after 15 years or later [46]. These findings led to a more conservative approach [48]. An exception to this more conservative attitude is the high grade forms of LN including pleomorphic LN, which are more often segmental in distribution, have a relatively high local recurrence rate and should therefore be treated with surgery (like DCIS) [145]. A strict conservative trend in the management of LN has been favored by some authors to follow the principle of avoiding unnecessary surgery. In this approach patients are only physically examined annually and monitored regularly by mammography [146,147]. Criteria for the choice of surgical excision or another approach are still under discussion and differ between clinical centers. There are certain arguments for choosing surgical excision after diagnosis with LN such as significant risk of invasive cancer [15,148], underestimation of the diagnosis due to limitations of core needle biopsies [149,150], presence of suspicious microcalcifications [151,152] and specific histopathological features (discussed above). Generally, surgery of the ipsilateral or contralateral breast is not performed after the diagnosis of LN.

Invasive Lobular Cancer

Surgery is usually the first choice of treatment for ILC patients and is performed by a wide local excision for the area of cancer and surrounding healthy tissue. Since the disease may have spread to multiple areas within the breast, a mastectomy can be preferred in selected cases. ILC regularly may metastasize to the axillary lymph nodes and a sentinel node biopsy is therefore routinely in place, like for all major epithelial breast cancer subtypes [153], generally followed by axillary lymph node dissection only in case of macrometastases to the sentinel node. In contrast to IDC, ILC lesions tend to be larger due to their diffuse or multifocal growth and infiltration pattern. As a consequence, breast conserving surgery and heat ablation are more likely to be irradical in ILC [154]. Radiation therapy, chemotherapy and endocrine therapy are therefore also applied individually or as a combination therapy.

Radiotherapy

It has been shown that radiotherapy reduces the risk of local recurrence substantially in LN patients who underwent breast conserving therapy, but not in patients that underwent mastectomy [155]. However, the effect of radiotherapy on long term survival seems only marginal on patients that underwent breast conserving therapy, while node-positive patients that underwent mastectomy do benefit from radiotherapy. In ILC patients, radiotherapy is generally applied in combination with breast conserving therapy. However, classic ILC may not be the

most sensitive form of breast cancer as it usually shown little proliferation. Pleomorphic ILC, which shows a higher proliferation rate, may however be more sensitive [156,157].

Chemo- and Hormonal Therapy

Systemic treatment (especially with anti-hormonal drugs like tamoxifen) can be used to prevent progression of LN. Unfortunately we currently have limited evidence showing the efficacy of such adjuvant therapies in the pre-malignant stages of the disease [158]. As most lobular tumors are hormone receptor positive, treatment with tamoxifen or aromatase inhibitors is an option. It has previously been shown that both pre- and postmenopausal women with LCIS benefit from tamoxifen treatment [159,160], although this has been associated with an increased risk of endometrial cancer [161]. ILC patient's pathologic response to neoadjuvant therapy tends to be poorer compared to the response IDC patients, while long-term response to chemotherapy tends to better compared to IDC patients [162,163]. The beneficial long-term outcome is most likely due to the often hormone-responsive nature of ILC. Systemic treatment in case of distant metastases is usually based on hormone and HER2 receptor status. Although still controversial, most guidelines [164], indicate that these receptors should preferentially be assessed in biopsies of the metastases [165].

Targeted Intervention

Because little is currently known about the pathways that regulate ILC tumor progression, targeted therapy is still in its infancy. Prominent candidates for clinical intervention are pathways controlling actin remodeling, as ILC cells appear susceptible for Rock inhibition under anchorage-independent conditions [33,127]. Another important signaling pathway in ILC tumors is the PI3K pathway and the associated AKT, mTOR and NF-κB pathways, which are currently subject of clinical trials in breast cancer patients [166]. The majority of lobular breast tumors harbor mutations in multiple pathways [78], which suggests that combined and targeted intervention strategies may be beneficial in these tumors. An example of this is the fact that mutations in proteins involved in the PI3K pathway can affect ER signaling, suggesting that PI3K-targeted therapy may sensitize ILC for hormonal therapy [167].

Clinically, the distinction between classic and pleomorphic lobular tumors is important as the latter has a poorer prognosis [23]. Especially age and negative hormone receptor status are indicative for worse clinical outcome in pleomorphic ILC patients [24]. What the best treatment options are for pleomorphic lobular tumors and whether they should be treated with trastuzumab, as most of them are ERBB2-positive, is still unknown, but has been suggested by Mahtani and Vogel [168] as well. Interestingly, enhanced PI3K/AKT signaling in ERBB2 positive tumors correlated with trastuzumab resistance [169], which suggests that ERBB2 positive pleomorphic ILC patients might benefit from combined ERBB2 and PI3K-targeted therapy. Finally, we recently showed that pleomorphic ILC shows high HIF-1α expression, a feature that is correlated with high mortality [170]. Thus, although drug delivery to hypoxic areas is challenging [171], pleomorphic ILC may perhaps benefit from treatment with HIF1 inhibitors.

Conclusions and Future Perspectives

Lobular breast carcinoma is a distinct breast cancer subtype based on pathology, genetic aberrations and cellular biochemistry. Detection and resection of lobular breast cancer is relatively difficult due to its diffuse growth pattern, leaving the pre-existing breast architecture largely intact, and a low tendency to induce a desmoplastic reaction. While ILC tumors harbor a more favorable histological status compared to IDC patients, ILC patients display the same prognosis compared to IDC patients [2]. In the past it has been under debate whether LN is an ILC precursor and whether pleomorphic ILC should be considered as a lobular or a ductal tumor. It has now become apparent that based on genetic analysis LN can be classified as a lobular precursor, pleomorphic LN is a pleomorphic ILC precursor and pleomorphic ILC should be considered as a progressed lobular tumor. Importantly, pleomorphic ILC is characterized by a higher frequency of p53 mutations, hormone receptor negativity and ERBB2 positivity, suggesting that future treatment of pleomorphic ILC should be designed accordingly. In ILC patients hormone therapy benefits patient survival, but since distant recurrences often occur with long latency [172], additional targeted therapies are needed. Unfortunately, although much progress has been made, little is known about the signaling pathways that drive ILC development and progression. It has become evident that mutations in E-cadherin are causal to ILC development and progression through aberrant activation of Rho and Rock, rendering this pathway eligable for clinical intervention using Rock inhibitors. Moreover, a substantial ILC subgroup harbors mutations affecting the PI3K/AKT pathway. Therapies targeting these pathways represent an attractive option to enhance survival of ILC patients. Finally, advances in sequencing techniques have enabled identification of mutations in systemic tumor cells found in patient plasma samples. This technique might facilitate tumor classification based on genetic aberrations and enable targeted therapy in the future [173,174].

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Chapter 3

Methylation biomarkers for pleomorphic lobular breast cancer

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Abstract

Pleomorphic invasive lobular cancer (pleomorphic ILC) is a rare variant of ILC that is characterized by a classic ILC-like growth pattern combined with an infiltrative ductal cancer (IDC)-like high nuclear atypicality. It is therefore an ongoing discussion whether pleomorphic ILC is a dedifferentiated form of ILC or in origin an IDC with secondary loss of cohesion. Since promoter hypermethylation is an early event in breast carcinogenesis and may give information on tumor progression, we compared the methylation patterns of pleomorphic ILC, classic ILC and IDC. Besides, we aimed to analyze the methylation status of pleomorphic ILC for the first time. We performed methylation profiling by methylation-specific multiplex ligation-dependent probe amplification analysis to compare 20 classical ILC, 16 pleomorphic ILC and 20 IDC cases. Pleomorphic ILC showed relatively low *TP73* and *MLH1* methylation and relatively high *RASSF1A* methylation compared to classic ILC. Furthermore, compared to IDC, pleomorphic ILC showed relatively low *MLH1* and *BRCA1* methylation. *TP73*, *RASSF1A*, *MLH1* and *BRCA1 m*ethylation and mRNA expression data from the Cancer Genome Atlas showed an inverse correlation. Hierarchical cluster analysis revealed a similar methylation pattern for classic ILC and IDC, while the methylation pattern of pleomorphic ILC was mildly different from these breast cancer subtypes. In conclusion, this is the first report to identify *TP73*, *RASSF1A*, *MLH1* and *BRCA1* as possible biomarkers to help distinguish pleomorphic ILC from classic ILC and IDC.

Keywords: Sporadic breast cancer; lobular breast cancer; pleomorphic lobular breast cancer; DNA hypermethylation; MS-MLPA; epigenetics

Introduction

Invasive lobular breast cancer (ILC) is the second most prevalent histological breast cancer type that accounts for 10-15% of all breast cancers [1,2]. ILC differs from invasive ductal carcinoma (IDC) in biology, histology, clinical presentation and response to therapy (reviewed in [3]). In contrast to ductal tumors, most lobular tumors show loss of E-cadherin expression, which is often due to inactivating mutations and subsequent loss of heterozygosity or promoter hypermethylation [4]. Indeed, conditional knock-out mouse models have demonstrated that somatic inactivation of E-cadherin leads to ILC development and progression [5,6]. Among the eight different ILC variants described Classic ILC and pleomorphic ILC are the most common variants [3,7]. Although the frequency of these ILC subtypes has not extensively been documented, approximately 60% of all ILC cases is classic and 13% is pleomorphic (reviewed in [3]). Phenotypically, classic ILC is composed of small regular low grade and dissociated cells with intracytoplasmic vacuoles and small nuclei that exhibit a highly trabecular infiltrative growth pattern, often distributed in targetoid patterns around uninvolved ducts [8]. Pleomorphic ILC shows a similar growth and invasion pattern, but exhibits high grade polygonal cells with eccentric and highly pleomorphic nuclei [7,9]. Furthermore, pleomorphic ILC has been reported to be significantly larger than classic ILC tumors [10], and pleomorphic ILC patients often present lymph node involvement and a higher rate of metastatic disease compared to classical ILC [10]. Moreover, overall survival and recurrence of pleomorphic ILC patients is worse compared with classic ILC patients [7,11], indicating that pleomorphic ILC is a more aggressive form of breast cancer compared to classic ILC.

Classic and pleomorphic ILC show similarities and differences. Both variants lack expression of basal markers like cytokeratin (CK)5 and CK14, but expresses the luminal epithelial markers CK8 and CK18 [12,13]. Considering their molecular profile, ILCs are usually "luminal" type breast cancers that express estrogen receptor (ER) and genes involved in ER activation including the progesterone receptor (PR) [14,15]. Cytosolic translocation of p120-catenin due to inactivation of E-cadherin is an ILC hallmark, but classic and pleomorphic ILC do not overexpress the Epidermal Growth Factor Receptor (EGFR) [1,9,16,17]. While most classic ILC lack expression of HER2 (*ERBB2*) [1,18] up to 81% of pleomorphic ILC have been reported to express HER2 [9,19,20]. Moreover, although the somatic *TP53* mutation percentage in pleomorphic ILC may be as high as 46%, this is a rare event in classic ILC (approximately 6%) suggesting a role for p53 loss in pleomorphic ILC etiology [19-22]. Supporting this are findings in mammary-specific E-cadherin and p53 knock-out mice that develop a mouse variant of pleomorphic ILC [6]. Furthermore, in contrast to classic ILC, pleomorphic ILC often expresses the apocrine differentiation marker Gross Cystic Disease Fluid Protein 15 and the androgen receptor [19,23]. The origin of pleomorphic ILC tumors is still under debate. It is currently unclear whether pleomorphic ILC is a dedifferentiated classic ILC or if it has evolved from a ductal type tumors. Differential diagnosis between these tumor types is important because surgery planning of ILC requires pre-operative MRI [24], due to a more often diffuse and multifocal growth pattern of lobular tumors and a higher incidence of contralateral tumors [1].

In cancer, DNA methylation is often disturbed and can be a driving force during tumor progression (reviewed in [25]). DNA methylation occurs by the enzymatic transfer of a methyl group onto the carbon-5 position of a cytosine (often part of a cytosine phosphate guanosine (CpG) dinucleotide), which influences histone modifications and can result in gene silencing [26]. During DNA replication the parental DNA strand retains methylated, while the newly

synthesized strand is not (reviewed in [27]). DNA cytosine methyltransferases (DNMTs) can recognize hemimethylated DNA strands and restore methylation on the opposing strand. This way DNA methylation can be inherited by the daughter cell. While DNA hypomethylation is fairly unexplored, the DNA promoter hypermethylation field emerges relatively quickly. DNA promoter hypermethylation of tumor suppressor genes is considered to be a very early event in carcinogenesis [28,29]. For instance, high methylation was frequently found in columnar cell lesions, the earliest recognized breast cancer precursors [30]. As gene methylation is an early event in tumor development and affects tumor progression, methylation patterns give insight in tumor progression and may therefore shed light on the precursor of pleomorphic ILC tumors. Because of the possible future extrapolation to methylation detection in biopsies, blood, nipple fluid and urine, DNA hypermethylation is a promising area in the clinical biomarker field. Unlike gene expression arrays, DNA hypermethylation analysis can be performed on formalin-fixed tissues and thus provide an alternative way for molecular profiling and the identification of prognostic markers that predict therapeutic responsiveness [28].

Here we have studied promoter methylation patterns in pleomorphic ILC in relation to ILC and IDC to identify pleomorphic ILC biomarkers. We performed methylation-specific multiplex ligation-dependent probe amplification (MS–MLPA), as this technique requires only small amounts of short DNA fragments and has been shown to be highly reliable and reproducible [31,32]. We assessed promoter methylation of a large set of tumor suppressor genes and compared 16 pleomorphic ILC, 20 classic ILC and 20 IDC cases. We found that the methylation patterns of classic ILC and IDC were comparable, while classic ILC and IDC profiles were mildly different from pleomorphic ILC. Furthermore, we observed that the methylation status of RAS Association (RalGDS/AF-6) domain Family member 1 (RASSF1A), Tumor Protein p73 (TP73), MutL Homolog 1 (MLH1) and Breast Cancer 1 (BRCA1) can be used as stratification markers to separate pleomorphic ILC from classic ILC and IDC.

Material & Methods

Patient Material

Patient samples were derived from the archives of the Departments of Pathology at the University Medical Centre in Utrecht, the Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands, the Institute of Pathology, Paderborn, Germany, and the Department of Pathology, Bács-Kiskun County Teaching Hospital, Kecskemét, Hungary. Clinicopathological characteristics are shown in **Table 1.** Classic and pleomorphic ILC and IDC patients were selected based on examination of haematoxylin and eosin (H&E)-stained slides by at least two pathologists (see F**igure 1** for representative images). In this study, the use of left over material was approved by the Tissue Science Committee of the UMC Utrecht [33]. Histological grade was assessed according to the Nottingham modification of the Scarff-Bloom-Richardson grading system [34], ER and PR were considered positive when ≥10% of the cells showed positive nuclear staining. HER2 was scored according to the modified DAKO score, where only a score of 3+ was considered positive. The mitotic activity index (MAI) was assessed as before [35].

Table 1. Clinicopathological characteristics of breast cancer patients.

Feature	Grouping	Classic ILC N (%)	Pleomorphic ILC N (%)	IDC
N		20	16	20
	Range	52-88	43-80	44-87
Histological grade	1	8 (40.0)	0 (0.0)	5 (25.0)
	2	6 (30.0)	5 (31.3)	5 (25.0)
	3	5 (25.0)	11 (68.8)	10 (50.0)
	Not available	1 (5.0)	0 (0.0)	-
MAI (%)	Mean	3	20	16.5
	Range	0-26	9-100	0-8
	Not available	1 (5.0)	1 (6.3)	-
Lymph node staus	Negative *	11 (55.0)	8 (50.0)	7 (35.0)
	Positive **	7 (35.0)	8 (50.0)	13 (65.0)
	Not available	2 (10.0)	-	-
Receptor status	ER positive	19 (95.0)	14 (87.5)	15 (75.0)
	PR positive	10 (50.0)	10 (62.5)	13 (65.0)
	Her2 positive	0 (0.0)	0 (0.0)	3 (15.0)
Tumor size (cm)	≤ 2.0	1 (5.0)	4 (25.0)	8 (40.0)
	> 2.0	18 (90.0)	12 (75.0)	12 (60.0)
	Not available	1 (5.0)	-	-

*: negative = N0 or N0(i+); **: positive = ≥N1mi (according to TNM 7th edition, 2010)

Methylation-specific multiplex ligation-dependent probe amplification

Tumor tissue was derived from 5 to 10 4μm thick slides (formalin-fixed paraffin embedded (FFPE) samples) by scraping and DNA was isolated by overnight incubation in lysis buffer (50mM Tris-HCL, pH 8.0; 0.5% Tween 20) with proteinase K (10 mg/ml, Roche) at 56 °C, followed by boiling for 10 minutes. After a 5 minutes centrifugation step (12,000g), 5 μl supernatant was used for MLPA analysis according to the manufacturer's instructions, using the ME001-C2 methylation kit (MRC-Holland). The principle of MS–MLPA has been described elsewhere [31]. In short, the MS–MLPA probes contain a recognition sequence for the methylation sensitive restriction enzyme *Hha*I, which specifically cleaves unmethylated DNA only. Half of the sample DNA-probe hybrids is cleaved with *Hha*I so that only methylated DNA remains intact, is successfully amplified and produces a signal. By comparing signals of the *Hha*I cleaved and the other uncleaved half one can calculate the methylation percentage

using Coffalyser software. The PCR procedure and data analysis were performed as described [29]. The ME001-C2 MS–MLPA probe mix contained 26 MS–MLPA probes, detecting the methylation status of promoter CpG sites of 24 probable tumor suppressor genes (**Table 2**) that are frequently silenced by methylation in tumors, but are unmethylated in blood-derived DNA of healthy individuals. In addition, we included 15 reference probes that are not affected by *Hha*I digestion. The cumulative methylation index (CMI) was calculated as the sum of the methylation percentage of all genes, as described before [36].

Table 2. Probes directed against the CpG islands of 24 tumor suppressor genes in the MS-MLPA kit (ME001-C2, MRC-Holland).

Length	Gene	Chromosome	Mapview	Full name
142	TIMP3	22q12.3	22-031.527795	TIMP metallopeptidase inhibitor 3
148	APC	5q22.2	05-112.101357	Adenomatosis Polyposis Coli
161	CDKN2A	9p21.3	09-021.985276	Cyclin-Dependent Kinase Inhibitor 2A
167	MLH1_a*	3p22.2	03-037.009621	MutL Homolog 1
184	ATM	11q22.3	11-107.599044	Ataxia Telangiectasia Mutated
193	RARB	3p24.2	03-025.444559	Retinoic Acid Receptor, beta
211	CDKN2B	9p21.3	09-021.998808	Cyclin-Dependent Kinase Inhibitor 2B
220	HIC1	17p13.3	17-001.905107	Hypermethylated In Cancer 1
238	CHFR	12q24.33	12-131.974372	Checkpoint with Forkhead and Ring finger domains
246	BRCA1	17q21.31	17-038.530811	Breast Cancer 1
265	CASP8	2q33.1	02-201.830871	Caspase 8
274	CDKN1B	12p13.1	12-012.761863	Cyclin-Dependent Kinase Inhibitor 1B
292	PTEN	10q23.3	10-089.612348	Phosphatase and Tensin homolog
301	BRCA2	13q12.3	13-031.787722	Breast Cancer 2
319	CD44	11p13	11-035.117389	CD44 molecule
328	RASSF1A_a*	3p21.31	03-050.353298	Ras Association (RalGDS/AF-6) domain Family member 1
346	DAPK1	9q21.33	09-089.303075	Death-Associated Protein Kinase 1
353	VHL	3p25.3	03-010.158426	Von Hippel-Lindau tumor suppressor
373	ESR1	6q25.1	06-152.170883	Estrogen Receptor 1
382	RASSF1A_b*	3p21.31	03-050.353347	Ras Association (RalGDS/AF-6) domain Family member 1
400	TP73	1p36.32	01-003.558977	Tumor Protein p73
409	FHIT	3p14.2	03-061.211918	Fragile Histidine Triad
427	CADM1	11q23.3	11-114.880585	Cell Adhesion Molecule 1
436	CDH13	16q23.3	16-081.218219	Cadherin 13
454	GSTP1	11q13.2	11-067.107774	Glutathione S-transferase pi 1
463	MLH1_b*	3p22.2	03-037.010000	MutL Homolog 1

* For these genes two probe sets against different CpG sites (a and b) are present.

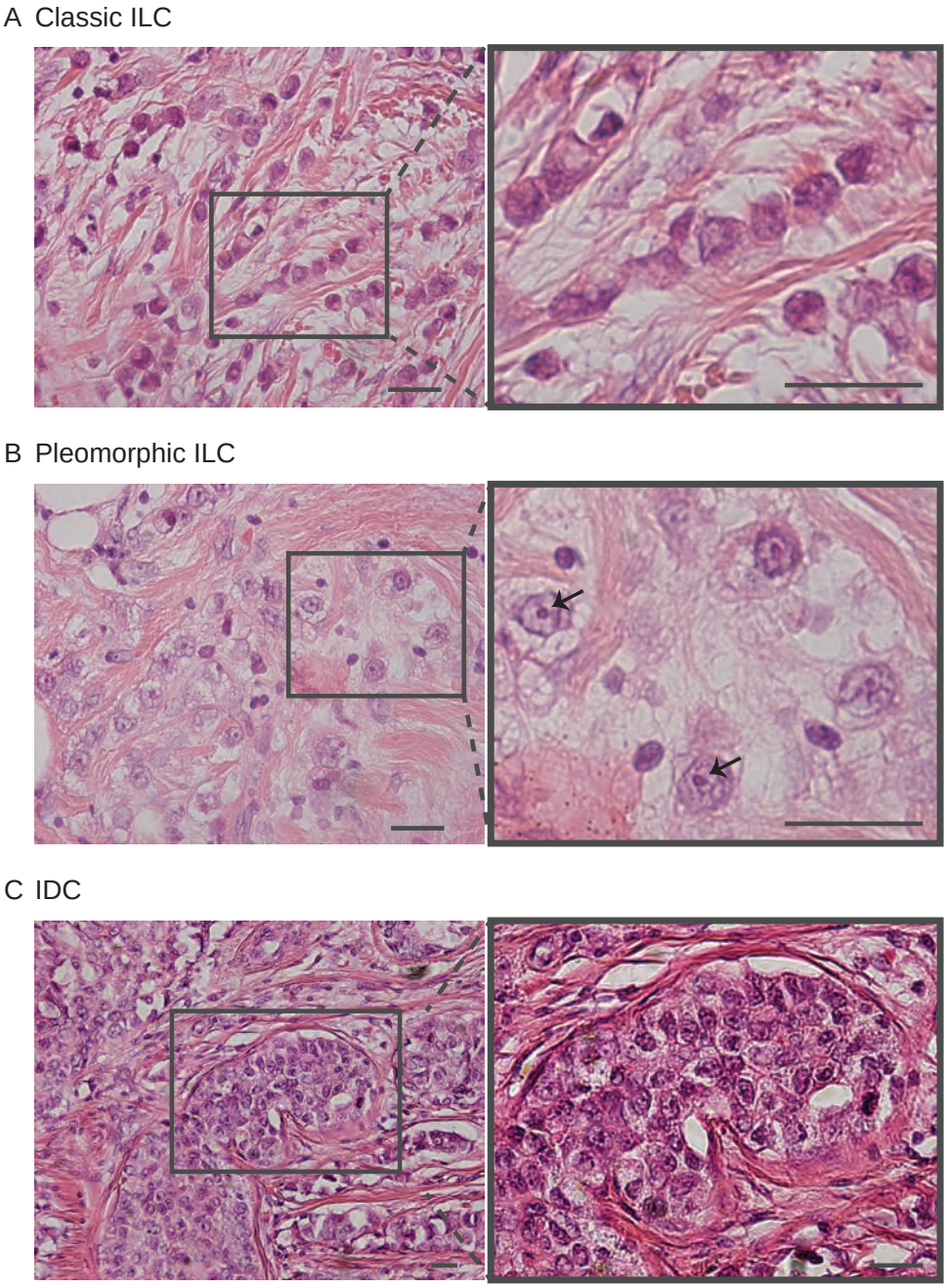


Figure 1. Representative H&E images of classic and pleomorphic ILC and IDC.
*(**A**) Classic ILC is characterized by small regular cells, small nuclei and a low mitotic rate. The formation of single ("indian") files is common in classic ILC (enlarged in right image). (**B**) Pleomorphic ILC exhibits polygonal cells and frequent mitoses. Nuclei are often eccentric, highly pleomorphic and show distinctive nucleoli (enlarged in right image, arrows). (**C**) Histological image of a low-grade IDC tumor. IDC tumors are not characterized by specific features like ILC. In contrast to ILC, IDC often shows formation of ducts within the tumor (left and right image). All size bars indicate 25 µm.*

Correlation between mRNA expression and promoter methylation by TCGA

TCGA Breast Invasive Carcinoma mRNA Expression z-Scores (RNA Seq V2 RSEM) data (n=1038) were downloaded via The cBioPortal for Cancer Genomics [37,38]. Illumina Infinium Human DNA Methylation 27 level 3 data (calculated beta values (M/M+U), gene symbols, chromosomes and genomic coordinates) were downloaded via TCGA Data Portal (n=313). We matched methylation specific CpG sites between MS-MLPA and TCGA (**Table 3,** only available online). Z-scores of mRNA sequence data were compared to percentages of DNA methylation by Pearson correlation.

Statistics

Statistical calculations and ROC curve analysis were performed using IBM SPSS statistics v20.0 (SPSS Inc., Chicago, IL, USA), regarding two-sided $p<0.05$ as significant. Absolute methylation values were used to calculate p-values upon comparing classic ILC, pleomorphic ILC and IDC samples, using the Student's *t*-test or Mann-Whitney U Test, and Kruskal-Wallis test. By Bonferroni-Holm correction on all p-values we counteracted false-positives caused by multiple comparisons. Logistic regression analysis was used to find the best (combination of) genes able to discriminate pleomorphic ILC from classic ILC and/or IDC. A backward stepwise method was used until the most predictive variables remained. Unsupervised hierarchical clustering (Euclidean metric) using the statistical program R was performed to identify relevant clusters, using the absolute MS-MLPA methylation values of the most differential genes.

