Cell–Cell Contacts in Melanoma and the Tumor Microenvironment

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Abbreviations

CDH   Cadherin
Cx   Connexin(s)
Dsc 1–3 Desmocollin
Dsg   Desmoglein
GJ   Gap junction
GJIC   Gap junctional intercellular communication

9.1   Melanoma Microenvironment

This is an update of our previous reviews on this topic (Haass et al. 2004, 2005; Kuphal and Haass 2011; Brandner and Haass 2013).

The state of a cell – quiescence, proliferation, differentiation or cell death – is under normal conditions determined by homeostasis (Bissell and Radisky 2001). A symbiotic relationship between a melanocyte and approximately 36 associated

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keratinocytes, which forms the epidermal melanin unit, maintains this homeostatic balance of the human epidermis (Fitzpatrick and Breathnach 1963; Jimbow et al. 1976). Within the *stratum basale*, the melanocytes keep a lifelong stable ratio of 1:5 with the keratinocytes (Fitzpatrick et al. 1979). This balance is maintained through regulated induction of melanocyte division coordinated through intercellular communication, which can be endocrine and paracrine via soluble factors and/or by direct contact via cell–cell and cell–matrix adhesion, or gap junctional intercellular communication (GJIC) (Haass et al. 2004, 2005). Dysregulation of this homeostasis may cause an imbalance of the epidermal melanin unit and trigger uncontrolled proliferation of the melanocytes, which may lead to the development of a nevus and/ or a melanoma (Haass and Herlyn 2005).

Alterations in the interaction between neoplastic cells and their immediate microenvironment play a key role in these processes (Hanahan and Weinberg 2000, 2011; Park et al. 2000). The tumor microenvironment includes (1) the tumor stroma composed of fibroblasts, endothelial cells, immune cells, soluble molecules, and the extracellular matrix (ECM); (2) the tissue where the tumor had originated from; and (3) different sub-compartments within the tumor itself. Signals to and from the stroma via cell–cell and cell–matrix contact and/or via secretion of cytokines and growth factors may lead to a remodeling of the tumor microenvironment and consequently to promotion of melanoma development, growth, and metastasis by inducing angiogenesis, invasion, and migration (Villanueva and Herlyn 2008; Zigler et al. 2011). In addition to the interaction with the tumor stroma, primary melanoma progression as well as cutaneous melanoma metastases impact on the epidermal tumor microenvironment: the multilayered epithelium of the skin (Haass et al. 2010). Finally, different microenvironmental conditions within the tumor itself are created by differential access to nutrients and oxygen (Groebe and Mueller-Klieser 1991; Minchinton and Tannock 2006; Santiago-Walker et al. 2009; Haass et al. 2014; Haass 2015).

The microenvironment is not only important for the primary tumor, but also for colonization of a secondary organ. The "seed and soil" hypothesis implies that the metastatic process depends on the tumorigenic capacity of the cells and – again – on their interactions with the microenvironment (Fidler 2003).

9.2   Adherent Junction of Cadherins

Cross-talk between benign precursor cells, malignant cells, and surrounding host cells influences tumor development. Already in 1914, Theodor Boveri recognized the importance of changes in tumor cell adhesion for the development of cancer (Boveri 1914). Among the molecules involved in this intercellular communication are cadherins, which play a critical role for the homeostasis of normal skin and also during tumor formation and progression (Fig. 9.1). The identification of cadherins in the late 1970s and early 1980s was primarily motivated