Results

Methylation patterns in pleomorphic ILC, classic ILC and IDC

Clinicopathological characteristics are shown in **Table 1.** Kruskal-Wallis one-way ANOVA analysis was carried out to analyze differential methylation patterns in our non-parametric methylation data of the three breast cancer subtypes. Sixteen genes showed significant differences between the groups. However, after correction for multiple comparisons only the methylation patterns of *TP73* ($p<0.002$), *MLH1_b* ($p<0.002$) and *RASSF1A_a* ($p<0.002$) were found to be significantly different between the three breast cancer subtypes (**Figure 2A**).

Methylation in pleomorphic versus classic ILC

A (post hoc) Mann-Whitney test followed by multiple comparisons correction was carried out, using the 16 genes derived from Kruskall-Wallis analysis, to specify the differences between classic and pleomorphic ILC. Methylation of *TP73, MLH1_b* and *RASSF1A_a* were significantly different between classic and pleomorphic ILC. When compared to classic ILC, pleomorphic ILC showed less methylation in *MLH1_b* ($p=0.003$) and *TP73* ($p=0.001$) (**Figure 2A**), while methylation of *RASSF1A* was higher in pleomorphic ILC ($p=0.001$). CMI of pleomorphic ILC was not significantly different from classic ILC, (353.3 versus 390.0 respectively; $p=0.437$). In logistic regression analysis, *TP73* ($p=0.017$) and *RASSF1A* ($p=0.005$), had a joint independent discriminative value for pleomorphic ILC versus classic ILC (area under the curve (AUC) 0.888, CI 0.764 – 1.000, $p<0.001$), with a combined receiver operating characteristic (ROC) curve-based sensitivity and specificity of 81% and 100%, respectively.

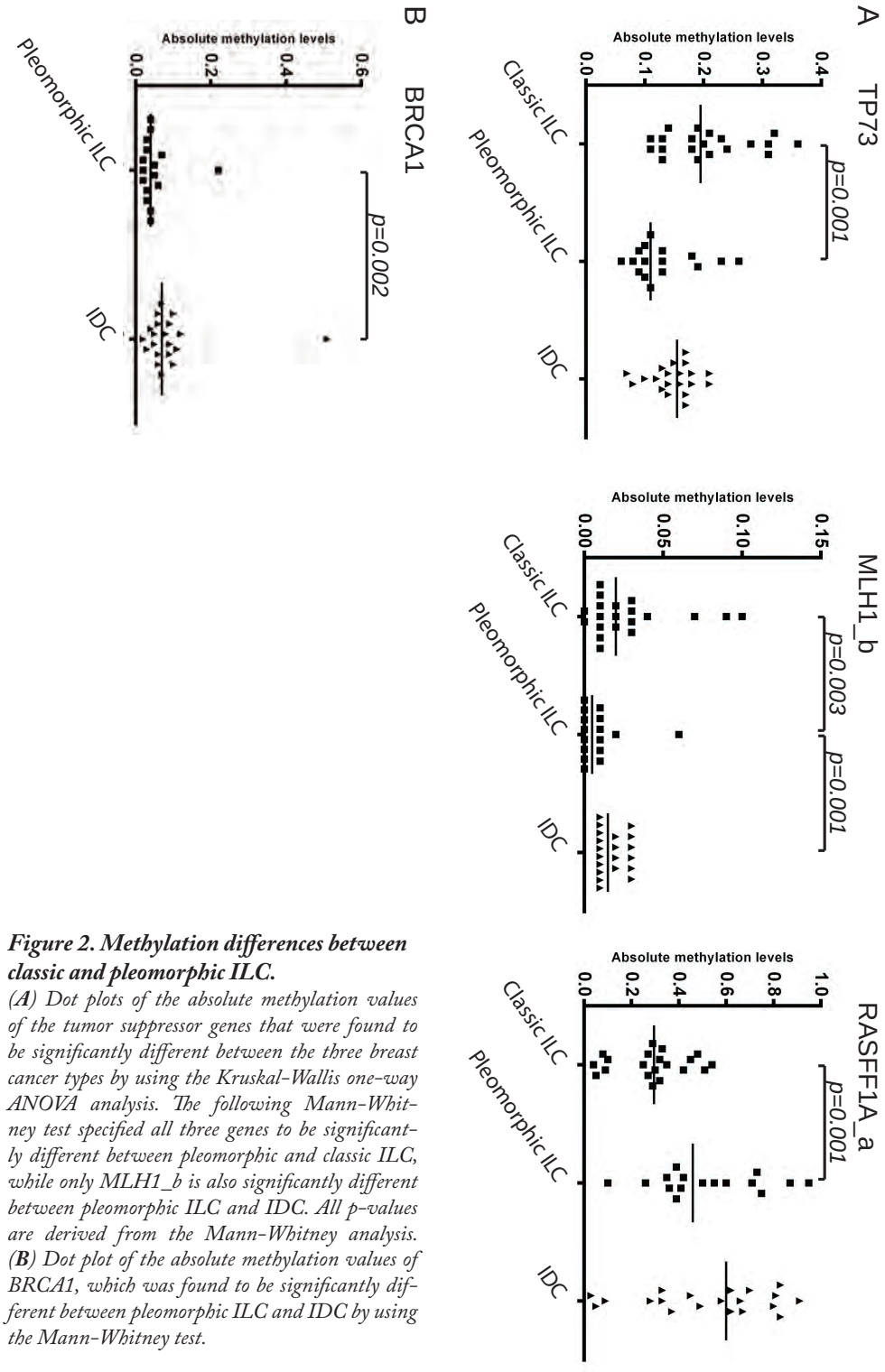


Figure 2. Methylation differences between classic and pleomorphic ILC.

*(**A**) Dot plots of the absolute methylation values of the tumor suppressor genes that were found to be significantly different between the three breast cancer types by using the Kruskal-Wallis one-way ANOVA analysis. The following Mann-Whitney test specified all three genes to be significantly different between pleomorphic and classic ILC, while only MLH1_b is also significantly different between pleomorphic ILC and IDC. All p-values are derived from the Mann-Whitney analysis. (**B**) Dot plot of the absolute methylation values of BRCA1, which was found to be significantly different between pleomorphic ILC and IDC by using the Mann-Whitney test.*

Methylation in pleomorphic ILC versus IDC

After correction for multiple comparisons, methylation of *MLH1_b* (p=0.001) and *BRCA1* (p=0.002) were significantly lower in pleomorphic ILC compared to IDC (**Figure 2A and B**). The mean CMI of pleomorphic ILC was not significantly different from IDC (353.3 vs. 392.6, respectively, p=0.357), indicating that the overall methylation patterns of the two breast cancer subgroups were similar. Logistic regression analysis showed that only *BRCA1* methylation (p=0.002) had an independent discriminative value for pleomorphic ILC versus IDC (area under the curve (AUC) 781, CI 0.623– 0.939, p=0.004), with a ROC curve based sensitivity and specificity of 75% and 81%, respectively.

Correlation between methylation and mRNA expression

Our analysis showed that *TP73*, *MLH1, RASSF1A* and *BRCA1* may serve as a biomarker to distinguish pleomorphic ILC from classic ILC or IDC. To see if the methylation of these genes actually reduces protein expression, we used the methylation and mRNA data of these genes from TCGA. **Table 3** shows higher methylation percentages of *MLH1* (p<0.0001 for one of the CpG locations), *TP73* (p=0.01), *RASSF1A* (p=0.046) and *BRCA1* (p<0.001) are indeed significantly correlated with lower mRNA expression z-scores.

Clustering analysis of the methylation data

In order to determine if the absolute MS-MLPA values of our samples clearly defined our three breast cancer subtypes, we performed a hierarchical Euclidean cluster analysis on all genes. This cluster analysis demonstrated that neither of the three groups formed a defined subgroup based on the absolute methylation levels of these genes (**Figure 3A**). Hierarchical cluster analysis on the four genes that significantly differed by the Mann-Whitney tests, showed that methylation levels of these genes lightly clustered the pleomorphic ILC samples into a separate group, while methylation levels did not stratify between classic ILC and IDC (**Figure 3B**).

Discussion

To our knowledge, this work represents the first study investigating the methylation status of pleomorphic ILC. In our search for methylation biomarkers that aid the differential diagnosis between pleomorphic ILC and classical ILC, we found significant differences in the methylation of *TP73*, *MLH1* and *RASSF1A*, while the cumulative methylation index (CMI) was not significantly different between the two breast cancer subtypes. Interestingly, logistic regression analysis on the methylation data of the analyzed tumor suppressor genes showed that the combined methylation data of *TP73* and *RASSF1A* was able to distinguish pleomorphic from classic ILC, while *BRCA1* was able to separate pleomorphic ILC from IDC. Overall our findings indicate that MS-MLPA facilitates the differential diagnosis between pleomorphic ILC and other breast cancer subtypes.

The different advantages and possibilities of DNA methylation towards disease stratification and prognostic capabilities have led to a growing amount of DNA methylation studies currently implicating the methylation of over a hundred genes in breast cancer tumorigenesis,

reviewed in [39]. However, the vast majority of these reports focus on IDC and we are unaware of any publications on DNA methylation in pleomorphic ILC. Interestingly, two reports compared the DNA methylation pattern in ILC and IDC, and showed that the patterns are highly similar in these breast cancer subtypes [40,41]. Our results confirm this similarity between IDC and classic ILC, but pleomorphic ILC appeared to have a somewhat different methylation pattern (**Figure 3A and B**), while the CMI was similar to that of classic ILC and IDC.

BRCA1, MLH1 and *RASSF1A* are established and *TP73* is a putative tumor suppressor gene. *BRCA1* and *MLH1* are involved in DNA repair and their loss causes accumulation of somatic genetic defects. While the function of *MLH1* as a tumor suppressor is still unclear, *BRCA1* is an established tumor suppressor and *BRCA1* methylation is observed in 10-15% of all sporadic breast cancer patients [42,43]. Only 4-5% of the lobular breast tumors carry a classified *BRCA1* deletion [44], and the *BRCA1* methylation status in ILC has only been analyzed once before, where none of the eleven ILC samples showed *BRCA1* methylation [43]. As we found *MLH1* and *BRCA1* methylation to be lower in pleomorphic ILC compared to IDC, their methylation is not a suitable therapeutic target, but can better be used as a biomarker. As the absolute values of *MLH1* and *BRCA1* methylation were low in our patient series, some reservation is in order regarding their possible discriminative value.

The putative tumor suppressor *TP73* is subject of alternative splicing, and the presence of an alternative promoter yields many different p73 isoforms that exhibit contrasting effects on tumor development [45]. Paradoxically, although *TP73* methylation has been correlated with poor survival of breast cancer patients [46], *TP73* methylation also impairs binding of the transcriptional repressor ZEB1, which may thereby increase *TP73* expression [47]. Unfortunately, studies analyzing methylation levels of *TP73* in normal breast tissue are scarce and are not combined with protein or RNA expression studies [30,48]. Furthermore, *TP73* hypermethylation has not been studied in ILC yet. As we found *TP73* promoter methylation to be relatively low in pleomorphic ILC compared to classic ILC, it may serve as a good biomarker, but it will be less suited for targeted therapy in pleomorphic ILC. Further studies are needed to determine the effect of *TP73* methylation on protein expression levels and to determine the functional effect in pleomorphic ILC.

RASSF1A methylation was higher in pleomorphic ILC compared to classic ILC. Although *RASSF1A* deletion is uncommon, polymorphisms and deletions have been found and *RASSF1A* hypermethylation frequently occurs in many different tumor types [49]. About 70-85% of ILC as well as IDC cases show *RASSF1A* methylation [41,50]. Also, hypermethylation of *RASSF1A* in pre-operative serum of breast cancer patients was an independent prognostic marker and correlated with poor overall survival [51]. As *RASSF1A* hypermethylation is rarely found in normal breast tissue it is considered an early event in breast tumorigenesis [51,52], and therefore a promising breast cancer biomarker. The *RASSF1* gene is a member of the RASSF family (RASSF 1-8), and gives rise to 8 different isoforms due to alternative splicing and alternative promoter usage [53]. Besides the RASSF proteins, Raf and phosphatidylinositol 3-kinase (PI3K) are also known as Ras effectors. Ras effectors are proteins that specifically bind the GTP-bound form of RAS. In contrast to RAF and PI3K, which control proliferation and survival, the *RASSF* genes are known tumor suppressors [53]. *RASSF1A*

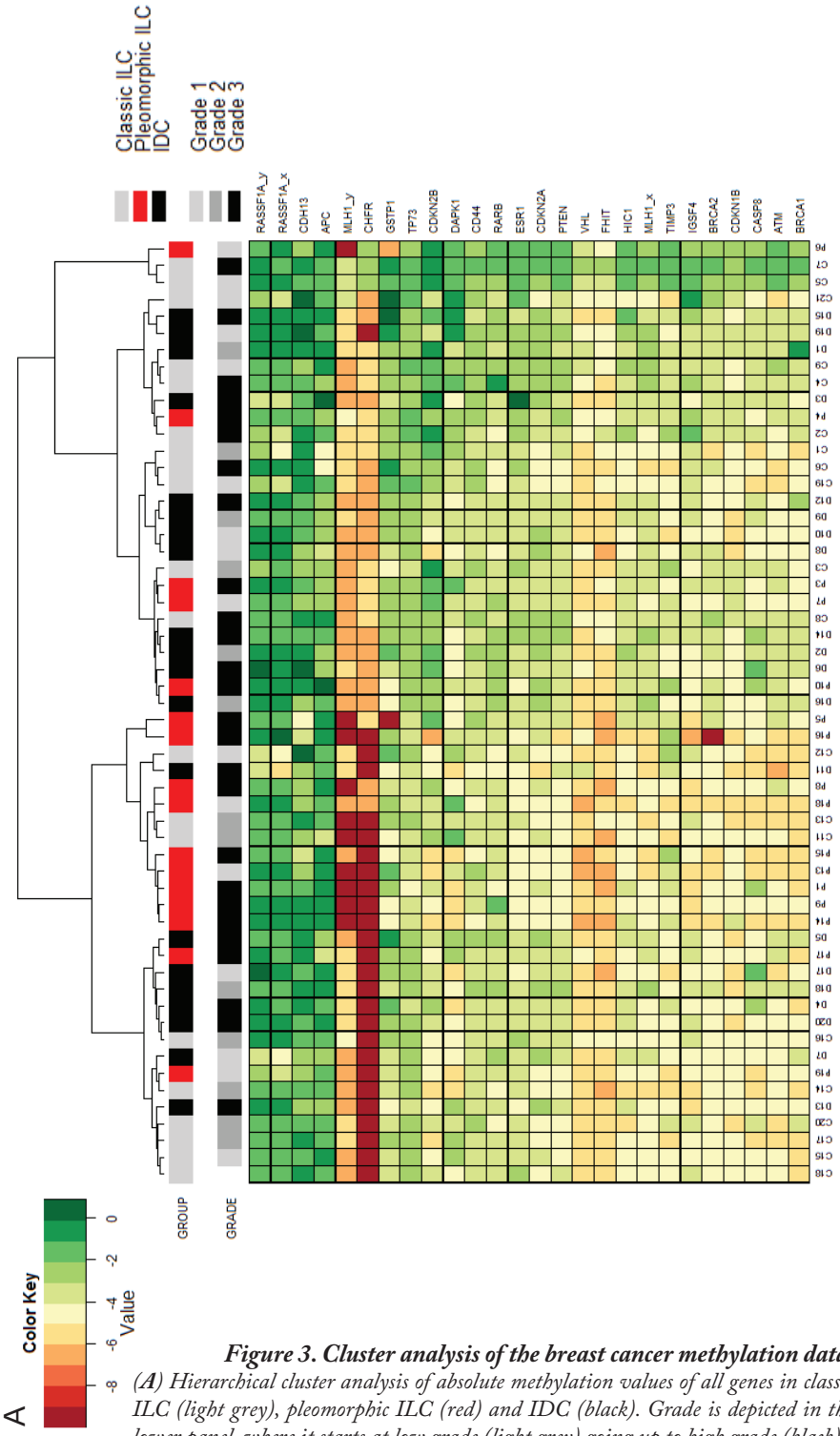
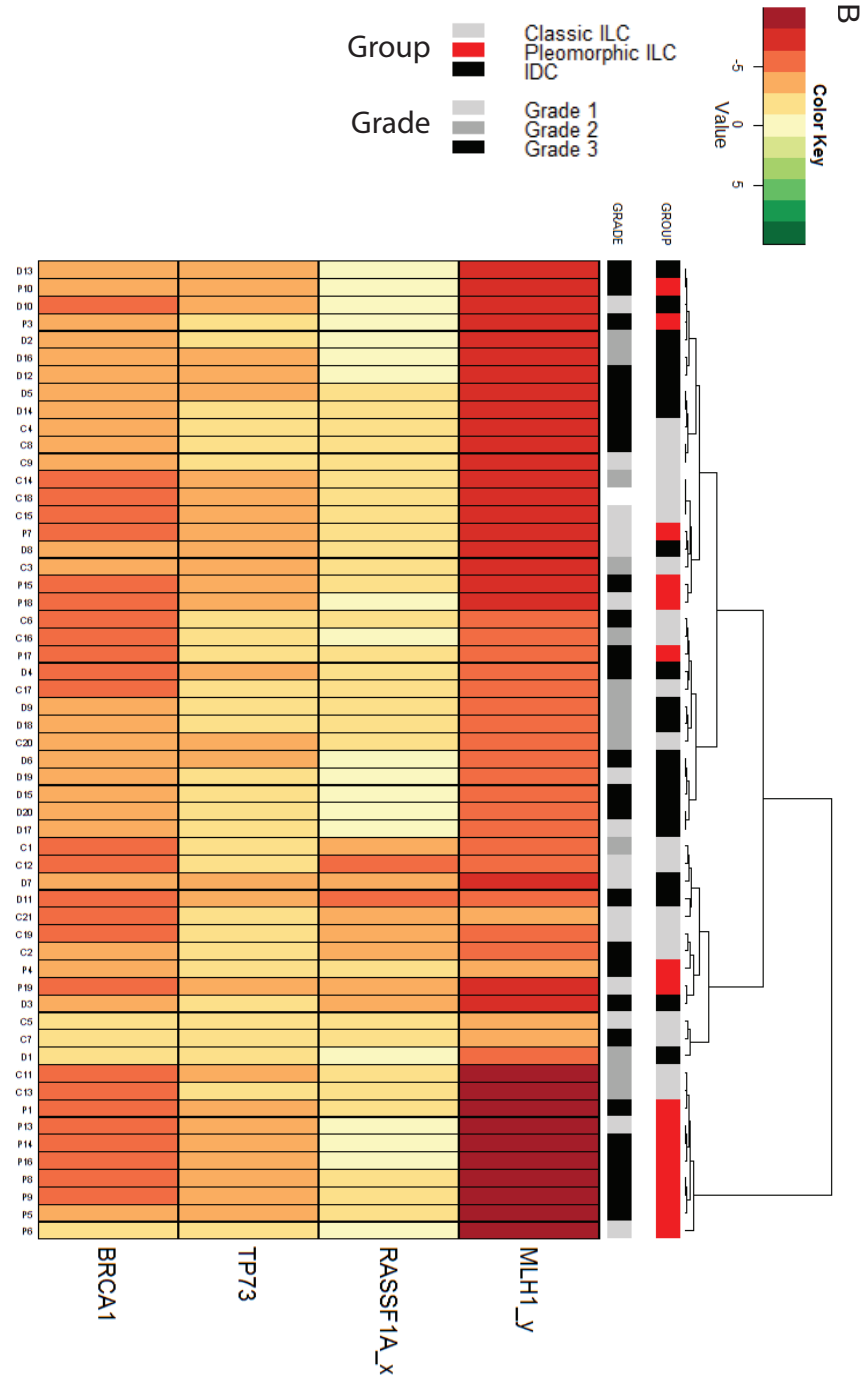


Figure 3. Cluster analysis of the breast cancer methylation data.
(A) Hierarchical cluster analysis of absolute methylation values of all genes in classic ILC (light grey), pleomorphic ILC (red) and IDC (black). Grade is depicted in the lower panel, where it starts at low grade (light grey) going up to high grade (black)



*(**B**) Hierarchical cluster analysis of absolute methylation values of the 4 significantly different genes according to the Mann-Whitney analysis. Pleomorphic ILC cases (red) seem to have a slightly different pattern compared to the other breast cancer cases.*

null-mice show an increased incidence of spontaneous tumor formation, decreased survival rate and an increased susceptibility for mutagens (reviewed in [53]). Forced expressions of *RASSF1A* in different tumor cell lines reduces their viability, proliferation and invasion [53]. These findings combined with our data showing increased *RASSF1A* promoter methylation in pleomorphic ILC, renders *RASSF1A* an interesting and functional biomarker in lobular breast cancer.

In order to evaluate if the pathways in which *MLH1, BRCA1, TP73* and *RASSF1A* are involved can be used as therapeutic targets it is important to know if their methylation actually correlates with lower mRNA and protein expression. Interestingly, for *MLH1, BRCA1, TP73* and *RASSF1A*, TCGA mRNA expression levels significantly correlated with the amount of promoter methylation present, indicating the functional significance of methylation in these CpG regions. These results should however be interpreted with caution as there was no exact match between CpG sites by MS-MLPA and CpG sites evaluated by TCGA (distance between CpG sites for both platforms varied between 53 and 367 nucleotides, **Table 3**).

In conclusion, our data indicate that the methylation status of *TP73*, *MLH1* and *RASSF1A* and *BRCA1* may be used as a biomarker for the differential diagnosis to distinguish pleomorphic ILC from classic ILC and IDC. As pleomorphic ILC is considered to be an aggressive breast cancer variant and as pre-operative MRI is favorable for ILC, but not for IDC patients, having pleomorphic ILC biomarkers can be useful for treatment design in cases where the pathological distinction between ILC and IDC is questionable. Future research is needed to confirm our findings in an independent patient group and to evaluate their possible role as a therapeutic target.

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Author Contributions

Experiments were conceived and designed by EJV, PJD and PWBD. EJV performed the experiments. EJV, CE and PJD performed the pathological analyses and scoring of human tumor samples. EJV and CM analyzed the methylation data. EJV, CM, PJD and PWBD wrote the paper.

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Chapter 4

αE–catenin inactivation leads to loss of cell-cell adhesion and Rock-dependent anoikis resistance in breast cancer

Eva J. Vlug, Mijanou Niklaas, Tanja Eisemann and Patrick W.B. Derksen

Under construction

Abstract

Loss of E-cadherin function and the subsequent acquisition of anchorage independence underlie the development and progression of Invasive Lobular Carcinoma (ILC), the second most common type of breast cancer. Interestingly, although mutational inactivation of E-cadherin is the main driver of ILC, approximately 10% of all ILCs retain membrane-localized E-cadherin, despite the presence of an apparent non-cohesive and invasive lobular growth pattern. The recent identification of αE-catenin (*CTNNA1*) inactivating mutations in breast cancer cell lines and isolated cases of invasive gastric and breast cancer have advocated α-catenin as a tumor suppressor in ILC. In this study we investigated the consequences of αE-catenin loss in the regulation of anchorage independence of non-invasive breast cancer cells. Our data show that inducible loss of αE-catenin in mouse and human E-cadherin expressing breast cancer cell lines induced aberrant localization of the adherens junction, a rounded cell morphology and anoikis resistance, which could be reverted upon reconstitution of a non-targetable αE-catenin cDNA. Pharmacological inhibition experiments subsequently revealed that anoikis resistance induced by αE-catenin loss was dependent on activation of the Rho/Rock-dependent actomyosin contractility, alike E-cadherin mutant ILC. In conclusion, our results show that αE-catenin loss leads to lobular morphological features and Rock-dependent anchorage independence in the presence of membrane-localized E-cadherin. As such it suggests that loss of αE-catenin may represent an alternative AJ-inactivating event leading to the development and progression of E-cadherin expressing ILC.

Introduction

Invasive Lobular Carcinoma (ILC) is a breast cancer subtype characterized by non-cohesive infiltrative growth patterns, diffuse dissemination and loss of E-cadherin expression (reviewed in [1]). E-cadherin (*CDH1*) controls epithelial integrity through formation of the adherens junction (AJ), through indirect linkage to the actin and microtubule cytoskeleton via αE-catenin (from hereon; α-catenin), α-catenin and p120-catenin (p120) [2]. While the exact mechanisms remain unclear, α-catenin connects the AJ with the actin cytoskeleton through binding of α-catenin and actin filaments (reviewed in: [3]).

Evidence from human cancer and conditional mouse models have established that mutational inactivation of E-cadherin underlies the formation and progression of invasive lobular breast cancer (ILC) and hereditary diffuse-type gastric cancer [4-7]. In ILC, p120 translocates to the cytosol where it binds and inactivates the RhoA antagonist myosin phosphatase Rho–interacting protein (Mrip), which results in the activation of RhoA/Rock1 signaling and subsequent anoikis resistance [8]. Sequencing efforts have shown that *CDH1* is mutationally inactivated in approximately half of the lobular breast cancer cases analyzed, confirming that loss of *CDH1* is a driver even in lobular breast cancer. However, a minority of ILC patients have retained E-cadherin expression without truncating or frame shift mutations, indicating that in these cases functional inactivation of AJ junction must have occurred through means other than somatic loss or epigenetic silencing of E-cadherin.

α-catenin is essential for proper cell-cell adhesion through control of actin dynamics (reviewed in: [9]). Underlying these functions is the fact that α-catenin drives AJ maturation through formin-dependent radial actin formation [10,11], which facilitates a robust increase in tensile strength [12]. Next to binding and bundling of actin filaments, α-catenin also inhibits actin branching by competing with the Arp2/3 complex for actin binding [13]. Moreover, α-catenin can enhance p120-catenin binding to E-cadherin, thereby facilitating junctional stability [14]. Studies in different organ systems have suggested that α-catenin functions as a tumor suppressor. For instance, conditional ablation of α-catenin in the skin or cerebral cortex of mice caused epidermal and cerebral hyper proliferation [15,16]. Also, loss of α-catenin expression is a prognostic factor for poor survival of breast and other cancers (reviewed in: [17]). Supporting this tumor suppressor scenario are recent studies that identified inactivating *CTNNA1* mutations in lobular-type breast cancer cell lines [18,19]) and a case of diffuse gastric cancer [20].

In this study we examined whether loss of α-catenin in nonmetastatic breast cancer cell lines expressing a functional AJ leads to the acquisition of prometastatic features. We observed that α-catenin loss induced a switch from a classical epithelial to a rounded non-cohesive growth pattern while retaining expression of wild-type E-cadherin. Importantly, α-catenin loss induced anoikis resistance in mouse and human breast cancer cell lines, a cancer progression hallmark that was dependent on active Rho/Rock-dependent actomyosin contractility.

Materials and Methods

Cell lines and Cell culture

Mouse cell lines Trp53$^{\Delta/\Delta}$-3 (KP6) and Trp53$^{\Delta/\Delta}$-7 (WP6) were derived from tumors that developed in *K14cre;Trp53$^{F/F}$* and *Wcre;Trp53$^{F/F}$* female mice and cultured as described [6,21]. MCF7 was obtained from the American Type Culture Collection (ATCC), STR type verified by PCR and cultured as described previously [22].

Constructs, Viral production and Transduction

SiRNA sequences targeting mouse (5'-GTCACATGCTTCACTCAAA-3') and human (5'-GTCACTGTTCGTCACTCAA-3') α-catenin were cloned as shRNA in the lentiviral vector pFUTG as described [8]. The shRNA against mouse α-catenin is positioned in the '5-UTR. Lentiviral particles were produced in COS-7 and used for transduction as described [8]. To induce knock-down, cells were treated with 2mg/ml doxycycline (1:5000, Sigma-Aldrich, D9891) for at least 3 days.

Western Blotting

Samples were lysed in sample buffer (50 mM Tris-Cl (pH 6.8), 0.5% β-mercaptoethanol, 2% SDS, 0.005% bromophenolblue, and 10% glycerol) (all Sigma-Aldrich), heated for 10 minutes at 100˚C and proteins were separated and western blotted as described [23]. Antibodies used were: Gapdh (1: 2,000; Millipore, Mab374), Akt1 (1:1000; Santa Cruz, Sc-1618), α-catenin (1:2000; Sigma-Aldrich, C2081), E-cadherin (1:2,000; BD Biosciences, 610182), p120-catenin (1:2,000; BD Biosciences, 610134), β-catenin (1:2,000; BD Biosciences, 610154), phospho-cofilin (Ser3) (1:2,000; Cell Signaling, 3311) and Cofilin (1:500; Cell Signaling, 3312). All blots were incubated for 30 min with either rabbit anti-goat-PO (DAKO, P160), goat anti-rabbit-PO (Bio-Rad, 170-6515) or goat anti-mouse-PO (Bio-Rad, 170-6516) secondary antibodies.

Immunofluorescence, Confocal Microscopy and Phase Contrast Microscopy

Glass coverslips were coated with 10μg/mL Collagen 1 (BD Biosciences, 354236) and residual coating was removed by washing with PBS. Cells were cultured on coverslips, fixed with 100% Methanol for 10 minutes on ice, permeabilized using 0.3% Triton-X100/PBS and subsequently blocked with 4% Bovine Serum Albumin (BSA) Fraction V (GE-Healthcare, K45-001) in PBS. Samples were incubated with antibodies against α-catenin (1:1,000; Sigma-Aldrich, C2081), or (clone 15D1/1:100; Enzo Life Sciences, Alx-804-101), anti-E-cadherin-TRITC (1:300, BD Biosciences, 612130), anti-p120-catenin-TRITC (1:300, BD Biosciences, 610137) or mouse anti-β-catenin (1:50, BD Biosciences, 610154) at room temperature for 1 hour. After washing slides were incubated with secondary antibodies: goat-anti-mouse Alexa-405 (1:600, Invitrogen, A31553) or antibodies goat-anti-rabbit Alexa-405 (1:600, Invitrogen, A31556) for 1 hour. DNA was stained with ToPro-3 (Life Technologies). Cover slips were mounted using Vectashield (Vector Laboratories, X1215) and analyzed with a Zeiss LSM 700 confocal laser microscope using a 63X 1.4 objective. Phase contrast pictures were produced with a Leica DMI 4000B microscope (20x objective).

Anoikis Resistance and Inhibitors

Anoikis Resistance was determined as described [8]. Cell survival was defined as the Annexin-V and Propidium Iodide negative population after FACS analysis. Survival of control cells was normalized to 1. For inhibition studies cells were treated with: 0.02μg/mL C3 transferase (Cytoskeleton, CT04-A), 10 μM Y27632 (Selleckchem, S1049), 1 μM GSK-429286A (Selleckchem, S1474) or 3 μM Blebbistatin (VWR, 203390). Standard deviation calculations were performed to calculate the error bars. A two-sided student's t-test was performed to calculate statistical significance, values of $p \leq 0.05$ were considered to be significant.

Results

Loss of α-catenin leads to a dysfunctional epithelial adherens junction complex and a rounded cell morphology.

To investigate how loss of α-catenin affects AJ formation in noninvasive breast cancer cell lines, we made use of E-cadherin expressing breast cancer cell lines (mouse $Trp53^{\Delta/\Delta}$ and human MCF7). These cell lines form mature epithelial AJs and show a classical cobblestone-type morphology under normal culture conditions. To enable inducible loss of function studies we cloned shRNA sequences targeting α-catenin into a doxycycline (Dox) inducible lentiviral knock-down (iKD) system and transduced the cell lines. Addition of Dox induced a strong reduction in the levels of α-catenin, while the expression of E-cadherin, p120 and α-catenin remained unaffected (**Fig. 1a and Supplemental Fig. 1a**). Culturing iKD cells in the presence of Dox led to a rounded cell morphology, indicating a reduction of cell-cell and cell-matrix dependent adhesion (**Fig. 1b and Supplemental Fig. 1b**). Loss of α-catenin also induced a non-cohesive growth pattern that prevented confluence and formation of an epithelial sheet (**Fig. 1b and Supplemental Fig. 1b**). Although we did not observe quantitative protein differences using western blot, α-catenin loss induced a marked change in E-cadherin distribution and AJ formation in mouse and human breast cancer cells (**Fig. 1c and Supplemental Fig. 1c**). At sites of cell-cell adhesion, E-cadherin, α-catenin and p120 still localized to the cell membrane, but maturation of the AJ appeared attenuated, which was characterized by aberrant localization of the AJ members in distinct puncta on the plasma membrane (Fig. 1c, indicated by arrows). We furthermore observed an increase in the cytosolic localization of the core AJ members, suggesting active recycling of the AJ (**Fig. 1c, indicated by arrow heads**). To determine specificity of our targeting approach and control for off-target shRNA effects, we performed a reconstitution assay by introducing a nontargetable and GFP-tagged α-catenin cDNA (Rescue). Reconstituted iKD cells expressed α-catenin at wild-type levels, which restored AJ formation and function, leading to formation of a classical epithelial morphology in the presence of Dox (**Fig. 1; + Rescue**). These results show that α-catenin loss prevents proper AJ formation, which results in a rounded and detached cell morphology in the presence of membrane-localized E-cadherin.

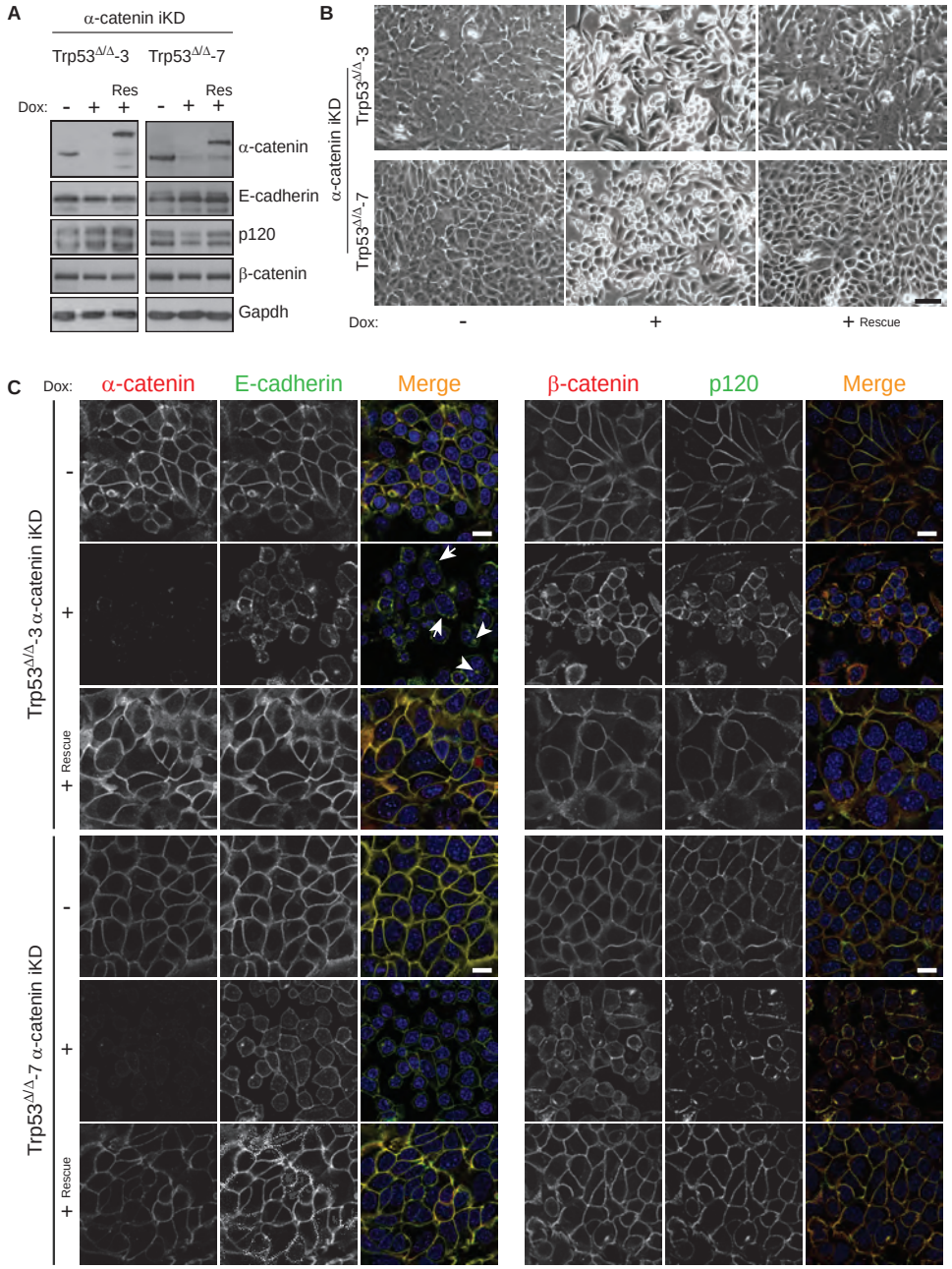

Figure 1. Loss of α-catenin induces loss of epithelial cell morphology and leads to aberrant localization of AJ members.

*(**A**) Inducible knock-down of α-catenin (α-cat iKD) does not lead to inhibition of AJ complex member expression levels. Western blot showing the extent of α-catenin iKD (+ Dox) on E-cadherin, p120 and β-catenin. Right lanes (+ Rescue) shows the effects of an α-catenin-GFP cDNA reconstitution. Gapdh levels were used as loading control. (**B**) Loss of α-catenin induces a rounded and non-adherent cell morphology. Phase-con-*

*trast images of α-catenin iKD and rescue cell lines. Size bar indicates 50 μm. (**C**) Dysfunctional formation of the AJ upon α-catenin loss. Shown are immunofluorescence images for the AJ complex members α-catenin, E-cadherin, p120 and β-catenin in control (- Dox), α-catenin iKD (+ Dox) and Rescue cells (+ Rescue). Note the distinct clustering of the AJ in membrane-localized puncta upon α-catenin loss (arrows) and the cytosolic localization of E-cadherin (arrow heads). Size bar indicates 10 μm.*

Loss of α-catenin results in anoikis resistance of E-cadherin expressing breast cancer cells.

We have previously established that E-cadherin loss is causal to the acquisition of anoikis resistance in breast cancer cells, a phenotype that coincided with metastases formation in mice [6]. Because *in vitro* anoikis resistance predicts metastatic potential in these cell systems, we investigated the effect of α-catenin iKD on anchorage independence. After culturing mouse and human α-catenin iKD cell lines under non-adherent conditions in the presence of Dox for 4 days, we analyzed cell survival by FACS analysis. While the majority of the mouse and human control iKD cell lines (no Dox) underwent anoikis, α-catenin loss induced a significant 3 to 5-fold increase in anoikis resistance (**Fig. 2a and 2b**). Reconstitution with α-catenin resulted in a full reversal of anoikis resistance to wild type levels (**Fig. 2a**). Together, these data indicate that α-catenin exerts tumor suppressor activity in nonmetastatic breast cancer cell lines.

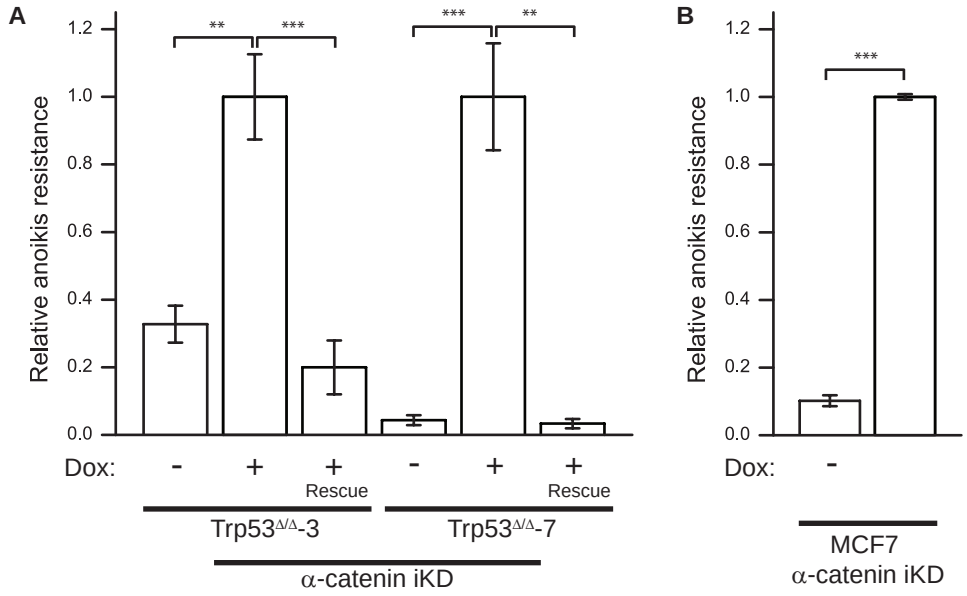

Figure 2. Loss of α-catenin induces anoikis resistance in mouse and human cell lines.
*Anoikis resistance was determined in mouse (**A**) and human (**B**) breast cancer cell lines upon α-catenin iKD by FACS analysis using binding to Annexin-V and propidium iodide. Note the reversal to anoikis sensitivity upon reconstitution of α-catenin in the mouse Trp53Δ/Δ cell lines (+ Dox Rescue). Error bars represent the standard deviation of three independent experiments. * p<0.05; ** p=0.01; *** p<0.01.*

Anoikis resistance upon loss of α-catenin is dependent on an active Rho-Rock-Actin pathway.

Although the vast majority of p120 localized to sites of E-cadherin expression, we observed a slight increase in cytosolic p120 levels upon α-catenin iKD (**Fig. 1c and Supplemental Fig. 1c**). We therefore asked whether this cytosolic p120 pool could promote anoikis resistance in the context of α-catenin loss. Hereto we performed a dual iKD for p120 and α-catenin, assessed anoikis resistance and observed that concomitant p120 and α-catenin loss did not reduce anoikis resistance (**Supplemental Fig. 2a and b**). Since we have recently demonstrated that (i) p120 inactivation in E-cadherin expressing cells leads to anoikis resistance (Schackmann, van Amersfoort et al. 2011), (ii) p120 loss does not induce additional anoikis resistance in α-catenin iKD cells (**Supplemental Fig. 2b**), and (iii) the vast majority of p120 is complexed with E-cadherin, our data indicate that loss of α-catenin is sufficient to induce functional inactivation of the AJ. Because ILC depends on Rock activity for anchorage-independent tumor growth and metastasis, we assayed the effect of α-catenin loss on Rho-Rock pathway activity. Using Serine phosphorylation of Cofilin as a surrogate read-out for the activity of Rho and Rock, we observed that α-catenin loss resulted in an increase in phosphorylated Cofilin in human and mouse breast cancer cells (**Fig. 3a**), indicating activation through Rock1. Indeed, treatment of the α-catenin iKD cell lines with the pharmacological Rock inhibitor GSK-429286A [24], decreased phosphorylation of Cofilin in the presence of Dox and, surprisingly, reverted the rounded cell morphology when cells were cultured in 2D (**Fig. 3a and b**). Because α-catenin negative cells are unable to form mature AJ (**Fig. 1**), and the α-catenin iKD cells did not show a restoration of epithelial junctions upon Rock inhibition (**Fig. 3B; right panels**), our data suggest that detachment in 2D upon α-catenin loss is due to inhibition of an integrin-based cell-matrix adhesion, rather than a cadherin-dependent homotypic interaction. To further investigate the dependency of the Rho-Rock pathway during anoikis resistance in α-catenin iKD cells, we tested the effect of several inhibitors that act upstream or downstream of Rock. Pharmacological inhibition of Rho (C3 transferase), Rock (GSK-429286A and Y-27632) and myosin II (Blebbistatin) all resulted in a robust reduction of anoikis resistance (**Fig. 3C and 3D**), indicating that –alike E-cadherin mutant ILC– Rock-dependent actomyosin contraction underpins anoikis resistance in the absence of α-catenin.

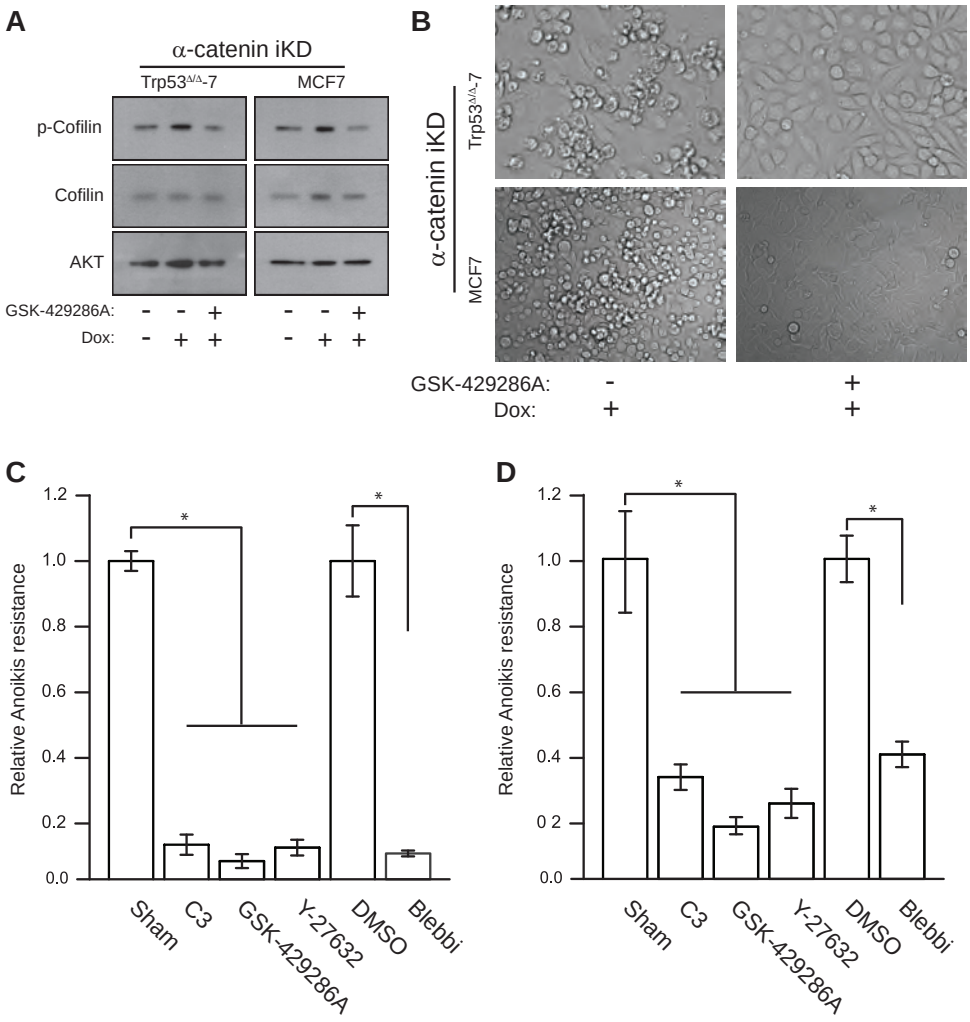

Figure 3. Rho-Rock actomyosin contractility controls anoikis resistance upon loss of α-catenin.
*(**A**) Western blot showing Cofilin levels in α-catenin iKD cell lines in the absence or presence of Dox and the Rock inhibitor GSK-429286A. AKT levels were used as a loading control. (**B**) Inhibition of Rock in Dox-treated α-catenin iKD cells restores AJ independent cell spreading in 2D. Phase-contrast pictures of α-catenin iKD cells after inhibition of Rock using 1μM GSK-429286A. (**C and D**) Rho-Rock-dependent actomyosin contraction controls anoikis resistance in the absence of α-catenin. Anoikis resistance was determined by FACS analysis after culturing mouse (**C**) or human (**D**) breast cancer cells in the presence of Dox and the indicated inhibitors. Controls without inhibitors (Sham or DMSO) were set to 1. Error bars represent the standard deviation of three independent experiments. * p<0.05.*

Discussion

Inactivation of the AJ through loss of E-cadherin underlies tumor development and progression in ILC and hereditary diffuse gastric cancer. Our previous findings in mice and man have pointed toward aberrant activity of Rho/Rock-dependent actomyosin contractility as a driving force of ILC tumor progression. Interestingly, E-cadherin mutant breast cancer cells seem specific in the sense that they use p120 to bind and dampen the Rho antagonist MRIP, resulting in activation of Rho and Rock [8]. Interestingly, inactivation of the AJ *per se* is not sufficient to drive formation of a specific breast cancer subtype. Whereas somatic ablation of E-cadherin in the mammary gland results in metastatic lobular breast cancer, inactivation of p120 induces formation of invasive metaplastic and basal-like IDC [6,22]. We anticipate that lobular breast cancer may uniquely depend on Rho/Rock-driven actin remodeling, given the fact that p120 null cells from the p120 knockout model and IDC-derived human tumor cell lines (*eg.* MDA-MB-231) do not rely on active Rock signals for their anchorage-independent survival [8,25]. Second, although p120 regulates Rho signaling at the cell cortex, it is not directly physically connected to the actin cytoskeleton (for a review see [26], which may explain why loss of p120 in mice (although it leads to inactivation of the AJ and induces metastasis) does not result in a lobular tumor phenotype, but leads to a metaplastic and ductal-type breast cancer [22]. Since p120 (*CTNND1*) mutations are extremely rare in breast cancer and breast cancer cell lines, the available data point to a scenario where p120 inactivation is not a breast cancer driver in ILC, but instead a potent tumor progression suppressor that is inactivated at later stages of non-lobular breast cancer development.

In contrast, bi-allelic inactivating mutations in *CTNNA1* have been described in several breast cancer cell lines (Hazan, 1997; Hollestelle, 2009). Because these cell lines are luminal and express membrane-bound wild-type E-cadherin, these findings suggest that disruption of the link between E-cadherin, α-catenin and actin may underpin lobular carcinoma development. Furthermore, a frame-shift mutation in *CTNNA1* was recently described in a family with hereditary diffuse gastric cancer (HDGC) without detectable E-cadherin defects [20]. Alike ILC, HDGC development is causally linked to mutational inactivation of E-cadherin, which implies that there may be a causal link between inactivation of α-catenin and the development of ILC.

Despite the low abundance of *CTNNA1* mutations in breast cancer, we probed its potential tumor suppressor functions. Hereto we silenced α-catenin in nonmetastatic and anchorage-dependent breast cancer cells from human and mouse origin, and assessed acquisition of a key metastatic trait; anoikis resistance. In line with the published findings in human α-catenin mutant breast cancer cell lines we observed that α-cat iKD resulted in a rounding of cells in cell culture. Given that α-catenin is necessary for proper lateral E-cadherin clustering and subsequent AJ maturation, and the fact that this phenotype in 2D could be fully reversed upon inhibition of the Rho/Rock pathway, it appears that inactivation of α-catenin leads to detachment of cells through an integrin/ECM-dependent adhesion mechanism. While we have not probed the underlying mechanism, we hypothesize that the mechanical forces induced by activation of Rho/Rock-dependent actomyosin contraction upon loss of α-catenin result in detachment of the ECM/integrin linkage, which may have substantial consequences for migration and invasion of tumor cells. Second, α-catenin loss induced anchorage independence, a cancer hallmark that prognosticates metastatic potential [6,27]. Given that α-catenin iKD cells acquire anoikis resistance and show detachment from the ECM, we envisage

that loss of α-catenin in cancer may result in a double-edged sword that strongly drives tumor progression and development through constitutive activation of anchorage-independent cell survival and cellular motility. While the exact mechanism remains to be clarified, it is clear that activation of Rho/Rock-driven actin contraction is key to anchorage independence and ECM detachment, and as such may underlie ILC tumor etiology.

Loss of α-catenin in the skin leads to hyper-proliferation of epidermal epithelial cells through activation of the RAS-MAP kinase pathway [15]. Cell accumulation in this context was recently attributed to negative regulation of YAP1 by α-catenin [28,29]. Here it was proposed that the α-catenin/YAP1 connection controls 'crowd sensing', leading to unrestricted growth upon α-catenin loss though nuclear localization of YAP, which is known to induce subsequent transcriptional activation of proliferative target genes [30]. In this scenario, F-actin bundling induced by active Rho-Rock signals cause nuclear localization of YAP, YAP-dependent transcriptional activity and subsequent cellular proliferation [31]. In contrast to these findings we have not observed a clear nuclear translocation of YAP upon α-catenin iKD. Whereas mouse Trp53$^{\Delta/\Delta}$-3 showed a switch from cytosolic to nuclear YAP, Trp53$^{\Delta/\Delta}$-7 and human MCF-7 did not (data not shown). Given that loss of AJ function leads to sensitization of growth factor receptor signaling [22,32], we envisage that YAP may aid anchorage independence because it can be regulated by AKT, leading to cytosolic retention and thereby preventing expression of pro-apoptotic targets [33]. Cell-specific differences may exist upstream of YAP, especially regarding the formation and function of the AJ. For example, while ablation of either E-cadherin, p120 or α-catenin in the skin induces aberrant hair follicle formation, differentiation defects and proliferation [15,34,35], somatic inactivation of these AJ members in the postnatal mammary gland is not tolerated [21,36-38]. As such, inactivation of the AJ in breast cancer needs to be preceded by activation of an oncogene or inhibition of a tumor suppressor. Indeed, our previous work confirmed this by showing that conditional inactivation of p53 results in tolerance to mammary specific loss of E-cadherin or p120 [6,22]. Although these models have not been generated in the context of α-catenin loss, we foresee that loss of α-catenin in a p53-deficient background may lead to ILC. However, the lack of somatic inactivation *CTNNA1* mutations in primary breast cancer samples implies that α-catenin may have additional roles that (if inhibited) may prevent a survival advantage and subsequent Darwinian selection in the formation of breast cancer. Considering this with the key roles of α-catenin in actin remodeling, regulation of integrin-based adhesion and transduction of YAP signals, it is not surprising that mutational inactivation of α-catenin is a very rare event, especially in breast cancer.

Evidently, inactivation of the AJ results in activation of Rho and Rock. While maintenance of Rho-Rock activity may be conferred by cytosolic p120 in E-cadherin mutant ILC, this is not the case in breast cancer cells that have inactivated α-catenin. Here, the p120 does not contribute to activation of anoikis resistance, but instead mainly resides in a complex with E-cadherin. In this context p120 is not a player in tumor progression because (i); E-cadherin function is already fully impaired due to α-catenin loss and (ii); cytosolic p120 levels may not be sufficient to confer control Rho activation. Thus, although we have not identified the upstream cue that instigates RhoA activity upon AJ dismantling, it is clear that actomyosin contraction is aberrantly activated upon α-catenin loss and activation of the Rho-Rock-actin axis underpins anchorage independence. Apart from autocrine factors promoting Rho/Rock activation, we think that Rho activity is most likely caused by intracellular cytoskeletal ten-

sion. This can be caused by cellular rounding due to the disruption of the E-cadherin linkage to actin, which could potentially induce a positive feed-back loop resulting in high Rho-GTP, as has been shown previously by others [39,40].

In closing, we show that α-catenin functions as a tumor suppressor in E-cadherin expressing nonmetastatic tumor cells though loss of cell-cell and cell-matrix interactions and the acquisition of anchorage-independence. Importantly, α-catenin loss confers anoikis resistance through activation of Rho and Rock-dependent actomyosin contraction, a hallmark of E-cadherin mutant ILC. We have thereby advocated loss of α-catenin as a potential driving mechanism underpinning ILC tumor etiology. Since E-cadherin links to the actin cytoskeleton through α-catenin, our data suggests that the mechanisms leading to ILC development and progression are dependent on common downstream effectors that activate Rock-dependent actomyosin contraction. Our findings emphasize the potential clinical ramifications of Rock inhibition in the treatment of ILC.

Acknowledgements

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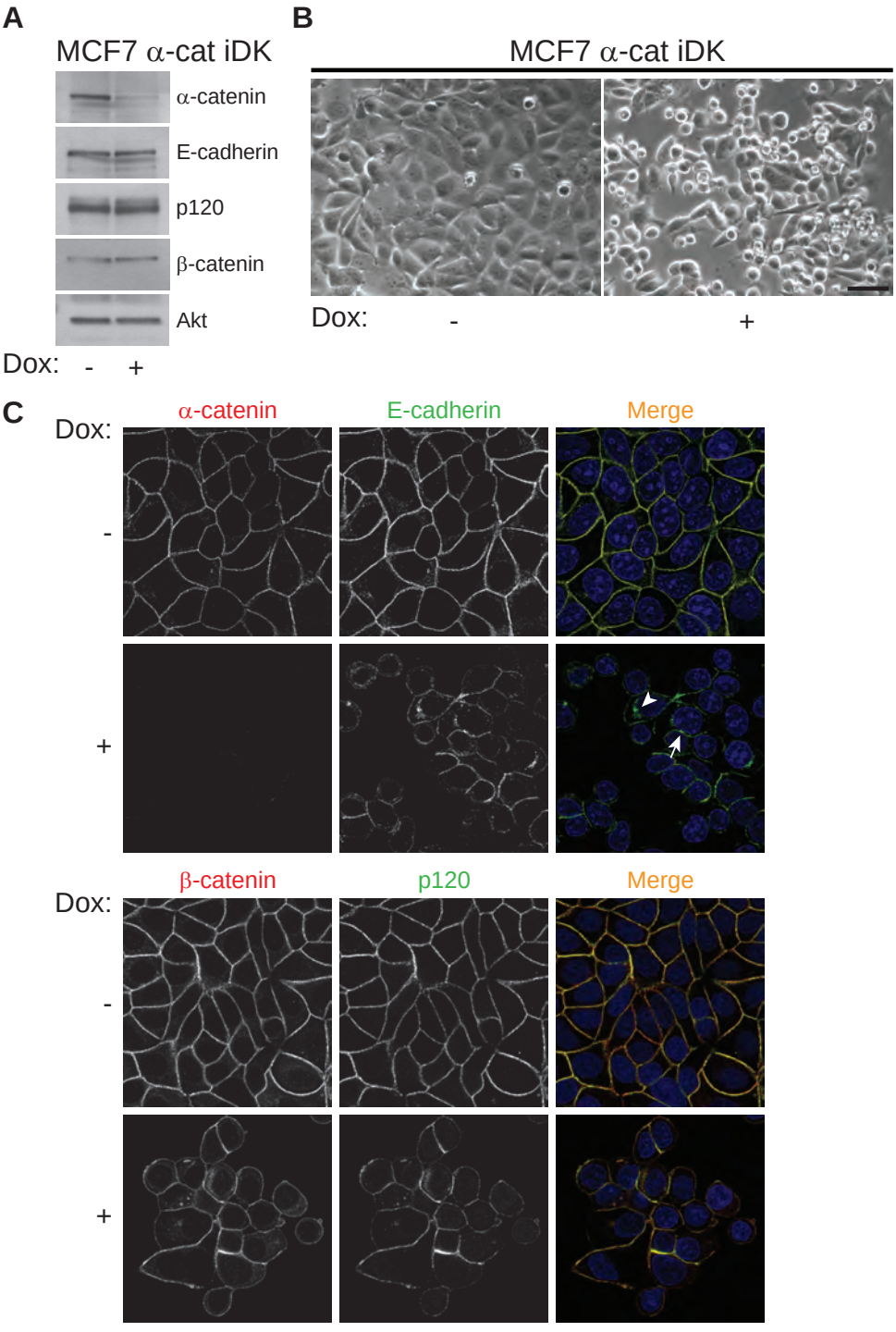
Author Contributions

Experiments were conceived and designed by EJV and PWBD. EJV, MN and TE performed the experiments. EJV and PWBD wrote the paper.

Supplemental Figure 1. Loss of α-catenin induces loss of epithelial cell morphology and leads to aberrant localization of AJ members in human breast cancer cells.

*α-catenin knockdown in the human breast cancer cell line MCF7. (**A**) Inducible knock-down of α-catenin (iKD α-cat) does not lead to inhibition of AJ complex member expression levels. Western blot showing the extent of α-catenin KD (+ dox) on E-cadherin, p120 and β-catenin. AKT levels were used as loading control. (**B**) Loss of α-catenin induces a rounded and nonadherent cell morphology. Phase-contrast images of control (- Dox) and α-catenin knock-down cells (+ Dox). Size bar indicates 50 μm. Immunofluorescence pictures of control (-) and α-catenin knock-down (+) and rescue cell lines (+ Rescue). (**C**) Dysfunctional formation of the AJ upon α-catenin loss. Shown are immunofluorescence images for the AJ complex members α-catenin, E-cadherin, p120 and β-catenin in control (- Dox) and α-catenin iKD (+ Dox). Note the distinct clustering of the AJ in membrane-localized puncta upon α-catenin loss (arrows) and the cytosolic localization of E-cadherin (arrow heads).*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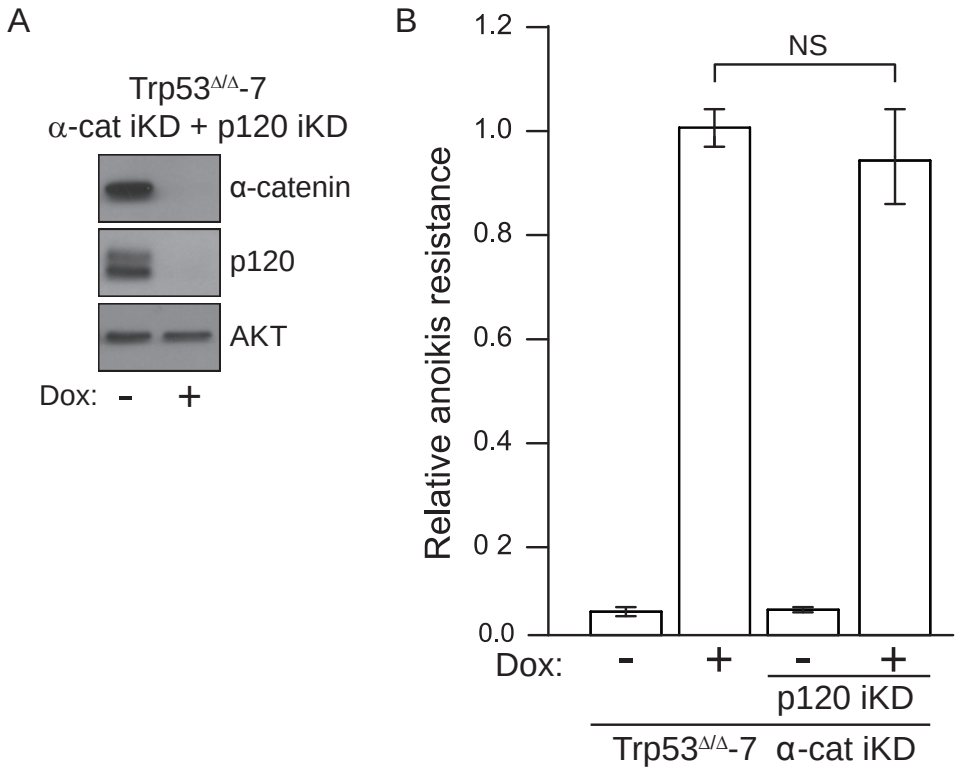

Supplemental Figure 2. Induction of Rho/Rock signaling upon α-catenin loss is p120 independent.
(***A***) Concomitant α-catenin and p120 iKD. Western blot of α-catenin and p120 expression levels in an α-catenin and p120 double inducible knock-down cell line. AKT levels are used as a loading control. (***B***) p120 is not necessary for anoikis resistance in the absence of α-catenin. Anoikis resistance was determined by FACS analysis upon after culturing cells in the presence of Dox to induce dual iKD of α-catenin and p120. Error bars represent the standard deviation of three independent experiments.

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Chapter 5

Nuclear localization of the transcriptional coactivator YAP is associated with ivasive lobular breast cancer

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Abstract

Yes Associated Protein (YAP) has been implicated in the control of organ size by regulating cell proliferation and survival. YAP is a transcriptional co-activator that controls cellular responses through interaction with TEAD transcription factors in the nucleus, while its transcriptional functions are inhibited by phosphorylation-dependent translocation to the cytosol. YAP overexpression was associated with different types of cancer, such as lung, skin, prostate, ovary and liver cancer. Recently, YAP was linked to E-cadherin-dependent regulation of contact inhibition in breast cancer cells. In this study we examined YAP protein expression and cellular localization in 237 cases of human invasive breast cancer by immunohistochemistry and related its expression to clinicopathological features and E-cadherin expression. We show that invasive lobular carcinoma is characterized by higher expression levels of both nuclear- and cytosolic YAP ($p<0.001$). Nuclear YAP expression did not associate with other variables such as lymph node involvement, tumor grade, tumor size, mitotic activity or the molecular sub-types of invasive breast cancer. We observed that high nuclear and cytosolic YAP expression are associated with the E-cadherin deficient breast cancer subtype ILC ($p<0.001$), and cell lines derived from human breast cancers and conditional mouse models of human lobular breast cancer. Since our data indicate that nuclear YAP localization is more common in breast cancers lacking functional adherens junctions, it suggests that YAP-mediated transcription may be involved in the development and progression of invasive lobular breast cancer.

Introduction

Breast cancer prognosis strongly depends on the capacity of tumor cells to invade and colonize foreign tissues, a process that has been linked to the functional loss of cell-cell adhesion. In breast cancer, expression of the tumor suppressor E-cadherin - a key component of the adherens junction (AJ) - is widely used to facilitate differential diagnosis between invasive ductal (IDC) and lobular breast cancer (ILC) [1,2]. While mutational inactivation of E-cadherin is a causal event in the formation of ILC [4,5,6], IDC often expresses E-cadherin [3-5]. In IDC, (epigenetic) inactivation of the AJ occurs at later stages, which is thought to induce an epithelial to mesenchymal transition (EMT) and subsequent tumor progression [6,7]. Despite this, little is known regarding the prometastatic downstream molecular processes that are aberrantly regulated upon loss of E-cadherin-mediated cell-cell adhesion.

Recently, a transcriptional coactivator named Yorkie was identified in *Drosophila* as an important regulator of proliferation and apoptosis [8,9]. Yorkie is a downstream component of the Hippo pathway, consisting of the kinase complexes Hippo-Salvador and Warts-Mats [9-11]. The kinases of the Hippo pathway inhibit Yorkie activity through phosphorylation (Serine 168 in *Drosophila*) [12]. Subsequent dephosphorylation of Yorkie induces translocation to the nucleus, where it activates transcription to regulate organ size [13,14]. Yorkie has two homologues in humans, called YAP (Yes Associated Protein) and TAZ (transcriptional coactivator with PDZ-binding motif, also known as WWTR1) [15]. Two YAP splice variants can be identified, containing either one or two dual WW domains (YAP1 and YAP2 respectively)[16]. Although Hippo pathway components are conserved in mammals, regulation of YAP signaling seems to be tissue-specific. In mouse liver, Mst1/2 (Hippo in Drosophila) inhibits activation of the YAP orthologue Yorkie [12]. However, in mammalian skin neither Mst1/2 nor Lats1/2 (Hippo and Warts respectively in *Drosophila*) influence YAP-mediated signaling [13]. Instead, the AJ member αE-catenin appears to regulate YAP activity. In this setting, αE-catenin binds to phosphorylated YAP (Serine 127 in humans) via the 14-3-3 adaptor protein, which prevents YAP binding to and subsequent dephosphorylation by the phosphatase PP2A [13]. Upon disruption of the AJ complex, YAP can be activated by PP2A and subsequently translocate into the nucleus where it drives transcriptional activation [13].

Interestingly, little is known about the regulation mechanism of YAP in breast tissue. Although it is clear that YAP may be differently regulated in breast tissue compared to its orthologue Yorkie, it is still under debate how YAP is phosphorylated and whether this affects YAP activity [17-22]. In breast cancer cell lines, E-cadherin regulates contact-inhibited proliferation through regulation of YAP activation [18,23]. Here, cell proliferation is inhibited by cell density via α-catenin and αE-catenin-dependent phosphorylation of YAP at serine residue 127 in humans (S112 in mouse) and subsequent translocation of YAP into the cytosol [18,23]. Moreover, recent data indicate that YAP may also be regulated by the actin and microtubule cytoskeleton [17,20,22], although the exact mechanism is largely unknown.

Several observations suggest an oncogenic role for YAP signaling in breast cancer. First, the YAP locus was found amplified in a mammary tumor that developed in a *MMTVcre;Brca1*$^{\Delta 11/ co}$*;Trp53*$^{+/-}$ conditional mouse model [24]. Second, cytosolic YAP was found overexpressed in 31% of human luminal ductal breast cancers [25]. Also, YAP overexpression resulted in transforming abilities in E-cadherin positive human breast cancer cell lines [22,24-26]. Finally, YAP/TAZ-mediated transcriptional activity was linked to the maintenance of a cancer

stem cell phenotype in breast cancer [27]. In contrast, loss of YAP expression correlated with estrogen receptor α (ERα) and progesterone receptor (PR) negativity in breast cancer [28], suggesting that YAP may also function as a tumor suppressor. Furthermore, loss of heterozygosity (LOH) of the YAP gene locus (located at 11q22.2), was frequently found in sporadic breast cancer [29-33]. Finally, loss of YAP expression was implicated in the induction of anoikis resistance and increased invasiveness [34].

Overall, the current literature indicates a role for YAP in breast cancer and suggests that the AJ and its downstream effectors may regulate YAP. Here, we analyzed 237 invasive human breast cancer samples by relating expression and localization of YAP to clinicopathological features and E-cadherin expression. Our data indicate that high nuclear and cytosolic YAP expression are associated with the E-cadherin deficient breast cancer subtype ILC. Furthermore, using human breast cancer cell lines and conditional mouse models of human ILC, we substantiate that nuclear localization of YAP is linked to loss of E-cadherin expression. Our data thus indicate that nuclear YAP is a feature of invasive breast cancers lacking a functional AJ, which suggests a role for YAP signaling in ILC.

Materials and Methods

Patients

The study population was derived from the archives of the Departments of Pathology of the University Medical Center Utrecht, Utrecht, and the Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands. These comprised 237 cases of invasive breast cancer (operated between 2003-2007). Histological grade was assessed according to the Nottingham scheme [35], and mitotic activity index (MAI) was assessed as before [36]. The clinicopathologic characteristics are shown in **Table 1**.

From representative donor paraffin blocks of the primary tumors, tissue microarrays were constructed by transferring tissue cylinders of 0.6 mm (3 cylinders per tumor) from the tumor area, determined by a pathologist based on haematoxylin-eosin stained slides, using a tissue arrayer (Beecher Instruments, Sun Prairie, WI, USA) as described before [37]. Normal breast tissue was obtained from patients that underwent mammoplasty, and was thus tumour-free. The use of anonymous or coded left over material for scientific purposes is part of the standard treatment contract with patients in The Netherlands [38], and no ethical approval is required according to Dutch legislation (as is stated by the Dutch committee for research on patient material 'Centrale Commissie Mensgebonden Onderzoek').

Immunohistochemistry

Immunohistochemistry was carried out on 4μm thick sections. After deparaffination and rehydration, endogenous peroxidase activity was blocked for 15 min in a 46mM citric acid-100mM sodium phosphate buffer solution (pH5.8) containing 0.3% hydrogen peroxide. After antigen retrieval, *i.e.* boiling for 20 min in 10mM citrate pH6.0 (PR, YAP), Tris/EDTA pH9.0 (E-cadherin, ERα, HER2), a cooling period of 30 min preceded the primary antibody incubation. Primary antibodies against E-cadherin (clone 4A2C7, Zymed, Invitrogen,

Table 1. Clinicopathological characteristics of the 237 invasive breast cancer patients studied for the expression of YAP.

Feature	Grouping	N or value	%
Age (years)	Mean	60	
	Range	28 to 88	
Histologic type	IDC	187	78.9
	ILC	50	21.1
Tumor size (cm)	≤2	108	45.6
	>2 and ≤5	98	41.4
	>5	30	12.7
	Not available	1	0.4
Histologic grade	1	36	15.2
	2	76	32.1
	3	125	52.7
MAI	≤ 12	103	43.5
	≥ 13	134	56.5
Lymph node status	Negative*	98	41.4
	Positive**	125	52.7
	Not available	14	5.9

= per 2mm^2 *: negative = N0 or N0(i+); **: positive = ≥N1mi (according to TNM 7th edition, 2010)

Breda, The Netherlands) 1:200; ERα (clone ID5, DAKO, Glostrup Denmark) 1:80; PR (clone PgR636, DAKO) 1:25; HER2 (SP3, Neomarkers, Duiven, The Netherlands) 1:100 were diluted in PBS containing 2%BSA and for 1h at room temperature. Primary antibodies against YAP (1:50, cat 4912, Cell Signaling), (YAP-IHC), was incubated over night at 4°C. The signal was amplified using Powervision poly-HRP anti-mouse, rabbit, rat (DPVO-HRP, Immunologic, Duiven, The Netherlands), followed by counterstaining with haematoxylin, dehydration in alcohol, and mounting.

Scoring of immunohistochemistry

All scoring was done blinded to patient characteristics and results of other stainings by two individual observers. E-cadherin expression was scored using the DAKO/HER2 scoring system for membranous staining. Membranous scores 1+, 2+, and 3+ were considered positive,

except for HER2 where only a score of 3+ was considered positive. Percentages of cells with nuclear YAP staining were estimated, and samples with more than 20% positive tumor nuclei were considered positive. Intensity of cytosolic YAP expression was semi-quantitatively scored as 0, 1, 2 or 3, regarding scores 2 and 3 as high. Based on ER, PR, and HER2 immunohistochemistry, tumors were classified as luminal (ERα and/or PR positive), HER2-driven (ER-, PR-, HER2+), or basal-like/triple negative (ER-, PR-, HER2- with or without EGFR expression), the immunohistochemical surrogate [39] of the original Sorlie/Perou classification [40].

Statistics

Statistical analysis was performed using SPSS Statistics version 18.0 (SPSS Inc., Chicago, IL, USA). Associations between categorical variables were examined using the Pearson's Chi-square test. P-values <0.05 were considered to be statistically significant.

Cell culture

Origin and culture of the mouse cell lines $Trp53^{\Delta/\Delta}$-4, $Trp53^{\Delta/\Delta}$-7, mILC-1 and mILC-3 were described before [41]. ILC cell line IPH-926 was a kind gift from Dr. M. Christgen (Hannover Medical School, Hannover, Germany) and cultured as described [42]. Human breast cancer cell line MCF10A was obtained from American Type Culture Collection (ATCC), while T47D and SKBR-3 were a kind gift from Dr. J. Martens (Erasmus Medical Center, Rotterdam, The Netherlands) and originate from the American Type Culture Collection (ATCC). These cell lines were cultured in DMEM-F12 (Sigma), and validated by Short Tandem Repeat (STR) profiling. All media contained 10% fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin, and maintained at 37˚C in a 5% CO_2 humidified atmosphere.

Western Blotting

Samples were lysed in sample buffer containing 50 mM Tris-Cl (pH 6.8), 0.5% β-mercaptoethanol, 2% SDS, 0.005% bromophenolblue, and 10% glycerol (all Sigma-Aldrich). Samples were heated for 10 minutes at 100˚C and proteins were separated using standard PAGE protocols and blotting as described previously [43].

Nuclear Fractionation

Cells were grown to confluence, washed with PBS containing Mg2+ and Ca2+ (PBS+), scraped from the plate and suspended in buffer A (10mM HEPES pH 7.9, 1.5mM MgCL2, 10mM KCl and freshly added 1mM DTT, 0.5mM PMSF, 5ug/ml leupeptin, 5ug/ml aprotinin). Cells were centrifuged at 400g at 4˚C for 5min, resuspended in 1 ml of PBS+, and centrifuged. Cells were resuspended in buffer A, centrifuged and resuspended again in buffer A. Cells were incubated for 10 min on ice, centrifuged, resuspended in buffer A and mechanically lysed with 50-75 strokes in a glass 2mL-douncer. Cell lysates were centrifuged for 10 min at 500g at 4˚C. Cell pellets (nuclear fraction) were separated from the supernatant (cytosolic fraction). Both fractions were submitted to another round of washing and centrifugation (10 min, 500g at 4oC) and used for western blotting. Rabbit anti-TAF5, [44], was used as a nuclear marker, goat anti-AKT (1:1000; cat sc-1618, Santa Cruz Biotechnology) was used

as a cytosolic marker. The primary antibody against YAP was mouse anti-YAP (1:200; cat sz101199, Santa Cruz), (YAP-IF).

Constructs, Viral production and Transduction

Cos-7 cells were transfected using X-tremeGene9 (cat 06365809001, Roche), and lentiviral particles were produced using third-generation packaging constructs as described [41]. For knockdown of YAP we used pLKO1-shYAP1 (cat 27368 addgene). Supernatant containing viral particles was harvested 48 hours after transfection, passed through a 45-µm filter, and concentrated 15- to 20-fold by centrifugation (175,000 g; 150 minutes). Cells were transduced overnight in the presence of 4 µg/ml polybrene (Sigma-Aldrich).

Immunofluorescence

Cells were cultured on glass coverslips and fixed in 1% paraformaldehyde in PBS for 10 minutes on ice, permeabilized using 0.3% Triton-X100/PBS and subsequently blocked with 4% BSA in PBS (Roche, Woerden, The Netherlands). Formalin fixed and paraffin embedded (FFPE) tissue slides were treated as described for immunohistochemistry staining. Blocking was done with 4% BSA in PBS after antigen retrieval. FFPE slides were incubated with rabbit anti YAP (1:50; cat 4912, Cell Signaling), (YAP-IHC), at 4°C overnight. The mouse anti-p63 antibody (1:400; MS-115-P, Neomarkers) was incubated for 1 hour at room temperature. Directly conjugated antibodies against E-cadherin (1:150, 612130 BD and 560062 BD Biosciences), were incubated for 1-3 hours at room temperature. All antibodies were diluted in 4% BSA/PBS. Secondary antibodies were incubated in 4% BSA/PBS for 1 hour (goat anti-rabbit Alexa-555, cat A21428, goat anti-mouse highly cross-adsorbed Alexa-488 and Alexa-568, cat A11029 and A11031, and goat anti-rabbit highly cross-adsorbed Alexa-488 and Alexa-568, cat A11036 and A11034, all from Invitrogen). Cell lines grown on glass coverslips were incubated with mouse anti-YAP (1:50; cat sz101199, Santa Cruz), (YAP-IF), in 4% BSA 4°C over night. Subsequently, cells were incubated in 4% BSA with goat anti-mouse Alexa-555 (1:600; cat A21422, Invitrogen) for 1 hour. DNA was stained with DAPI (1:1000; cat D1306, Invitrogen) for 5 min at room temperature. Cover slips were mounted using Vectashield (Vector Laboratories, Burlingame, USA) and analyzed either by Zeiss LSM 510 Meta confocal laser microscope using a 63X 1.4 objective or by using the Zeiss LSM 700 confocal laser microscope using the 63X 1.4 objective.

Figure 1. YAP expression in human and mouse normal breast tissue.
(A) YAP expression in normal human breast tissue. Shown are immunofluorescence for YAP (middle panel, red) and E-cadherin (left panel, green). Nuclei were visualized using DAPI. Right panel depicts the merged image. Luminal epithelial cells form clear AJ and are characterized by low cytosolic YAP expression. Note the predominant nuclear YAP localization in myoepithelial cells (arrowheads) and expression of YAP in apical snouts (arrow). Size bar = 5 μm. (B) YAP expression in mouse mammary glands. Shown are immunofluorescence for YAP (middle panel, red) and E-cadherin (left panel, green). Arrowheads depict nuclear YAP expression in mouse myoepithelial cells. Nuclei were visualized using DAPI. Right panel depicts the merged image. Size bar = 5 μm.

Results

YAP expression in human and mouse normal breast tissue

In normal human breast tissue, we found YAP to be predominantly expressed in the outer layer of the ducts, the myoepithelium (**Fig. 1a and Online Resource Fig. 1**). While YAP was expressed at low levels in the cytosol, we detected prominent nuclear YAP staining using immunofluorescence in this cell type (**Fig. 1a, arrowheads and Online Resource Fig. 1**). In contrast, E-cadherin-expressing luminal cells showed low YAP expression that was mainly localized to the apical snouts (**Fig. 1a, arrow and and Online Resource Fig. 1**). Next, we assessed YAP expression and localization in mouse mammary epithelium. As in the human tissue, mouse myoepithelial cells showed a stronger nuclear YAP localization pattern (arrowheads), whereas luminal mammary epithelial cells mostly expressed cytoplasmic YAP (**Fig. 1b**). Specificity of the YAP-IF antibody used was confirmed by performing shRNA-mediated knock-down (**Online Resource Fig. 2**, middle panel).

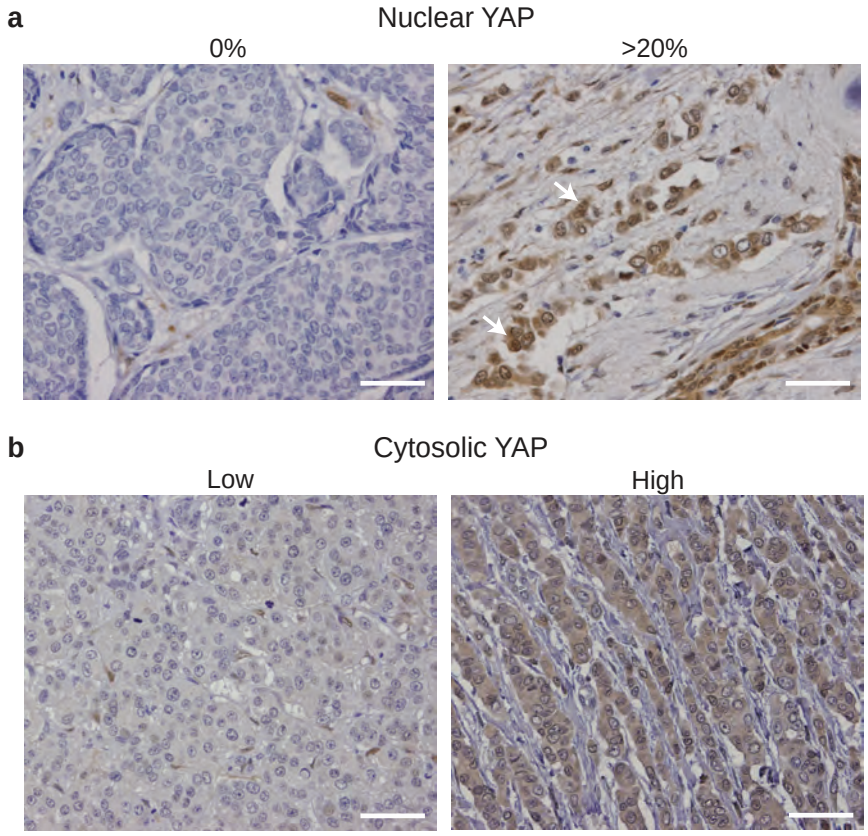

Figure 2. YAP expression in human invasive breast cancer.
(A) Nuclear YAP expression patterns. Shown are representative examples of immunohistochemistry of YAP (IHC). The percentage of nuclei that showed YAP expression was determined and scored as 0% (left panel) or more than 20% (right panel). Arrows denote nuclear staining. (B) Cytosolic intensities of YAP expression. Shown are representative examples of immunohistochemistry of YAP (IHC). Cytosolic YAP expression was scored as either high YAP (left panel) or low YAP (right panel). The sample shown in the right panel was also scored <20% (15–20%) for nuclear YAP localization. Size bar = 25 µm.

Table 2. Correlations of cytosolic YAP with clinicopathological features in invasive breast cancer.

Feature	N	Cytosolic YAP expression		
		Negative N (%)	Positive N (%)	p-value
Histologic type				
IDC	187	126 (67.4)	61 (32.6)	
ILC	49	21 (42.9)	28 (57.1)	**0.002**
Histologic grade				
1	36	25 (69.4)	11 (30.6)	
2	76	46 (60.5)	30 (39.5)	
3	124	76 (61.3)	48 (38.7)	0.626
Tumor size (cm)				
≤2	108	67 (62.0)	41 (38.0)	
>2 and ≤5	97	65 (67.0)	32 (33.0)	
>5	30	15 (50.0)	15 (50.0)	0.240
MAI (per $2mm^2$)				
≤ 12	103	64 (62.1)	39 (37.9)	
≥ 13	133	83 (62.4)	50 (37.6)	0.966
Lymph node status				
Negative	97	57 (58.8)	40 (41.2)	
Positive	125	80 (64.0)	45 (36.0)	0.426

Nuclear YAP localization in Invasive Lobular Carcinoma

To investigate the expression pattern of YAP in invasive breast cancer, we stained a tissue micro array (TMA) containing 237 invasive breast cancer samples using immunohistochemistry (IHC). The clinicopathological characteristics of these tumors are shown in Table 1. Because YAP functions as a mediator of transcriptional activation, we based our score on an estimation of the percentage of YAP positive nuclei. Representative pictures of nuclear YAP staining are shown in **Figure 2A**. Moreover, since YAP shuttles between the nucleus and the cytoplasm, we scored the intensity of cytoplasmic YAP as well (**Fig 2b**). Since a different antibody was used for IHC (YAP-IHC), we also confirmed specificity for this antibody by using a YAP knock-down approach (**Online Resource Fig. 2, top panel**). Analysis of the YAP expression patters showed that both high cytoplasmic levels and nuclear YAP localization correlated with the histological type of breast cancer invasive lobular carcinoma (ILC) ($p=0.002$ and $p<0.001$ respectively; **Table 2 and 3**). We used 20% as cut-off value for positive nuclear YAP staining, although 5% and 10% cut-off were also statistically significant ($p<0.001$ for both 5% and 10%). Neither cytosolic nor nuclear YAP expression correlated with histological grade, mitotic activity or lymph node status (**Tables 2 and 3**).

Table 3. Correlations of nuclear YAP with clinicopathological features in invasive breast cancer.

Feature	N	Nuclear YAP expression		
		Low (<20%) N (%)	High (≥20%) N (%)	p-value
Histologic type				
IDC	187	140 (74.9)	47 (25.1)	
ILC	50	12 (24.0)	38 (76.0)	**<0.001***
Histologic grade				
1	36	19 (52.8)	17 (47.2)	
2	76	51 (67.1)	25 (32.9)	
3	125	82 (65.6)	43 (34.4)	0.297
Tumor size (cm)				
≤2	108	79 (73.1)	29 (26.9)	
>2 and ≤5	98	60 (61.2)	38 (38.8)	
>5	30	13 (43.3)	17 (56.7)	**1.451***
MAI (per 2mm^2)				
≤ 12	103	61 (59.2)	42 (40.8)	
≥ 13	134	91 (67.9)	43 (32.1)	0.167
Lymph node status				
Negative	98	65 (66.3)	33 (33.7)	
Positive	125	78 (62.4)	47 (37.6)	0.544

[#] = these data have been corrected for the difference in tumor size between IDC and ILC tumors.

We also analyzed the relationship between YAP expression and tumor size. Because ILC tumors were significantly larger compared to the IDC tumors, we corrected for this and found that nuclear YAP expression was not correlated with tumor size (odd ratio 1.451, 95% confidence interval of 0.790 to 2.667), while high nuclear YAP localization remained significantly correlated with ILC tumors (odd ratio 8.829, 95% confidence interval of 4.208 to 18.523) (**Table 3**). In contrast to previously published data [28], we did not find a correlation between nuclear or cytosolic YAP localization and hormone receptor status, HER2 expression, molecular breast cancer subtypes or overall survival (**Online Resource Table 1 and Table 2**). In conclusion, increased cytosolic YAP expression and nuclear YAP localization are associated with ILC.

High YAP expression is correlated with human and mouse ILC

Since E-cadherin has been implicated in the regulation of YAP activity in human breast cell lines [17,18,23], we analyzed whether E-cadherin expression was correlated with YAP expression in our invasive breast cancer samples. Indeed, we found that high cytoplasmic as well

as high nuclear YAP expression inversely correlated with E-cadherin expression (p=0.024 and p<0.001 respectively, **Table 4, Table 5 and Fig 3**). We substantiated the correlation between E-cadherin loss and nuclear YAP localization in ILC by analyzing YAP expression in a set of E-cadherin expressing and E-cadherin mutant (lobular) breast cancer cell lines. In agreement with the findings in our invasive breast cancer cohort we observed that the E-cadherin positive cell lines T47D and MCF10A showed cytosolic YAP staining (**Fig 4a**), whereas the E-cadherin mutant cell lines SKBR-3 and lobular breast cancer cell line IPH-926 [42,45], showed predominately nuclear YAP localization (**Fig 4c**). Next, we used tumor cell lines generated from mammary tumors that developed in conditional mouse models based on tissue-specific and conditional inactivation of E-cadherin and/or p53. In these mice, inactivation of E-cadherin is causal to the formation of invasive and metastatic tumors that mimic human ILC [4,5]. Using immunofluorescence we compared YAP localization in $Trp53^{\Delta/\Delta}$ (E-cadherin positive, p53 negative) and mouse ILC (mILC) (E-cadherin and p53 negative) cell lines. Similar to their human counterparts, mILC cells showed an increase in nuclear YAP localization. Furthermore, while both mILC and Trp53Δ/Δ cells showed cytosolic YAP, YAP expression in Trp53Δ/Δ cell lines was mainly cytoplasmic (**Fig 4b and d**). In order to corroborate our finding that nuclear YAP expression is a hallmark of E-cadherin mutant breast cancer, we performed nuclear fractionation of human and mouse cell lines, which were compared using western blot analyses. Indeed, using TAF5 as a nuclear marker we observed that in both species E-cadherin negative cell lines showed an enrichment of nuclear YAP when compared to their E-cadherin expressing counterparts (**Online Resource Fig 3**). In conclusion, our data indicate that YAP localization is inversely correlated with E-cadherin expression, since nuclear YAP is a characteristic of E-cadherin negative ILC from mice and man.

Table 4. Correlation between E-cadherin expression and cytosolic YAP expression in invasive breast cancer.

Feature	N	Cytosolic YAP expression		
		Negative N (%)	Positive N (%)	p-value
E-cadherin				
Positive	176	114 (64.8)	62 (35.2)	
Negative	49	23 (46.9)	26 (53.1)	**0.024**

Table 5. Correlation between E-cadherin expression and nuclear YAP expression in invasive breast cancer.

Feature	N	Nuclear YAP expression		
		Low (<20%) N (%)	High (≥20%) N (%)	p-value
E-cadherin				
Positive	176	127 (72.2)	49 (27.8)	
Negative	50	15 (30.0)	35 (70.0)	**<0.001**

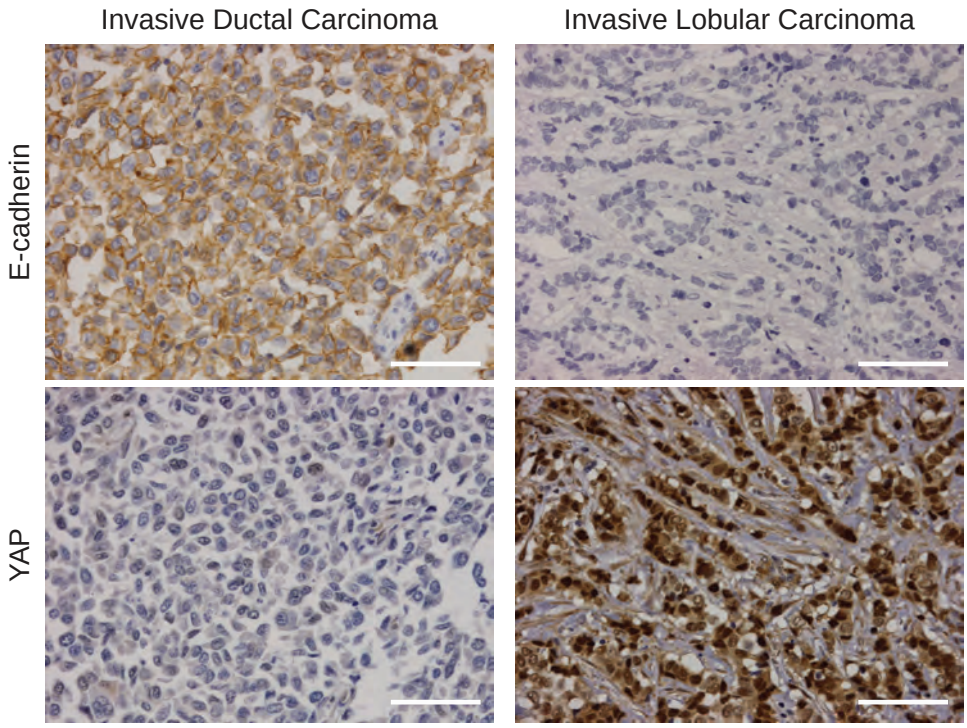

Figure 3. Nuclear YAP expression is a feature of E-cadherin negative ILC.
E-cadherin status correlates with nuclear YAP expression. IDC (left panels) and ILC (right panels) samples were stained for E-cadherin (top panels) and YAP (IHC) (bottom panels). Note the striking correlation between nuclear YAP and absence of E-cadherin expression. Size bar = 50 µm.

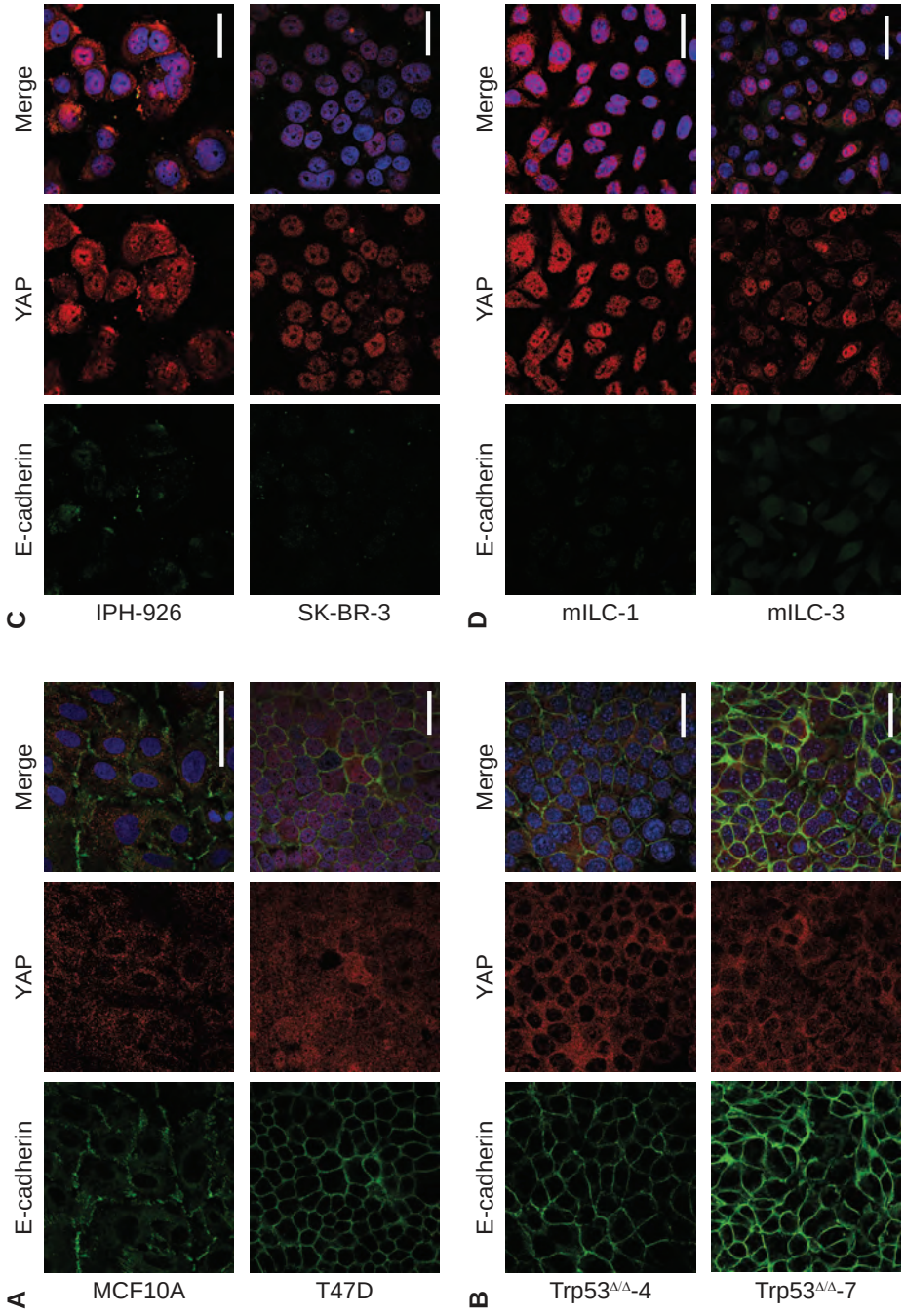

Figure 4. Nuclear localization of YAP in human and mouse ILC.
Immunofluorescence for E-cadherin (left panels, green) and YAP (YAP-IF, middle panels, red). Nuclei were visualized using DAPI (blue). Right panels depict the merged image. In E-cadherin positive human (A) and mouse (B) cell lines YAP expression is predominantly cytosolic. E-cadherin negative human (C) and mouse ILC (D) cell lines are characterized by prominent nuclear YAP expression. Size bar = 15 μm.

Discussion

This study demonstrates for the first time that nuclear and cytosol YAP localization are correlated with E-cadherin negative invasive lobular breast cancer. As YAP is a transcriptional co-activator that shuttles between the cytosol and nucleus, we analyzed cytosolic intensity of YAP expression and the number of YAP positive nuclei. Our results suggest that YAP does not only translocate into the nucleus E-cadherin negative breast cancer, but also that total YAP levels are increased in this situation. While we have not tested whether YAP expression levels correlate with YAP activity, it was recently demonstrated that YAP signaling may also be regulated through YAP degradation [21]. It is currently unknown whether E-cadherin expression regulates YAP degradation. A possibility might be that loss of E-cadherin inhibits YAP phosphorylation and subsequent ubiquitin-mediated degradation, leading to increased levels of YAP. However, while we observed a correlation between membranous E-cadherin expression and cytoplasmic YAP localization, total YAP protein levels did not seem to correlate with E-cadherin status in the human breast cell lines (data not shown). This discrepancy may reflect the differences between cultured cell lines and clinical breast cancer specimens.

In contrast to previous studies [19,28], we did not find a correlation between YAP expression/localization and overall survival of breast cancer patients. We did not observe a statistically significant correlation between YAP expression/localization and overall survival (data not shown). We think the lack of correlation in our cohort is not surprising, since YAP is strongly linked to ILC, and differences in survival rates for ILC and IDC patients are only observed when comparing large numbers of patients over long time periods of more than 10 years [46].

An interesting yet unanswered question is the possible link between YAP localization/function and E-cadherin negative IDC. In contrast to early mutational inactivation of E-cadherin in ILC, IDC tends to show loss of E-cadherin by epigenetic mechanisms during later stages of tumor progression [1,47,48]. Since almost all our IDC cases showed membranous expression of E-cadherin (97%), this prevented an in-depth survey into the localization of YAP in E-cadherin negative IDC.

Our current findings are in line with recent data that addressed a scenario whereby stress fiber formation resulted in a reduction of YAP phosphorylation and subsequent nuclear localization [17,20,22]. Although the impact of cell-cell adhesion was not directly addressed in these studies, it was recently shown that E-cadherin-dependent contact inhibition and a resulting reduction in proliferation may be regulated through Lats-dependent phosphorylation of YAP [18]. Cell-type specific differences may play a role, as morphology-dependent F-actin bundling can also regulate YAP in a Lats-independent manner [17]. Regardless of the exact mechanism, a common denominator in the nuclear localization of YAP seems the activation of Rho-Rock-driven actin polymerization. As such, both cellular morphology and cadherin-mediated cell-cell binding may regulate YAP-mediated transcriptional activity in a tension-dependent manner. Interestingly, YAP overexpression can also cause transformation of E-cadherin expressing breast cancer cells, which is accompanied by loss of epithelial characteristics, expression of mesenchymal markers and acquisition of invasiveness and anchorage-independence [22,24,49]. Thus, irrespective of the upstream signaling cascade, nuclear YAP may play a central role in the regulation of breast cancer invasiveness.
We have previously demonstrated that loss of junctional integrity through mutational inactivation of E-cadherin leads to a p120-catenin and MRIP-dependent activation of the Rho-

Rock pathway in metastatic ILC [41]. In this setting, the functional consequence of active Rock signaling is the regulation of anchorage-independent tumor growth and metastasis. Because activation of Rock directs actin polymerization, this may result in dephosphorylation and subsequent nuclear membranous expression of E-cadherin and nuclear localization of YAP appear to be largely mutually exclusive in mouse and human breast cancer. We think that actin-dependent nuclear translocation of YAP is a plausible mode of action since several Hippo pathway components bind actin and recent data indicated that F-actin may regulate YAP donwstream of cell morphology [20, 50-53]. Our recent finding that p120 controls anchorage independence through MRIP-dependent F-actin bundling [41], may provide an alternative mode of regulation. Whether p120 links actin polymerization to subsequent nuclear YAP expression in ILC, and how these events contribute to ILC development and metastasis, is subject of further research.

To summarize, our results show that nuclear YAP localization and overall YAP expression levels significantly correlate with the ILC breast cancer subtype, a malignancy that is causally related to mutational inactivation of E-cadherin. These findings were validated in human and mouse E-cadherin mutant cell lines, which showed an increase in nuclear YAP localization compared to E-cadherin expressing cell lines. We hypothesize that upon loss of E-cadherin and subsequent inactivation of the AJ complex, YAP translocates to the nucleus where it may induce a transcriptional program favoring ILC tumor development and progression. In conclusion, our data suggest that YAP translocation to the nucleus is a consequence of early mutational inactivation of E-cadherin and subsequent p120-mediated activation of Rock-dependent actin polymerization. As such, YAP and its target genes hold promise for the development of novel intervention strategies to better treat metastatic ILC.

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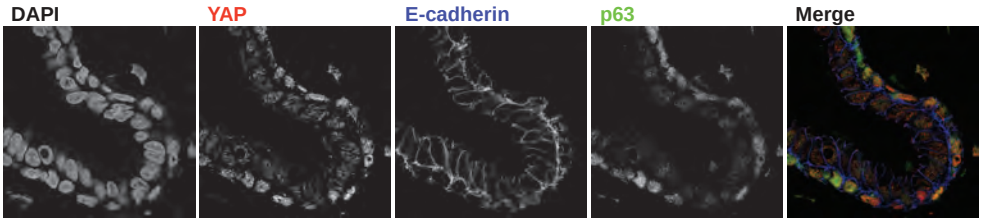
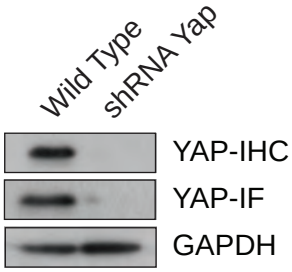
Author Contributions

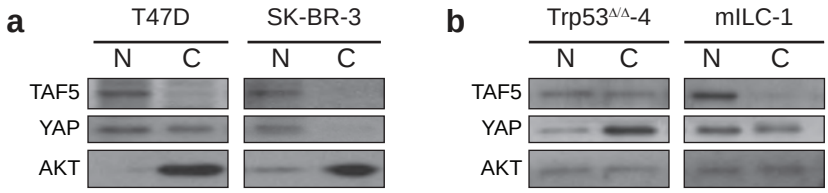
Experiments were conceived and designed by EJV and PWBD. EJV, RvdV and JFV performed the experiments. EJV, PB and PVD performed the pathological analyses and scoring of human tumor samples. EJV and PWBD wrote the paper.



Online Resource Figure 1. Myoepithelial cells express nuclear YAP .
Normal human breast tissue was sectioned and triple-stained using immunofluorescence for YAP, E-cadherin and p63 to determine YAP expression in luminal and myoepithelial mammary cells. DAPI depicts the nuclei (left panel). The right panel shows a merged image of YAP (red), E-cadherin (blue), and p63 (green) signals.



Online Resource Figure 2. Validation of the YAP antibodies.
YAP knock down in the T47D cell line. The YAP-IHC and the YAP-IF antibody were used to analyze YAP expression levels. Both antibodies clearly show a reduction of YAP expression upon shRNA-mediated knock down and thereby establish specificity for the antibodies used .



Online Resource Figure 3. E-cadherin mutant breast cancer cells are characterized by nuclear YAP localization.
*Biochemical fractionation to study YAP localization in breast cancer cell lines. Nuclear (N) and cytosolic (C) compartments from E-cadherin expressing (left panels) and E-cadherin mutant (right panels) human (**A**) and mouse (**B**) breast cancer cell lines were western blotted and probed with the YAP-IF antibody. TAF5 was used as a nuclear marker. Note the enrichment of YAP in the nuclear fractions of E-cadherin mutant breast cancer cell lines SKBR-3 (**A**) and mILC-1 (**B**) compared to their E-cadherin expressing counterparts. AKT was used as cytosolic marker.*

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Chapter 6

The transcriptional coactivator YAP is overexpressed in lobular carcinoma in situ

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Submitted for publication

Abstract

Yes-Associated Protein (YAP) is a transcriptional coactivator that becomes active upon dephosphorylation and nuclear translocation. Although YAP has been implicated in tumour initiation, its activation cues and control of breast cancer development and progression are still largely unknown. To study the expression and localisation of YAP during breast cancer development we examined 38 lobular carcinoma *in situ* (LCIS) and 66 ductal carcinoma *in situ* (DCIS) cases by immunohistochemistry. Adjacent invasive cancer was available for 27 LCIS and 45 DCIS cases, allowing to study the potential role of YAP in breast cancer progression.

High nuclear YAP expression was significantly more frequent in LCIS (92.6%) in contrast to DCIS (15.6%) ($p<0.001$), and in ILC compared to IDC lesions (88.9% *versus* 24.4%, $p<0.001$). Nuclear YAP expression did not significantly differ when comparing LCIS and ILC, or DCIS and IDC lesions within the same patient.

In conclusion, the LCIS stage of lobular breast is characterised by nuclear YAP expression,while high nuclear YAP expression is rare in DCIS, which can aid to differentiate LCIS from low grade DCIS. We conclude that nuclear YAP is a hallmark of lobular neoplasia that may play an early role in ILC development.

Keywords: Lobular breast cancer, in situ cancer, YAP, E-cadherin, tumour progression

Introduction

Yes Associated Protein (YAP) has emerged as a focal point of processes regulating organ size, stem cell renewal and mitosis [1]. YAP and its homologue transcriptional co-activator with PDZ-binding motif (TAZ) function as the downstream effectors of the Hippo signalling cascade [2]. YAP shuttles between the cytosol and the nucleus, where it binds PPXY motifs present in several transcription factors [3]. In addition, YAP binds the family of transcription factors called TEAD [4], and hereby induces cell growth, oncogenic transformation and epithelial-to-mesenchymal transition (EMT) [5]. YAP activity can be modulated through phosphorylation. For instance, YAP phosphorylation at S127 by the cell cycle kinase CDK1 leads to inactivation of YAP [6], and phosphorylation of S381 primes YAP for ubiquitin-dependent degradation [7]. In addition, YAP activity can be negatively regulated by binding to Angiomotin in the cytoplasm [8,9].

Hippo signalling can also be controlled by the atypical cadherin Fat and the actin binding proteins Expanded and Merlin [10,11], although it is unknown whether this mechanism is conserved in mammals. Expanded and Merlin localise at the adherens junction (AJ), a structure that may link α-catenin and Angiomotin to YAP through binding of Merlin (transcriptional product of Neurofibromatosis 2 (NF2) [12]. The fact that α-catenin can bind YAP through the scaffold 14-3-3, suggests an intricate regulation of YAP activity by the AJ[9,16]. Indeed, disruption of the connection between YAP and the AJ leads to nuclear YAP localisation and the induction of target gene expression [13]. Finally, cell contact inhibition, a trait that is controlled by the AJ complex, is mediated via YAP [14]. Overall, these findings show that cell-cell adhesion and YAP signalling are intricately linked.

Recent findings have implicated YAP in tumour development and progression. For instance, hyper activation of YAP leads to tumour formation in mouse tissues such as liver and skin [13,15,16]. The YAP locus was found amplified in mammary tumours that developed in a *MMTVcre;Brca1*$^{\Delta 11/\omega}$*;Trp53*$^{+/-}$ conditional mouse model, suggesting an oncogenic role for YAP [17]. Also, YAP is overexpressed in 31% of human luminal ductal breast cancers, and drives the tumour formation and proliferation of breast cancer cell lines in mice [18]. In conjunction with these findings, YAP regulates anoikis resistance and migration in breast cancer cells, which are processes known to function downstream of AJ-dependent cell-cell adhesion stimulate tumor initiation and development [17-19].

We have previously demonstrated that YAP is overexpressed in invasive lobular breast carcinoma (ILC), but not in invasive ductal breast carcinoma (IDC) [20], a disease that is causally linked to loss of the cell-cell adhesion molecule E-cadherin [19,21,22]. Recently, it was shown that E-cadherin controls cell contact inhibition by phosphorylation-based sequestration of YAP within the cytosol [14]. As loss of E-cadherin is an early event in ILC development [22,23], we analysed YAP expression during breast cancer development and conclude that nuclear YAP expression is a hallmark of both *in situ* and invasive lobular breast cancer.

Material and methods

Tumour material and tissue micro array (TMA) construction

Patient tumour samples were collected from the archive of the Departments of Pathology of the University Medical Center Utrecht, Utrecht. Clinicopathological characteristics of the se tumours can be found in **Table 1**. Representative donor paraffin blocks of the primary tumours were used to construct a TMA by transferring tissue cylinders of 0.6 mm (3 cylinders per tumour) as described before[41]. The use of a computer-controlled tissue arrayer (TMA Master, 3D Histech,) allowed precise cylinder extraction based on marked haematoxylin-eosin stained slides of the primary tumour. The use of anonymous or coded left over material for scientific purposes is part of the standard treatment contract with patients in The Netherlands [24], and no informed consent is required according to Dutch legislation. DCIS was graded according to Holland *et al.* [25], and invasive cancers were graded according to the modified Bloom and Richardson score [26].

Table 1. Clinicopathological characteristics of lobular and ductal breast cancer patients.

		With adjacent invasive cancer		Without adjacent invasive cancer	
		Lobular	Ductal	LCIS	DCIS
Feature	Grouping	N (%)	N (%)	N (%)	N (%)
N		27	45	11	21
Age (years)	Mean	55	55	55	56
	Range	37-72	38-79	45-72	43-74
Grade *in situ*	1		7 (15.6)		3 (14.3)
	2		17 (37.8)		9 (42.9)
	3		20 (44.4)		9 (42.9)
	Not available		1 (2.2)		0 (0.0)
Lymph node status	Negative *	8 (29.6)	15 (33.3)	4 (36.4)	4 (19.0)
	Positive **	18 (66.7)	20 (44.5)	1 (9.1)	1 (23.8)
	Not available	11 (3.7)	10 (22.2)	6 (54.5)	16 (76.2)

*: negative = N0 or N0(i+); **: positive = ≥N1mi (according to TNM 7th edition, 2010)

Immunohistochemistry and used antibodies

Immunohistochemistry (IHC) was carried out as described before [20]. Briefly, 4 μm thick sections were de-paraffinised and rehydrated, followed by endogenous peroxidase activity and blocking for 15 min in a 46mM citric acid-100mM sodium phosphate buffer solution

(pH5.8) containing 0.3% hydrogen peroxide. After antigen retrieval, i.e. boiling for 20 minutes. in 10mM citrate pH6.0 (YAP) or Tris/EDTA pH9.0 (E-cadherin), a cooling period of 30 minute preceded the primary antibody incubation. Primary antibodies against YAP (1:50, cat 4912, Cell Signalling) or E-cadherin (1:200, clone 4A2C7, Zymed, Invitrogen) were diluted in PBS containing 2% BSA and were incubated for 1 hour at room temperature (E-cadherin) or overnight at 4°C (YAP). Signals were amplified using Brightvision poly-HRP anti-mouse, anti-rabbit, anti-rat (DPVO-HRP, Immunologic, Duiven, The Netherlands), followed by counterstaining with haematoxylin, dehydration in alcohol and mounting. The specificity of the YAP antibody has been validated previously[35].

Scoring of immunohistochemistry

All scoring was done blinded to patient characteristics and results of other stainings by two individual observers as described before [20]. YAP expression was examined as the cytosolic intensity and the percentage of nuclear YAP expression. Samples with more than 20% positive tumour nuclei were considered YAP high. E-cadherin expression was scored using the DAKO/HER2 scoring system for membranous staining. ER and PR expression was scored positive when 10% or more of nuclei stained.

Statistics

Statistical analysis was performed using SPSS Statistics version 21.0 (SPSS Inc., Chicago, IL, USA). Associations between categorical variables were examined using the Pearson's Chi-square or Fischer's exact test when appropriate. P-values <0.05 were considered to be statistically significant.

Results

We have recently published that the outer cell layer of normal mammary ducts, the myoepithelial (basal) cells, show high expression of nuclear YAP, while luminal cells do not [20]. We therefore scored the *in situ* lesions only for nuclear YAP staining within the luminal cells.
We selected our lobular carcinoma cohort (both the *in situ* and invasive lesions) based on the absence of E-cadherin expression. Nuclear YAP expression did not significantly differ when comparing LCIS versus ILC, or DCIS versus IDC lesions within the same patient (**Table 2**). However, the frequency of high nuclear YAP expression was significantly higher in LCIS compared to DCIS lesions (92.6% *versus*. 15.6%, p<0.001), and in ILC compared to IDC lesions (88.9% *versus* 24.4%, p<0.001) (**Fig. 1 and Table 3**).

High nuclear YAP was rare in pure DCIS (14.3%) but frequent in pure LCIS (81.8%, p<0.001) (**Figure 1 and Table 4**). However, although grade 1 DCIS (4/10) had a tendency to show elevated nuclear YAP expression levels than in grade 2 (3/31) and grade 3 DCIS (5/10), this was not statistically significant (p=0.069).

In conclusion, we demonstrate that both the non-invasive *LCIS* and the invasive ILC lesions are characterised by nuclear YAP expression. Thus, nuclear YAP expression correlates with the absence of E-cadherin (**Figure 2**), a feature that is causally linked to the development and progression of lobular breast cancer.

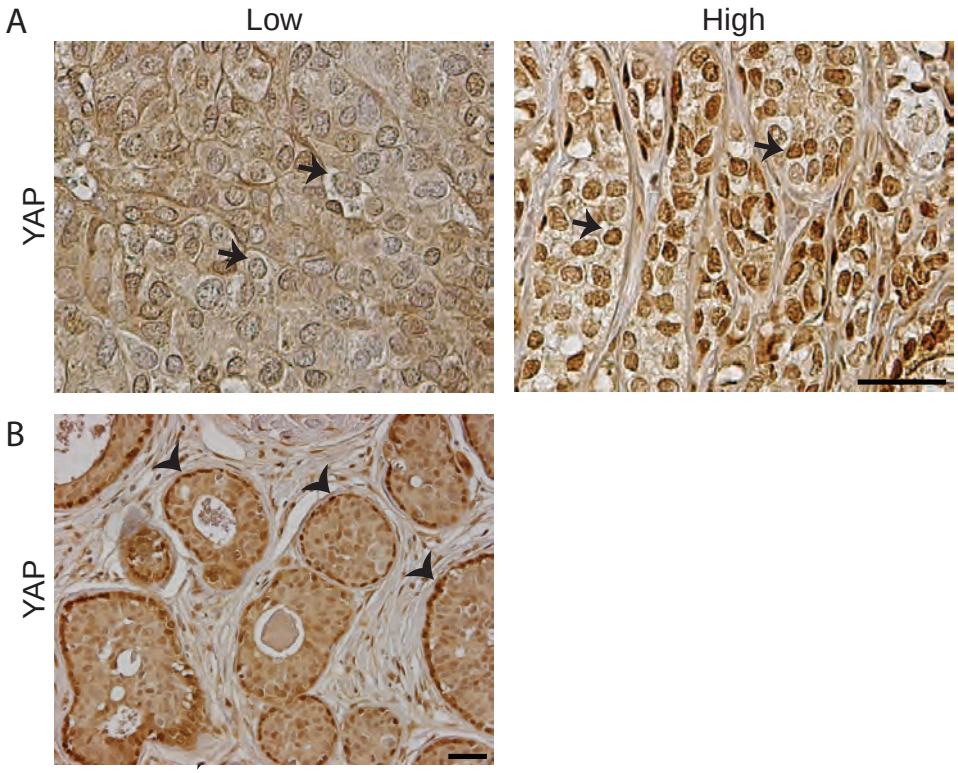

Figure 1. YAP and E-Cadherin in invasive and in situ breast cancer.

A. *Shown are representative images of low and high YAP expression (further indicated by arrows). The images were taken from a DCIS (left) and a LCIS (lright) specimen.* **B**. *Representative image showing myoepithelial cells tend to express high levels of YAP. Image was taken from a DCIS specimen. Arrow heads indicate high YAP expressing myoepithelial cells.*

Table 2. Comparison between nuclear YAP expression in the in situ and invasive components within breast cancer patient.

	Invasive breast cancer		
Feature	**Low**	**High**	
	N (%)	**N (%)**	**p-value**
LCIS			
Low	1 (50.0)	1 (50.0)	0.214
High	2 (8.0)	23 (92.0)	
DCIS			
Low	30 (78.9)	8 (21.1)	0.337
High	4 (57.1)	3 (42.9)	

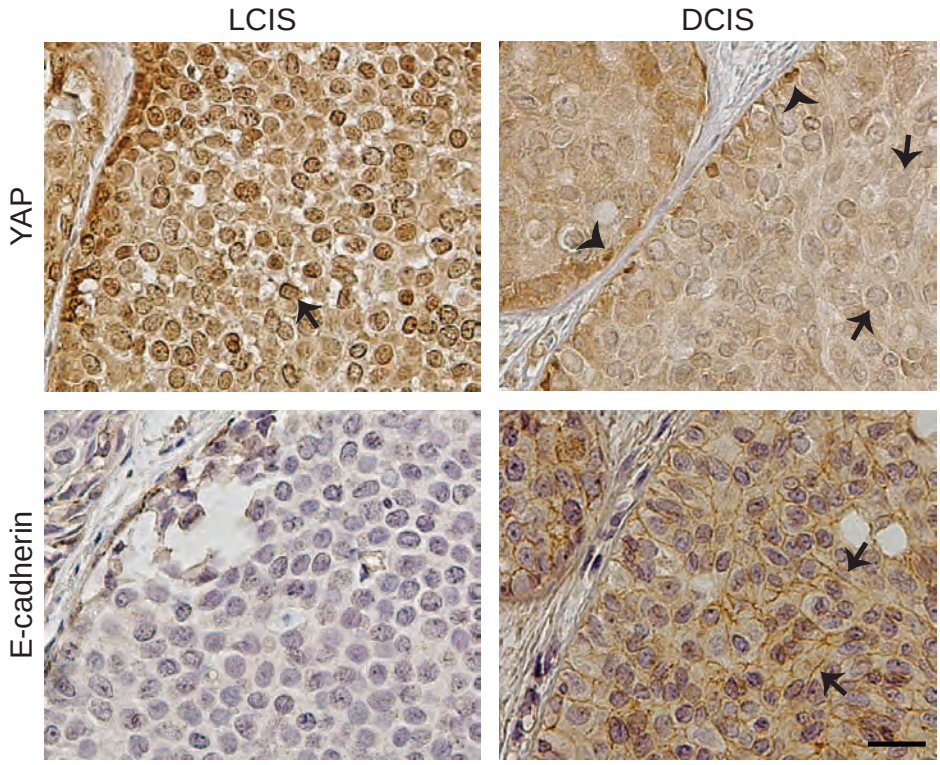

Figure 2. Loss of E-cadherin correlates with high nuclear YAP expression in LCIS.
Representative IHC images for YAP and E-cadherin in LCIS and DCIS. LCIS is characterised by low E-cadherin membrane expression (low left) and high nuclear YAP expression (top left). Inversely, DCIS shows high E-cadherin membrane expression (bottom right) and low nuclear YAP expression (top right). Size bar = 25 μm.

Table 3. Comparison between nuclear YAP expression in lobular (LCIS) and ductal in situ (DCIS) lesions and between invasive lobular (ILC) and ductal (IDC) lesions.

	Nuclear YAP expression			
Feature	**Low**	**High**		
	N (%)	**N (%)**	**Total**	**p-value**
LCIS	2 (7.4)	25 (92.6)	27	
DCIS	38 (84.4)	7 (15.6)	45	p<0.001
ILC	3 (11.1)	24 (88.9)	27	
IDC	34 (75.6)	11 (24.4)	45	p<0.001

Table 4. Nuclear YAP expression is significantly more frequent in pure LCIS compared to pure DCIS cases.

Feature	YAP expression in breast cancer *in situ*			
	negative	positive		
	N (%)	N (%)	Total	p-value
LCIS	2 (8.2)	9 (81.8)	11	
DCIS	18 (85.7)	3 (14.3)	21	p<0.001

Discussion

Our findings may help to differentiate LCIS from DCIS in daily pathology practice. This is clinically important, since DCIS will be radically excised by surgery, while the presence of LCIS is usually not an indication for radical surgical excision. Also, while E-cadherin is the obvious biomarker for the differential diagnosis between lobular and ductal breast cancer, E-cadherin may be non-functionally expressed in lobular breast cancer due to truncating mutations (ref) or rendered inactive due to inactivation of tight junction members such as Par3 [27-29]. Furthermore, Since YAP is not nuclear in normal luminal epithelial cells [20], its nuclear expression in *in situ* lesions may aid to distinguish DCIS from LICS.

We show that high nuclear YAP expression is frequent in both LCIS and ILC, while it is rare in DCIS and IDC. Because our data indicated that nuclear YAP expression is already high in LCIS, it suggests that nuclear YAP expression is probably an early event in lobular breast cancer development. We believe that loss of cell-cell adhesion through E-cadherin inactivation may induce nuclear translocation of YAP, because E-cadherin loss underpins lobular breast cancer etiology. Indeed, since E-cadherin loss and YAP activity have been functionally coupled [14], our findings suggest that E-cadherin inactivation in lobular breast cancer may instigate tumour progression through activation of YAP. Supporting this are findings that attenuation of AJ function through loss of α-catenin or components of the polarity complexes lead to activation of YAP [13]. An alternative is that YAP overexpression induces an EMT, which would lead to an inhibition of E-cadherin expression [17].

We have previously demonstrated that loss of E-cadherin in lobular breast cancer leads to constitutive activation of actomyosin contraction. In ILC, p120-catenin (p120) confers constitutive Rho and Rock-dependent tumour growth and metastasis through binding and inactivation of the Rho antagonist Mrip [30]. Since nuclear YAP has been coupled to cell morphology and F-actin polymerisation[31,32], we think that functional inhibition of the AJ in lobular breast cancer may instigate nuclear YAP localisation through constitutive and p120-dependent activation of Rho, Rock and F-actin. This scenario may therefore also explain the absence of contact inhibition or 'crowd-sensing' in the absence of E-cadherin and its effect on YAP localisation in breast cancer [14].
We have not addressed if the nuclear localisation leads to transcriptional activation of established target genes such as CTGF, CYR61 and AREG, established YAP targets genes [33-37]. Because these YAP targets have been strongly linked to breast cancer progression, it will be very interesting to determine whether expression of these factors correlate with the lobular breast cancer phenotype, and if their expression is dependent on nuclear YAP. Moreover,

future studies are needed to investigate whether YAP activation is sufficient for lobular breast cancer initiation and progression.

In conclusion, our results show that nuclear YAP expression is high in early and late stages of lobular breast tumours. Therefore, YAP activation may be important for the development and progression of lobular breast cancer. Future studies are needed to investigate if YAP confers transcriptional activity in LCIS/ILC and to further unravel functional relationships between the E-cadherin, the AJ members and YAP in lobular breast cancer.

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EJV, PvD and PWBD conceived the experiments and wrote the manuscript. EJV NtH and JFV performed the experiments.

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Chapter 7

General Discussion

General Discussion

Summarizing conclusions

In this thesis we focused on lobular breast carcinoma, the second most common breast cancer subtype based on histology. We aimed to enhance our understanding of the lobular breast cancer specific processes involved in tumor initiation and metastasis, to further define lobular breast cancer subtypes and to find subtype specific biomarkers. The ultimate goal of these efforts was to use the acquired knowledge to develop new or improve therapies for ILC patients.

Pleomorphic ILC is considered to be a more aggressive variant of ILC and biomarkers may help to correctly diagnose this tumor type in the future. In **chapter 3** we identify pleomorphic ILC specific methylation biomarkers for the first time, and we discover that the overall methylation patterns of IDC and ILC are relatively similar. Histologically lobular breast cancer shows a dyscohesive growth pattern, which suggests a flaw in cell-cell adhesion. Indeed, E-cadherin, which mediates cell-cell adhesion, was found to be absent in the majority of ILC tumors. The link between E-cadherin and ILC development was further underlined with an E-cadherin and p53 negative mammary specific conditional mouse model, which developed malignant breast tumors resembling human ILC. E-cadherin expression is lost in ILC due to genetic mutation, methylation or transcriptional repression. However, 10-15% of the ILC patients retain E-cadherin expression. Like E-cadherin, α-catenin, is required for proper AJ function and is an established tumor suppressor in the skin. Furthermore, α-catenin inactivating mutations have been found in breast cancer cell lines and in a family of hereditary diffuse gastric cancer. Besides, α-catenin expression is lost in the majority of ILC tumors and loss of α-catenin proved to be a strong prognostic factor for invasion and poor survival of breast cancer patients. To explore the possible role of α-catenin in E-cadherin positive ILC development, we investigated the effects of α-catenin loss in nonmetastatic breast cancer cell lines in **chapter 4**. We showed that upon α-catenin loss E-cadherin expression at the cell membrane is retained, but AJ maturation is impaired and cells show a rounded cell morphology. Furthermore, α-catenin loss lead to anoikis resistance in a Rho/Rock/actin dependent manner. Previously, we showed in vitro anoikis resistance and in vivo metastatic growth of E-cadherin mutant breast cancer cells is also largely controlled by Rho/Rock dependent actin contraction, which coincides with expression of downstream markers of active Rho/Rock signaling, phosphorylated myosin light chain (MLC) and cofilin, in ILC patients. Furthermore, high Rho GTPase or Rock expression has been linked with metastatic growth in mouse models and is correlated with invasive growth in cancer patients. Therefore, we conclude that active Rho/Rock signaling lies at the heart of both E-cadherin positive and negative ILC development and progression. In **chapter 5 and 6** we demonstrate that lobular carcinoma in situ (LCIS) and ILC are characterized by nuclear expression of the transcriptional coactivator YAP. Because LCIS, already expresses nuclear YAP, our findings indicate that YAP is activated during the early stages of lobular breast cancer development. E-cadherin loss is also an early event in ILC development and E-cadherin dependent regulation of proliferation is mediated by YAP. Besides, Rho activity and actin polymerization have been shown to be required for nuclear YAP localization and target gene expression. Because YAP acts down-

stream of E-cadherin and subsequent actin polymerization/contraction we conclude that loss of proper cell-cell adhesion by the AJ in the breast, is likely to cause nuclear translocation of YAP and induce target gene expression resulting in ILC development.

Summarizing discussion

ILC is characterized by a dyscohesive growth pattern, which suggests cell-cell adhesion is dysfunctional in these tumor cells, the loss of the key AJ complex member E-cadherin and frequent expression of the downstream Rho/Rock effector phosphorylated cofilin. These characteristics indicate cell-cell adhesion is dysfunctional and Rho/Rock signaling is activated in ILC. Although the majority of ILC is characterized by loss of E-cadherin expression, 10-15% of the ILC cases retains E-cadherin expression. α-catenin is also required for proper cell-cell adhesion, its expression is lost in the majority of ILC tumors and its absence causes anoikis resistance and Rho/Rock activation in breast cancer cell lines (chapter 4). Here, we will discuss by which mechanisms cell-cell adhesion loss can cause Rho/Rock activation. In chapter 5 and 6 we demonstrate that ILC is defined by high nuclear YAP expression. YAP was found to be critical for the regulation of contact inhibition of proliferation (CIP) by E-cadherin [2]. Moreover, Rho activity and actin polymerization stimulate nuclear YAP localization and target gene expression [3, 4]. Therefore, we will discuss YAP as a possible downstream effector of Rho/Rock signaling induced by AJ disruption in ILC. Finally we will discuss possible therapeutics inhibiting the Rho/Rock pathway and their possible effect on ILC patients.

Biological mechanisms

How is Rho/Rock/actin contraction regulated in ILC?

Normal breast epithelial cells form cell-cell adhesion structures, AJs, at the cell membrane by homotypic interactions between E-cadherin molecules on neighboring cells. The transmembrane protein E-cadherin is bound to β-catenin on the intracellular side of the cell. By linking the AJ to the actin cytoskeleton, α-catenin enables the relay of tension onto the AJ, which is essential for lateral clustering of E-cadherin molecules and for the maturation of the AJ. However, in ILC cell-cell adhesion is disrupted, which is caused by the loss of E-cadherin function. Previously, we demonstrated that within E-cadherin mutant mouse ILC cells, p120 is translocated to the cytosol [1]. Here p120 binds and inhibits the Rho/Rock antagonist Mrip, resulting in Rho/Rock activity, which is essential for anchorage independent tumor growth and metastasis formation in vivo. We also showed that 77% of the ILCs expresses phosphorylated cofilin, which is the downstream target of Rho/Rock signaling. Together with the fact that Rho/Rock expression is correlated with invasiveness and poor survival, these data indicate that Rho/Rock signaling lies at the heart of ILC development and progression. In chapter 4 we showed that in the presence of E-cadherin, α-catenin loss in p53 negative mouse breast cancer cell lines also induces Rho/Rock dependent anoikis resistance. However, we demonstrated that the activation of Rho/Rock signaling was independent of p120/Mrip signaling in α-catenin negative cells, which implies Rho/Rock activation has to be alternatively regulated. To understand how Rho/Rock activation is maintained in the absence of α-catenin, we will have to evaluate α-catenin function at the membrane and within the cytosol. While membranous α-catenin links F-actin with the AJ and enables AJ maturation, cytosolic α-catenin forms F-actin binding homodimers and regulates actin dynamics.

AJ formation is known to affect the composition of receptors within the membrane and to influence their activity [5, 6]. Others have shown that Rho activation is regulated by cell surface receptors, including G-protein coupled receptors (GPCRs) and receptor tyrosine kinases, and by Rho activating and Rho inactivating or inhibitory proteins. The inability to form an AJ could therefore influence Rho activation through changes in cell membrane composition. For example integrin engagement can activate focal adhesion kinase (FAK). As integrins are also localized near the AJ and FAK can interact with the AJ [7], FAK activity could be regulated by AJ formation. Activated FAK can phosphorylate p190RhoGAP which inhibits Rho [8]. Besides loss of FAK has indeed been associated with high Rho/Rock signaling in the breast [9]. Thus, disrupted AJ formation could attenuate inhibition of p190RhoGAP by FAK, leading to Rho/Rock activation. Second, cytoskeletal remodeling upon cell detachment has been reported to cause Rho activation in a feed-forward loop [10, 11]. Since loss of AJ-actin linkage by α-catenin knock down results in cell detachment and rounding, this could also induce a activation of Rho and cytoskeletal contraction upon cytoskeletal remodeling. Third, α-catenin is known to bind Merlin [12], and E-cadherin or α-catenin loss may prevent p21-activated kinase (PAK) inhibition by Merlin [13]. Like Rock, PAK is another kinase which has been linked to actin contraction and cofilin inhibition (reviewed in [14]). Indeed, depletion of α-catenin has been associated with PAK activation [15], suggesting PAK activation could cause Rho/Rock activation in ILC. In short, loss of E-cadherin-bound α-catenin could lead to Rho/Rock activation through aberrant activation of cell surface receptor signaling, the mechanical cue induced by loss of cell-matrix engagement and subsequent cytoskeletal remodeling or by release of PAK inhibition by Merlin.

Alternatively, the lack of cytosolic α-catenin could possibly increase Rho signaling. Within the cytosol α-catenin is present as a dimer and binds F-actin to regulate actin cytoskeletal remodeling by inhibiting Arp2/3 mediated actin branching and cofilin dependent actin severing [16-19]. Although sequestration of cytosolic α-catenin dimers to different locations within the cell does not affect cell-cell adhesion, it does reduce actin dynamics at the membrane [17, 20]. However, cytosolic α-catenin loss would cause stimulation of branched actin polymerization and actin severing by dephosphorylating and activating cofilin, which is opposite to the α-catenin-negative phenotype; cell rounding, lack of radial actin filaments and phosphorylated cofilin expression. Therefore, we hypothesize that the lack of membranous α-catenin is responsible for an actin cytoskeletal remodeling inducing Rho/Rock activation, which causes anoikis resistance and tumor progression (Figure 1). The fact that E-cadherin - α-catenin fusion constructs rescued AJ formation and contact inhibition of proliferation [2, 5], further underlines this hypothesis. To verify the different functions between cytosolic and membranous α-catenin future rescue experiments could be performed using α-catenin negative cells rescued either with an E-cadherin - α-catenin fusion construct, with an α-catenin truncation construct (deletion of first 64 amino acids) which favors dimerization over α-catenin binding [21], or with an α-catenin fusion construct which targets α-catenin to a membrane.

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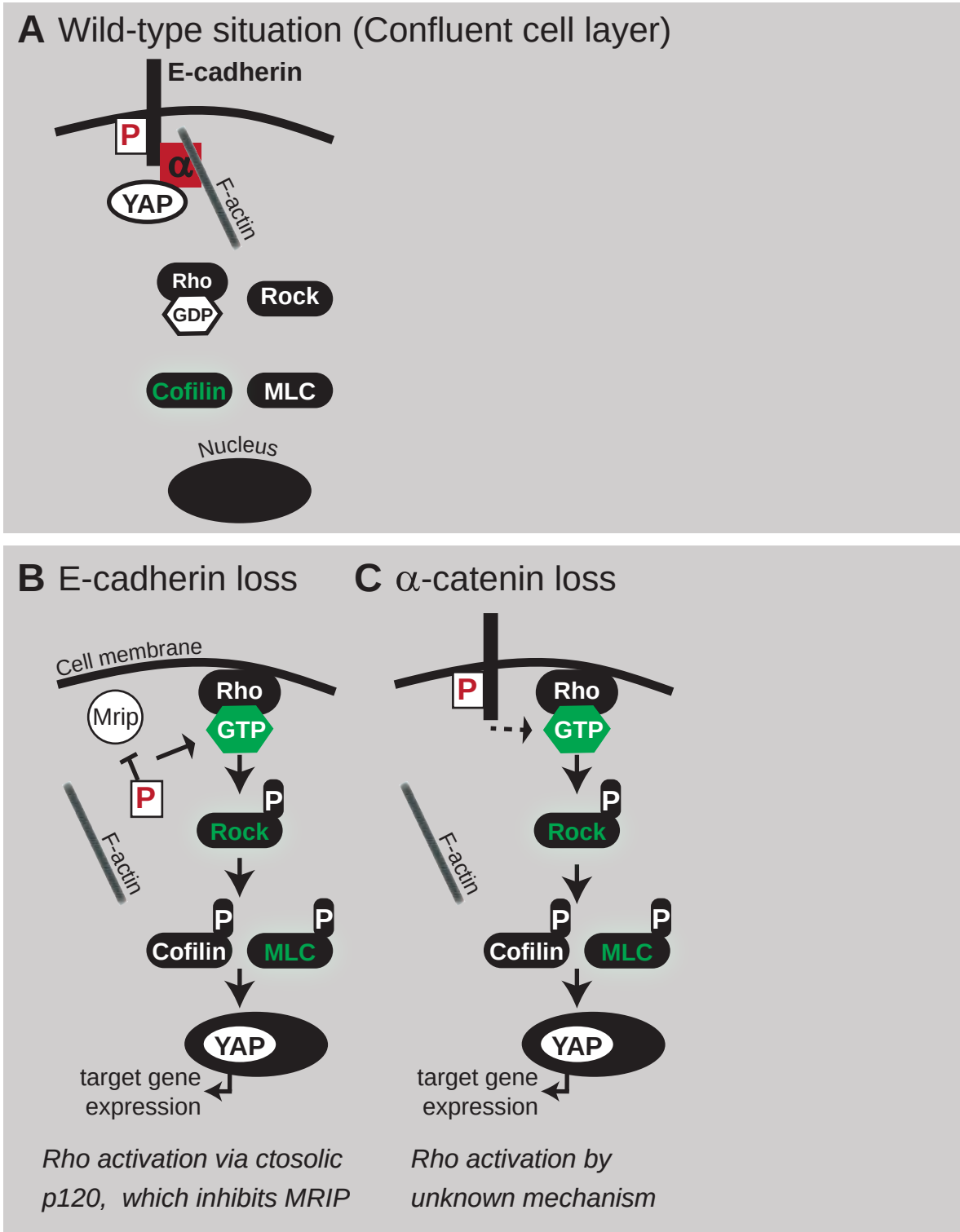
A Wild-type situation (Confluent cell layer)
E-cadherin
P
α
YAP
F-actin
Rho
GDP
Rock
Cofilin
MLC
Nucleus
B E-cadherin loss
C α-catenin loss
Cell membrane
Mrip
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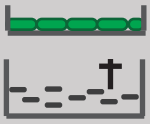
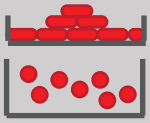


- F-actin linked to AJ via α-catenin
- Cytosolic YAP sequestration
- No Rho/Rock activation
- Actin severing and no actin tension
- Contact inhibition of proliferation
- Anoikis



- F-actin - AJ linkage is disrupted
- Rock activation
- Actin polymerization and tension
- Nuclear YAP translocation
- No contact inhibition of proliferation
- Anoikis Resistance

Figure 1. Effect of adherens junction disruption on contact inhibition of proliferation and anoikis resistance. *A. Wild-type situation (similar to cytosolic loss of α-catenin or an E-cadherin- α-catenin fusion construct). p120-catenin (indicated by a red P in the image) is bound to E-cadherin at the cell membrane and F-actin is linked to the AJ. Rho and Rock are not activated, causing Cofilin to be active due to its unphosphorylated state. Phosphorylated YAP is sequestered within the cytosol or bound by α-catenin or Angiomotin. Contact inhibition of proliferation will be well regulated and cells will die in suspension.* ***B****. Loss of E-cadherin prevents F-actin linkage to the AJ. E-cadherin loss will cause cytosolic localization of p120 and subsequent inhibition of the Rho antagonist Mrip (left cartoon) and result in Rho activation. Rho-GTP will cause phosphorylation of downstream effectors. Phosphorylated cofilin is inactive and will not be able to stimulate actin severing, while phosphorylated myosin light chain (MLC) will stimulate actin cytoskeletal tension. Overall this results in nuclear YAP translocation, expression of YAP target genes, proliferation and anoikis resistance. C. In case of α-catenin loss, E-cadherin and p120 will still be present at the membrane, but F-actin linkage to the AJ will be disrupted. In the absence of α-catenin, Rho is also activated, which may be caused by several signaling mechanisms (discussed in the paragraph above). Rho-GTP will have the same downstream effects as in the E-cadherin negative situation. Protein names written in green indicate their in an activated state due to the presence or absence of a phosphorylation group.*

Is YAP a downstream target of cell-cell adhesion loss and subsequent actin contraction in ILC?

Recently, YAP was found to regulate organ size through cell density sensitization [2, 22, 23], which stirred up an old debate about the mechanism responsible for contact inhibition of proliferation (CIP). CIP refers to the phenomenon that cells grown to confluence decrease their proliferative rate and eventually go into cell cycle arrest to form a monolayer. In contrast, cancer cells often lack CIP and continue to proliferate (reviewed in [24]). Although the phenomenon has been known from the 1960s, it is still debated what biological mechanism(s) regulate this process. Recent findings showed that both E-cadherin and α-catenin can regulate CIP in a YAP dependent manner, indicating cell-cell adhesion regulates CIP through YAP [2, 25]. In the skin, α-catenin is thought to bind phosphorylated YAP via a scaffolding protein called 14-3-3 and to hereby sequester YAP within the cytosol inhibiting YAP target gene expression stimulating proliferation and survival. Furthermore, proteins like Merlin and Angiomotin also associate with the AJ and regulate CIP [12, 26, 27]. Angiomotin binds Merlin and Merlin depletion disrupts AJ formation. FInally, Angiomotin can regulate YAP localization directly; at low cell density Angiomotin is recruited to the actin cytoskeleton. As F-actin and YAP compete for Angiomotin binding, YAP is released and translocates into the nucleus [27]. These findings further strengthened the thought that cell-cell adhesion mediates CIP via YAP. However, besides cell-cell adhesion many other YAP regulating cues have been discovered including Hippo signaling [23], actin cytoskeletal tension [2, 4], G-protein coupled Receptor (GPCR) signaling [28] and growth factor stimulation [26]. Furthermore, mechanical regulation of YAP, by cell stretching or extracellular matrix stiffness, was shown to overrule regulation by GPCR and Hippo signaling [29]. Interestingly, the actin severing and/or capping proteins cofilin, gelsolin and capz were identified as the mechanical effectors regulating YAP [29]. Experiments using cell detachment as a mechanical cue, showed YAP is differentially regulated in normal versus cancer cells [30]. While cell detachment caused apoptosis in non-cancerous cell lines, cancer cell lines survived the loss of cell-matrix interactions and therefore were anoikis resistant. Although YAP target gene expression was not analyzed, cell detachment dependent survival depended on Hippo signaling and nuclear YAP expression, and Hippo signaling was able to overrule mechanical cues in this setting [30]. The amount of cytoskeletal tension in cells in suspension is unknown, and as Rho/Rock/cofilin signaling was shown to be activated in our adherent E-cadherin and α-catenin negative cells, we expect this creates cytoskeletal tension inducing YAP activation, preventing anoikis resistance and stimulating tumor progression in ILC.

To understand more about how YAP may cause anoikis resistance and tumor progression in ILC, it is important to know which target genes it induces. YAP is a transcriptional co-activator and can associate with different transcription factors to induce target gene expression. YAP contains a WW domain (a sequence motif containing two conserved and consistently-positioned tryptophan (W) residues), which is able to bind a PPXY motif. There are many transcription factors that contain a PPXY motif and are therefore putative YAP binding partners (reviewed in [31]). Interestingly, the most renowned binding partner of YAP are the TEAD family of transcription factors, which do not contain a PPXY motif and are known to induce connective tissue growth factor (CTGF), KI67, c-Myc, Survivin, Axl and Cysteine-rich 61 (Cyr61) transcription ([32] and reviewed in [33]). CTGF induction is essential for YAP induced growth and colony formation and could therefore also have an important role in ILC [32]. Furthermore, Cyr61 and ankyrin repeat domain 1 (Ankrd1) have often been used as a read-out for YAP activation [29, 30], but their function is not well studied. To be able to

analyze the relationship between YAP and anoikis resistance, transcription factors bound by YAP and the induced target genes need to be identified in our cells.

We observed high nuclear as well as cytosolic YAP expression in ILC samples (chapter 5). YAP degradation is regulated via Hippo/SCFβ-TRCP dependent ubiquitination [34]. The lack of AJ formation in ILC will prevent Angiomotin recruitment to the AJ in ILC. Although Angiomotin-F-actin binding has not been proven to be stimulated by cytoskeletal tension, cytoskeletal tension in ILC could enhance F-actin-Angiomotin binding preventing cytosolic sequestration and degradation of YAP. We have not measured the Hippo signaling status or the expression of YAP transcriptional target genes in our cells, but as our cells are anoikis resistant and this is a trait proven to be inhibited by Hippo signaling [30], we expect that this pathway is inactive in ILC. Thus, Angiomotin dependent cytosolic sequestration of YAP and Hippo dependent degradation is probably inhibited in ILC, which can explain high YAP levels both in the nucleus and cytosol.

Alternatively to YAP mediating anoikis resistance in ILC, the phosphatidylinositol 3-kinase (PI3K) pathway may be responsible for cell survival in suspension. PI3K catalyzes the conversion of phosphatidylinositol (3,4)-bisphosphate (PIP2) into phosphatidylinositol (3,4,5)-trisphosphate (PIP3) located within the cell membrane, which stimulates Akt (also known as protein kinase B) dependent cell survival. PI3K activating mutations are found in about 33-46% of the ILC patients [35-37]. The Greek word anoikis means 'homelessness' and is a particular apoptotic death due to loss of cell-matrix adhesion [38]. Our previous findings demonstrated p120 depletion causes increased Akt and MAPK/ERK signaling and anoikis resistance in mouse and human breast cancer cell lines [39]. Furthermore, others have shown PI3K can induce Rho activation [40-42]. These findings suggest PI3K signaling can activate both Rho and Akt. Therefore, PI3K mutations could facilitate an alternative cell survival signal through Akt and cause Rho/Rock activation preventing anoikis and stimulating tumor progression in ILC.

Therapeutic intervention for ILC based on Rho/Rock inhibition

Many Rho and Rock inhibitors have been developed, albeit the orally available Rock inhibitor fasudil hydrochloride (fasudil, HA-1077 or AT877) is the only inhibitor that is clinically approved for the treatment of vasoconstriction [43]. Vasoconstriction is caused by contraction of the muscle cells in the blood vessels. Intra-arterial injection of fasudil will inhibit Rock and cause arterial muscle cell relaxation. Two Rock isoforms exist, Rock1 and 2. Fasudil (and the related version H-1152P) targets both Rock isoforms, and a range of other kinases with a ≥ 2-fold lower efficiency [44-46]. Other more potent and specific Rock inhibitors have been developed, but none have been clinically approved so for the treatment of breast cancer fasudil can be most readily used.

As Rock inhibitors/drugs will relax the arterial muscle cells they will cause hyperemia, which refers to a process where an increased/excessive amount of blood flow is induced. Tumors are known to stimulate angiogenesis to increase their blood supply, which will increase the availability of growth promoting molecules and stimulates metastasis as the newly formed blood vessels are of poor quality. Although the effect of a dilated arterial system on tumor progression is unknown, this effect is concerning and may enhance tumor growth and metastasis formation. Possibly, Rock inhibitors could be administrated via the nipple to directly target the breast epithelial cells. As blood vessels will only be reached indirectly by this method, the effect of Rock inhibitors on the blood vessels may be less pronounced. Alternatively, a treatment regimen where administration of a Rock inhibitor is followed by an angiogenesis inhibitor may be successful. Besides, fasudil administration could be combined with another chemotherapeutic agent as it may facilitate increased tumor delivery due to the increased blood flow. Chemotherapeutic agents targeting the PI3K/Akt pathway may make an effective combination with Rock inhibitors, as this is an alternative signaling pathway, stimulating cell survival, often mutated in ILC. Mammalian (or mechanistic) target of rapamycin (mTOR) is a downstream effector of the PI3K/Akt pathway. The mTOR inhibitor Everolimus could be used as it is FDA approved for the treatment of hormone-receptor positive breast cancer in postmenopausal women (reviewed in [47]). Furthermore, as several PI3K and Akt inhibitors are in clinical trials, any of these drugs may be used in the near future. Furthermore, a combination of Fasudil, Everolimus and Trastuzumab (Herceptin), which inhibits the ERBB2 (Her2/Neu) receptor, may be very beneficial in pleomorphic ILC patients as they are often Her2 positive and enhanced PI3K signaling has been implicated in Trastuzumab resistance [48].

The use of Rock inhibitors to treat cancer has only been tested in mouse models. For instance, fasudil significantly lowered tumor development in mice that were orthotopically transplanted with the breast cancer cell line MDA-MB-231 [49]. Previously, we showed that Y-27632 and fasudil significantly decreased anoikis resistance in cell lines derived from a mouse and human ILC cells [1]. Moreover, Rock1 knockdown in these orthotopically transplanted cell lines attenuated tumor growth and prevented metastatic dissemination. Finally, we demonstrated in chapter 4 that Y-27632 and GSK429286A can significantly reduce anoikis resistance induced by α-catenin knockdown. In contrast to the effect in suspended cells, Rock inhibition in adherent α-catenin knockdown cells did not cause cell death and instead rescued cell-matrix adhesion. Although this could indicate Rock inhibition would be ineffective in the presence of ECM proteins or could stimulate recolonization, these effects were not observed when treating E-cadherin negative tumors in mice. Instead, both primary tumor

growth and metastasis formation were inhibited [1]. These findings suggested that Rock as a targeted treatment in ILC patients might be beneficial for primary tumor growth reduction as well as metastasis formation. We advocated that α-catenin or E-cadherin loss both cause Rho/Rock activation inducing anoikis resistance and nuclear translocation of YAP. As Rho/Rock is known to be associated with metastasis formation and poor survival, inhibition of Rock will probably inhibit invasion and metastasis formation in patients. Secondly, inhibition of Rock is likely to prevent nuclear translocation of YAP and subsequent induction of cell proliferation. As E-cadherin loss and nuclear YAP expression are early events in ILC development, Rock is likely to be activated in early lesions like LCIS. This suggests lobular breast carcinomas in any progressive stage may profit from Rock inhibiting treatments. ILC is characterized by a non-cohesive growth pattern and these tumors often do not form a palpable tumor, but instead show invasive growth. Therefore, neo-adjuvant and adjuvant treatment would probable both be beneficial when treating ILC.

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Addendum

Nederlandse samenvatting

List of Abbreviations

Curriculum Vitae

List of Publications

Dankwoord (Acknowledgements)

Nederlandse samenvatting

Borstkanker

Borstkanker epidemiologie

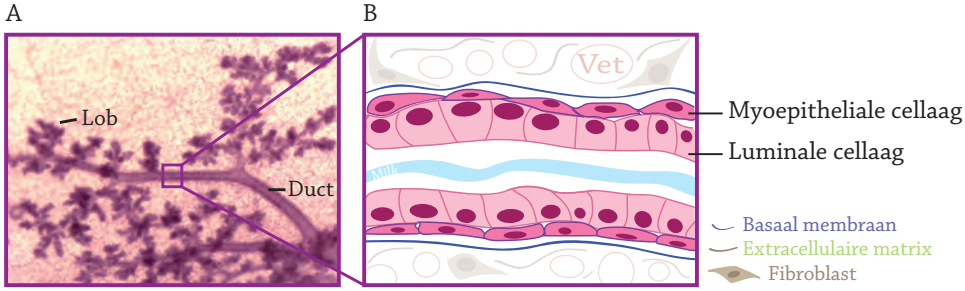
In Nederland wordt 1 op de 8 vrouwen geconfronteerd met borstkanker gedurende haar leven, en dit maakt borstkanker de meest prevalente kankervorm bij vrouwen. In 2011 werden dan ook 14 duizend vrouwen gediagnosticeerd met, en overleden er 3 duizend vrouwen aan een invasieve (kwaadaardige) vorm van borstkanker [1]. Borstkanker komt niet alleen voor bij vrouwen, maar ook bij een kleine groep mannen. Zo werd invasief borstkanker in 2011 bij 83 mannen gediagnosticeerd en overleden er 22 mannen aan deze ziekte [1]. De gemiddelde leeftijd waarop vrouwen gediagnosticeerd worden met borstkanker is 60 jaar, voor mannen 67 jaar. Risico factoren voor het krijgen van borstkanker zijn: erfelijkheid, leeftijd, geen of weinig kinderen, geen borstvoeding geven of heel kort, eerste kind op hoge leeftijd (boven 35), vroege menarche (eerste menstruatie op jonge leeftijd), late menopauze (overgang), hormoon substitutie, de pil (hormonale anticonceptie) alleen tijdens en niet na gebruik, overgewicht, sedentaire levensstijl (weinig beweging), stevige borsten en alcohol [2,3]. Ongeveer 5% van alle borstkankergevallen kunnen toegeschreven worden aan erfelijke mutaties in het gen BRCA1 of 2. Dragers van dit gen hebben een 40-85% verhoogd risico op het ontwikkelen van borstkanker [2]. De totale gezondheidskosten in 2011 in Nederland was 87 miljard euro. In datzelfde jaar werd 696 miljoen euro uitgegeven aan borstkanker, waarvan 79% werd gebruikt voor de ziekenhuiskosten, 9% voor preventie en 12% voor overige kosten [2].

Borstkanker behandeling

Sinds 1989 wordt er in Nederland aan borstkanker preventie gedaan doormiddel van X-ray mammografie bij vrouwen tussen de 50-70 jaar oud. In 1998 werd het programma uitgebreid en werd de maximum leeftijd verhoogd naar 75. De patiënten die gediagnosticeerd werden met borstkanker in 1990 hadden een 10-jaar overlevingskans van 65%. In 2002 was deze kans gestegen naar 77%, wat toegeschreven kan worden aan eerdere ontdekking en verbeterde behandel methoden. Ondanks dat de behandeling dus duidelijk verbeterd is, gaan er nog steeds veel vrouwen dood aan borstkanker en is er dus nog steeds een noodzaak voor het beter begrijpen van het ziekteproces en het verbeteren van de behandeling. De huidige behandeling is gebaseerd op tumor grootte, histologische graad (afgeleid van de kenmerken die te zien zijn in het biopt met behulp van een microscoop), hormoon receptor en HER2 (ook bekend als ERBB2 of Neu) gen expressie. Onderzoek heeft uitgewezen dat tumoren heterogeen (verschillend) van aard zijn, zowel histologisch (morfologie van de tumor onder de microscoop) als moleculair (eiwit expressie). Het huidige idee is dat we 'personalized medicine' moeten nastreven om de behandeling van borstkanker in de toekomst zoveel mogelijk te kunnen verbeteren. Bij personalized medicine zal er gekeken worden naar de tumor specifieke karakteristieken (welke signaleringsroutes zijn er actief) bij 1 patiënt, waar de behandeling dan vervolgens op aangepast wordt, genaamd 'targeted therapy'. Om dit te kunnen verwezenlijken zullen we in staat moeten zijn om belangrijke, drijvende signaleringsroutes aan te kunnen wijzen in iedere tumor. Ook zal een verregaande kennis over interacties tussen verschillende signaleringsroutes essentieel zijn voor het verbeteren van kankerbehandelingen in de toekomst.

Borstklier opbouw

De borstklier bestaat uit melk-producerende lobben die ontsloten worden door melk-geleidende ducten die naar de tepel lopen. De ducten en lobben bestaan uit een binnenste, luminale, cellaag bedekt met een myoepitheliale (basale) cellaag. Dit alles wordt afgedekt met een basaalmembraan (**Figuur 1**). De myoepitheliale cellaag kan zich samentrekken en daarmee de melk, die geproduceerd en uitgescheiden wordt door de alveolaire luminale cellen (binnenste cellaag in de lobben), richting de tepel duwen. Het is nog onduidelijk of borstkanker ontstaat in de luminale of de myoepitheliale cellen.

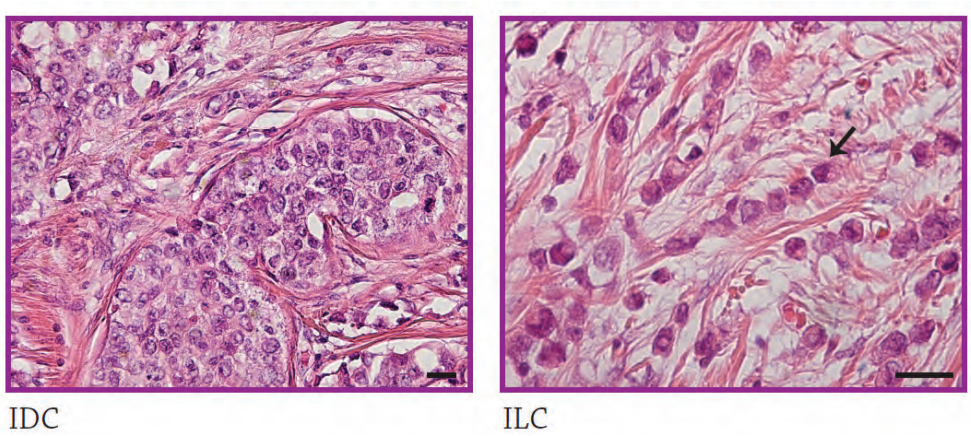


Figuur 1. Structuur van de borst.
A*. Beeld van borstmateriaal van een 10 weken oude muis gekleurd met carmine (speciale kleurstof). Melk wordt geproduceerd in de lobben en naar de tepel getransporteerd via de ducten.* ***B****. Schematische weergave van een duct in de borst. De epitheliale borstcellen worden omringt door een basaalmembraan en liggen gezamenlijk in het vetweefsel van de borst, waar fibroblasten en extracellulaire matrix eiwitten tussen liggen. De buitenste myoepitheliale cellaag kan zich samentrekken en zo het transport van de melk richting de tepel stimuleren. De binnenste luminale cellaag vormt de wand van het ductale lumen (de ruimte in de buis), die melk en andere vloeistoffen bevat.*

De meeste of alle organen in het menselijk lichaam bevatten stamcellen of progenitorcellen. Stamcellen kunnen nieuwe cellen produceren door te delen. Vervolgens differentiëren (specialiseren) de nieuwe cellen zich tot alle verschillende soorten cellen die in dat orgaan voorkomen. Progenitorcellen, kunnen slechts een beperkt aantal soorten gedifferentieerde cellen produceren. De borstklier in vrouwen is onderhevig aan continue cycli van celdeling, differentiatie en regressie, wat gereguleerd wordt door hormonen die samen gaan met de menstruatie cyclus, zwangerschap en borstvoeding. Misschien hebben stamcellen hierdoor een relatief grote rol in de borstklier vergeleken met andere organen. Muizenstudies hebben laten zien dat je een klein deel van een volwassen borstklier kan transplanteren in een lege, alleen uit vet bestaande ontvangende borst van een 3 weken oude muis, wat resulteert in een normale uitgroei van een volledige borstklier [4]. Deze bevindingen toonden aan dat er verspreid door de hele borstklier stamcellen aanwezig moeten zijn. Omdat stamcellen delende en regeneratieve capaciteiten bezitten, ontstaan tumoren misschien uit stamcellen of kunnen tumor cellen mogelijk (gedeeltelijk) dedifferentiëren (teruggaan in de ontwikkeling) naar een stamcel. Om deze redenen zijn wetenschappers erg geïnteresseerd in het identificeren en het begrijpen van de biologie van stamcellen. Het vinden van stamcel markers en hun exacte ligging in de borstklier bleek echter heel moeilijk. Recente vooruitgang in dit onderzoeksgebied wijst erop dat er zowel in de luminale als in de myoepitheliale cellaag verschillende soorten stam- en progenitorcellen te vinden zijn (reviewed in [5]).

Borstkanker subtypes

Ook al bestaat de borst slechts uit luminale en myoepitheliale cellen, toch bestaan er veel verschillende soorten borstkanker subtypes. Met 80% is invasief ductaal carcinoom (IDC) (ook wel 'not otherwise specified' (NOS) genoemd) de grootste op histologie-gebaseerde groep. Invasief lobulair carcinoom (ILC) vormt de op één na grootste groep met 10-15% (**Figuur 2**). De laatste 5-10% van de borstkankergevallen bestaat uit veel verschillende zeldzame borstkankertypen. IDC en ILC kunnen vervolgens weer onderverdeeld worden in subtypes (deze worden beschreven in **Hoofdstuk 2**). In tegenstelling tot IDC vormt ILC meestal geen tastbare knobbel, waardoor vroege diagnose moeilijk is. Tot 6 jaar na de diagnose is de ziektevrije overleving gunstiger voor ILC ten opzichte van IDC [6]. Echter na 10 jaar zijn de ILC patiënten slechter af vergeleken met de IDC patiënten [6].



Figuur 2. Representatieve H&E afbeeldingen van humaan ILC en IDC.
ILC wordt gekarakteriseerd door een niet-coherent groeipatroon van kleine, vergelijkbare cellen, die als alleenstaande cellen infiltreren of als een rijtje van cellen (1-cellaag dik, zie pijltje), vaak om een gezonde duct heen. Het groeipatroon van de cellen in IDC vertoond veel cohesie. Omdat de IDC groep tumoren bevat die niet tot een specifieke subgroep gerekend konden worden, is deze groep niet heel eenduidig en zijn er verder geen algemene histologische karakteristieken. De vergrotingsbalk geeft 20 µm aan.

De genetica achter Invasief Lobulair Carcinoom

Genen maken onderdeel uit van je DNA en zijn een soort recept voor het maken van een eiwit, en zonder eiwitten is er geen cel en dus geen leven. Daarom geeft het bestuderen van je genen belangrijke informatie over de eiwitten en processen in zowel gezond als ziek weefsel. In de overgrote meerderheid van de borsttumoren is er een verlies te zien van een hele chromosoomarm (een stuk DNA dat vele genen bevat) genaamd 16q [7]. Doordat we van ieder gen twee kopieën (allelen) hebben kan dit verlies opgevangen worden. Echter, alleen in ILC raakt de enige andere kopie van het *CDH1* gen ook verloren of corrupt door mutatie of methylatie [8,9]. Dit gen, gelegen op 16q22.1, zorgt voor de expressie/aanmaak van het eiwit E-cadherine en is dus afwezig bij >80% van de ILC patiënten[10], vergeleken bij ongeveer 7% van de IDC patiënten [11].

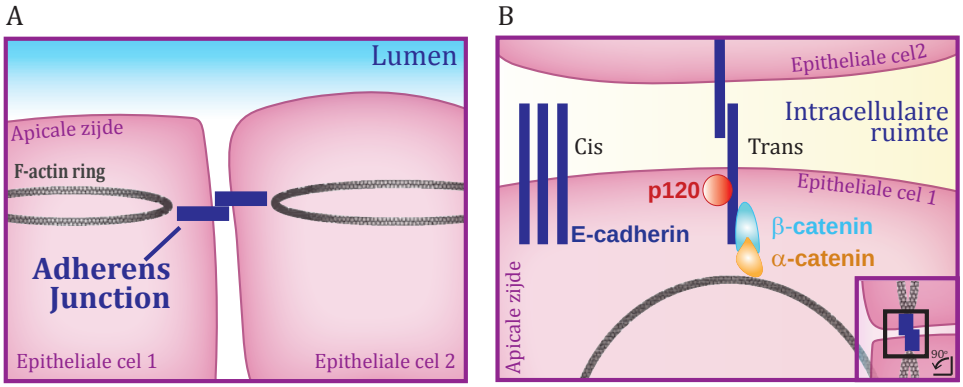
Cel-cel adhesie

Vorming van cel-cel adhesie knooppunten

Epitheliale cellen bedekken zowel de naar binnen gevouwen als de openlijke oppervlaktes van het lichaam. Om een beschermende barrière te vormen voor de buitenwereld vormen deze cellen een hechte laag ondersteund door onderlinge verbindingen genaamd cel-cel adhesie knooppunten (junctie's). Er zijn verschillende soorten knooppunten tussen cellen, maar aangezien E-cadherine onderdeel uitmaakt van de 'adherens junction (AJ)' zullen we alleen deze belichten in deze samenvatting. AJs zijn calcium afhankelijke complexen die de celmembraan (buitenkant van de cel) verbinden met het skelet van de cel, genaamd actinecytoskelet. E-cadherine is een transmembraaneiwit (steekt door de celmembraan heen) en vormt de basis van deze junctie (**Figuur 3**). Het extracellulaire domein bindt aan E-cadherine eiwitten op naburige cellen, die zo voor de verbinding, in 'trans', tussen verschillende cellen zorgen. Hiernaast maken E-cadherine moleculen in hetzelfde membraan ook verbindingen met elkaar, 'cis' verbindingen. Deze verbindingen zijn vrij zwak maar ze versterken weer de trans verbindingen [12,13]. De AJs bevinden zich niet overal maar vooral op een bepaalde hoogte in het membraan rondom de cel, waardoor ze een soort riem vormen om de cel heen, genaamd zonula adherens. Het intracellulaire domein van E-cadherine bindt aan de eiwitten p120-catenin (p120) en β-catenin, waarvan de laatste op zijn beurt weer aan α-catenin bindt (**Figuur 3**). De naam 'catenin' is afgeleid van het Latijnse woord 'catena' wat 'ketting' betekent [14]. α-catenin verbindt de AJ met het actinecytoskelet en vormt dus inderdaad een soort ketting, al is er nog steeds onduidelijkheid over hoe deze verbinding exact gevormd wordt [15,16]. Na de initiële verbinding zal het complex volwassen worden. Ook dit proces wordt nog niet goed begrepen, al is het wel duidelijk dat het volwassen worden van de junctie alleen mogelijk is als er spanning op staat via het actinecytoskelet. Daarnaast verandert de oriëntatie van de actinekabels t.o.v. de junctie [17]. Een grote familie van eiwitten genaamd RHO GTPase's kunnen de vorm en de spanning op het actinecytoskelet beïnvloeden. Eén van de eiwitten waarmee zij dit bereiken is Rho-associated kinase (Rock). Rock stimuleert de formatie van gebundelde actinekabels (10-30 actinekabels) en verhoogt de spanning op de kabels.

Verlies van α-catenin en kankerformatie

Bij afwezigheid van α-catenin kunnen E-cadherine moleculen nog wel trans verbindingen aangaan met andere E-cadherine moleculen, maar de stabiliteit van het complex en de spanningskracht tussen de E-cadherine moleculen verminderd, cis en trans, omdat de AJ geen verbinding meer heeft met het actine cytoskelet en het daarbij horende spanningsveld dus afwezig [18,19]. In tegenstelling tot het verlies van E-cadherine, is er nog geen uitgebreid onderzoek gedaan naar eventuele genetische afwijkingen of expressie niveaus van α-catenin in borstkanker tumoren. Wel is een mutatie in het α-catenin gen beschreven die mogelijk erfelijke diffuse maagkanker veroorzaakt bij een Nederlandse familie [20], en zijn er borsttumorcellijnen bekend die geen (functioneel) α-catenin eiwit tot expressie brengen door een mutatie [21]. Verder hebben muizenstudies aangetoond dat genetisch verlies van α-catenin in de huid of het hoofd (cerebrale cortex) leidt tot tumor formatie [22,23], maar of dit ook zo is in de borst is nog niet onderzocht. Zowel E-cadherine en α-catenin zijn nodig voor een fenomeen dat 'contact inhibitie van proliferatie' wordt genoemd [22,24]. Dit houdt in dat bij verlies van de AJ de cellen niet meer stoppen met delen zodra ze elkaar aanraken, wellicht doordat ze elkaars aanraking niet meer 'voelen'. Deze resultaten geven samen aan dat α-catenin functioneert als een tumor suppressor (eiwit dat tumor formatie remt) in veel organen en dus mogelijk dezelfde functie heeft in de borst.



Figuur 3. Schematische weergave van de adherens junction die zorgt voor cel-cel adhesie tussen naburige epitheliale borstcellen.

***A**. De apicale zijde van de epitheliale cellen is gelegen aan de kant van de cel die het lumen raakt. Binnen in de cel is de adherens junction (AJ) verbonden met een ring gemaakt van actine filamenten (F-actin ring), die een soort adhesie ring vormt rondom de cel en de zonula adherens wordt genoemd. **B**. Dit beeld laat zien dat de AJ bestaat uit een interactie tussen E-cadherine moleculen van 2 tegenover elkaar gestelde cellen (trans-interactie), die binnenin de cel verbonden zijn mijn p120, β-catenin en α-catenin. Dit laatste eiwit is verantwoordelijk voor de connectie tussen de AJ en het actine cytoskelet. De exacte aard van deze verbinding is nog onbekend. Zowel enkele E-cadherine moleculen als volledige AJ complexen kunnen ook een zijwaardse interactie met E-cadherine moleculen aangaan; een cis-interactie.*

Yes-associated protein

YAP regulatie

Yes-associated protein (YAP) is een transcriptie coactivator, wat inhoud dat het bindt aan verschillende transcriptie factoren en dat ze tezamen aan het DNA binden en de aanmaak van bepaalde eiwitten stimuleren. Ongeveer 8 jaar geleden werd ontdekt dat YAP in fruitvliegen (officieel *Drosophila melanogaster*) een hele belangrijke rol vervult door via celdeling (proliferatie) en celdood (apoptose) te bepalen hoe groot organen worden. Ook in de muis leidt YAP overexpressie (artificieel hoge expressie) in de lever tot een verhoogde proliferatie en orgaangrootte [25,26]. Bij het normaliseren van het YAP expressie niveau nam de lever weer een normale grootte aan, wat aantoont dat het effect van YAP reversibel is, alhoewel langdurige overexpressie van YAP niet reversibel is en tot tumorformatie leidt. In tegenstelling tot de lever leidt YAP overexpressie in de darmen van een muis al binnen een aantal dagen tot tumor formatie [26].

De activiteit van YAP wordt bepaald door meerdere dingen. Zo leidt de zogenaamde 'Hippo kinase cascade' tot phosphorylatie van YAP, wat ervoor zorgt dat het eiwit in het cytosol (celplasma) blijft en niet naar de celkern (met het DNA) getransporteerd kan worden. Verder kunnen andere eiwitten YAP ook phosphoryleren, kan YAP afgebroken worden en kan YAP in het cytosol gehouden worden door binding met bepaalde eiwitten. Angiomotin en 14-3-3 zijn voorbeelden van eiwitten die aan YAP kunnen binden en YAP zo in het cytoplasma vasthouden [25].

De link tussen YAP en α-catenin

Steeds meer onderzoek wijst erop dat α-catenin, YAP en het actine cytoskelet met elkaar in verband staan. Zo wordt α-catenin opengevouwen op het moment dat het actinecytoskelet aan het eiwit trekt en daarmee komt het bindingsdomein vrij voor vinculin, dit is een ander actine-bindend eiwit die de verbinding tussen het actinecytoskelet en de AJ helpt verstevigen [27,28]. Naast α-catenin wordt YAP ook beïnvloedt door kracht. Als je cellen namelijk kweekt op een stijve of grote ondergrond (waardoor de cellen uitrekken) dan verplaatst YAP naar de kern, terwijl deze verplaatsing voorkomen kan worden door remming van eiwitten die het actinecytoskelet en de contractie daarvan stimuleren, zoals het eiwit Rock [29-32]. Verder is het opvallend dat YAP overexpressie tot teveel celdeling leidt in de huid van een muis en hetzelfde resultaat is te zien bij α-catenin verlies, wat suggereert dat een gemeenschappelijke signaleringsroute misschien verantwoordelijk is voor deze uitkomst [22,33]. Zo blijkt dat α-catenin verlies in het brein van een muis ook tot teveel celdeling leidt en gepaard gaat met nucleaire lokalisatie van YAP [34]. Een andere overeenkomst is dat niet alleen α-catenin betrokken is bij 'contact inhibitie van proliferatie', maar YAP ook [22]. De interactie tussen YAP, de transcriptie factor genaamd TEAD en de inductie van de bijbehorende eiwitten, blijkt verantwoordelijk te zijn voor het verlies van 'contact inhibitie van proliferatie' [35].
Alles bij elkaar laten deze resultaten zien dat α-catenin een tumor suppressor is in de huid en het brein van de muis, en dat YAP en de transcriptie factor TEAD waarschijnlijk heel belangrijk zijn voor dit effect. Of α-catenin ook een tumor suppressor is in de borst van de muis en de mens en of dit ook afhankelijk is van signalering via YAP en TEAD moet nog onderzocht worden.

Resultaten van dit proefschrift

Dit proefschrift is een verdiepende studie naar de lobulaire vorm van borstkanker. Omdat het aantal lobulaire borstkanker patiënten klein is ten opzichte van de groep ductale patiënten, wordt er relatief weinig onderzoek naar dit borstkanker subtype gedaan. Ondanks dat de lobulaire tumoren genetisch en moleculair anders lijken te zijn dan de ductale tumoren en als een apart subtype worden beschouwd, worden ze tot op heden nog niet anders behandeld dan ductale tumoren omdat er nog geen lobulaire behandelingsmethoden bestaan. Meer kennis zou dus mogelijk kunnen leiden tot de ontwikkeling hiervan. Bovendien lijken de lobulaire tumoren qua genetische achtergrond en moleculaire status ook een meer uniforme groep te vormen dan de ductale groep, waardoor nieuw ontwikkelde behandelingen ook bij een groter gedeelte van de patiënten goed aan zouden kunnen slaan.

In **Hoofdstuk 2** is dan ook een 'Review' (overzichtsartikel) dat gaat over het lobulaire borstkanker type. Subtypes binnen deze borstkanker vorm, diagnose methodes en afwegingen, de genetische achtergrond en de huidige behandeling worden onder andere besproken.

In **Hoofdstuk 3** analyseren we bepaalde modificaties van het DNA, genaamd methylering. Deze modificatie zorgt meestal voor minder expressie van het eiwit dat gecodeerd wordt door het gen dat deze modificatie bevat. We identificeren verschillende methylatie markers specifiek voor een agressieve vorm van lobulair borstkanker, genaamd pleomorf lobulair borstkanker. Tevens vergelijken we de methylatie patronen in lobulair *versus* ductaal borstkanker en blijkt dat deze patronen maar weinig van elkaar verschillen. De methylatie markers kunnen in de toekomst misschien helpen als aanwijzing voor de identificering van mogelijk actieve signaleringsroutes in pleomorf lobulair borstkanker die niet actief zijn in klassiek lobulair borstkanker. Als deze belangrijke signaleringsroutes bekend zijn dan kunnen patiënten met pleomorf lobulair borstkanker in de toekomst misschien behandeld worden met medicijnen die juist deze routes remmen.

In **Hoofdstuk 4** onderzoeken we de rol van α-catenin in borstkanker progressie. We laten zien dat α-catenin verlies leidt tot onvolwassen AJ formatie, cellen hechten en spreiden niet goed meer aan de ondergrond en bollen daarom op. Het is bekend dat gezonde cellen doodgaan als ze zich niet kunnen hechten aan eiwitten om hen heen en deze vorm van celdood heet anoikis. Wij vinden echter dat in de afwezigheid van α-catenin cellen dit blijken te kunnen overleven; ze zijn anoikis resistent. Ook ontdekken we dat deze overlevingskracht afhankelijk blijkt te zijn van het actinecytoskelet en de contractie daarvan, want remming hiervan leidt tot celdood. Resultaten van een voorgaand onderzoek hebben laten zien dat anoikis resistentie in het lichaam correleert met de capaciteit om uit te zaaien naar andere delen in het lichaam. Het is dus mogelijk dat α-catenin verlies invasie en tumor progressie stimuleert. Of α-catenin ook echt afwezig is in een gedeelte van de E-cadherine positieve ILC tumoren zal nog moeten blijken.

In **Hoofdstuk 5** bepalen we het expressie patroon van YAP in ILC. Het bleek dat YAP heel hoog tot expressie komt in zowel het cytoplasma als de nucleus van cellen in ILC vergeleken met IDC. Ook bleek YAP vooral in het cytoplasma aanwezig te zijn van onze E-cadherine positieve humane en muizen cellijnen, terwijl de E-cadherine negatieve cellijnen relatief meer nucleair YAP hadden. Het is bekend dat artificiële re-expressie van E-cadherine in cellijnen heel moeilijk is omdat de cellen waarschijnlijk zo afhankelijk zijn geworden van het verlies van E-cadherine dat ze doodgaan zodra het weer aanwezig is. Re-expressie experimenten bleken dan ook niet haalbaar. Omdat E-cadherine niet tot expressie komt in

een grote meerderheid van de ILC tumoren, hadden we helaas ook te weinig ILC tumoren die E-cadherine nog wel tot expressie brachten om YAP expressie te kunnen correleren met E-cadherine verlies. Uiteindelijk bleek hoge cytoplasmatische en nucleaire YAP expressie dus significant te correleren met ILC tumoren, maar konden we de eventuele link met het verlies van E-cadherine expressie niet bewijzen.

In **Hoofdstuk 6** vergelijken we de YAP expressie patronen tussen de vroege niet invasieve vorm van lobulaire en ductale tumoren, lobulair carcinoom in situ (LCIS), ductaal carcinoom in situ (DCIS), met de invasieve vormen ILC en IDC. Hiervoor hebben we niet alleen losse patiënten monsters vergeleken, maar ook monsters genomen uit 38 lobulaire en 66 ductale patiënten die zowel de niet invasieve vorm als de invasieve vorm bij zich droegen en we hebben die met elkaar vergeleken. Het bleek dat hoge nucleaire YAP expressie significant vaker voorkwam in LCIS (92.6%) vergeleken met DCIS (15.6%) ($p<0.001$). Terwijl het YAP expressie patroon tussen LCIS en ILC, en tussen DCIS en IDC niet verschillend was. Hoge YAP expressie is dus kenmerkend voor alle stadia van de lobulaire borsttumoren en speelt daarom mogelijk een rol bij het ontstaan en bij de progressie van dit tumortype.

In **Hoofdstuk 7** geven we een samenvatting van de algemene conclusies en bediscussiëren we hoe het verlies van AJs kan leiden tot activatie van Rock signalering en of dit tot de nucleaire lokalisatie van YAP kan leiden. We sluiten af met een evaluatie van de bestaande Rock remmende medicijnen. En we bespreken het mogelijke effect van deze remmers bij een eventuele toekomstige behandeling van lobulaire borstkanker patiënten.

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List of abbreviations

AJ	adherens junction
ALH	atypical lobular hyperplasia
Ankrd1	ankyrin repeat domain 1
BRCA1/2	breast cancer gene ½
CAP102	cadherin associated protein 102 kDa (α-catenin)
CDH1	E-cadherin
CIP	contact inhibition of proliferation
CK	cytokeratin
CpG	cytosine phosphate guanosine
CTNNA1	α-catenin
Cyr61	cysteine-rich 61
DCIS	ductal carcinoma *in situ*
DNMTs	DNA cytosine methyltransferases
Dox	doxycycline
DRF	diaphanous-related formin
EC repeat	extracellular cadherin repeat
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
EMT	epithelial-to-mesenchymal transition
ER	estrogen receptor
FA	focal adhesion
F-actin	filamentous actin
FAJ	focal adherens junctions
FAK	focal adhesion kinase
FFPE	formalin-fixed paraffin embedded

GAP	GTPase-activating protein
GDI	guanine nucleotide exchange inhibitor
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GPCR	G-protein coupled receptor
H&E	haematoxylin and eosin
HDGC	hereditary diffuse gastric cancers
HER2	human epidermal growth factor receptor 2 (ERBB2, HER2/Neu)
IDC	invasive ductal carcinoma, or not otherwise specified (NOS)
iKD	inducible knock down
ILC	invasive lobular carcinoma
JAM	junctional adhesion molecule
LCIS	lobular carcinoma *in situ*
LIMK	LIM kinase
LN	lobular neoplasia
LOH	loss of heterozygosity
MAI	mitotic activity index
MAPK	mitogen-activated kinase
mDia	mammalian homologue of Drosophila diaphanous
MLC	myosin light chain
MLH1	MutL homolog 1
MMTV	mouse mammary tumor virus
Mrip	myosin phosphatase Rho–interacting protein
MS-MLPA	methylation-specific multiplex ligation dependent probe amplification
Necls	nectin-like molecules
NF2	neurofibromatosis 2 (NF2) (gives rise to Merlin)

p120	p120-catenin
PAK	p21-activated kinase
PALS	proteins associated with Lin seven
Par3	partition defective 3
PATJ	protein-associated with tight junction
PIP2	phosphatidylinositol-4,5-bisphosphate
PR	progesterone receptor
RASSF1A	RAS association domain family member 1 (RASSF1A)
Rock	rho-associated kinase
TAZ	transcriptional coactifator with PDZ-binding motif
TCGA	the cancer genome atlas
TEAD	TEA Domain
TJ	tight junction
TMA	tissue micro array
TP73	tumor protein p73
VH domain	vinculin homology domain
WAP	whey acidic protein
YAP	yes-associated protein
ZAJ	zonula adherens junctions

Curriculum Vitae

Eva J. Vlug was born November 3rd, 1983 in Amsterdam, the Netherlands. During high school, she followed a professional and intensive show musical dance training program at *Lucia Marthas, institute for performing arts*. She completed her secondary education (VWO, Natuur & Gezondheid) in 2002, at the *Montessori Lyceum Amsterdam*. Within the same year, she started the bachelor *Biomedical* Sciences at *Utrecht University*, and continued with the master program *Biology of Disease* in 2005. Her master included an internship at the *British Columbia Cancer Research Centre* in Vancouver, Canada under the supervision of dr. Aly Karsan, where she investigated the role of Slug in Notch-induced breast cancer. Under supervision of dr. Gerrit Jan Schuurhuis and prof. dr. ir. Theo H. Smit she wrote her master thesis on the role of the extracellular matrix on tumorigenesis at the *VU University Amsterdam*. After an extracurricular internship at the *Netherlands Cancer Institute* supervised by dr. Kees Jalink, she completed her master in 2008 and started her PhD training in 2009 with the group of dr. Patrick W.B. Derksen as part of the Pathology department lead by prof. dr. Paul van Diest at *Utrecht University*. Here she studied a breast cancer subtype called lobular breast cancer, with a particular interest for the role of cell-cell adhesion in tumor initiation and progression. The results are described in this thesis entitled 'Loss of cadherin-based cell adhesion and the progression of Invasive Lobular Breast Cancer'.

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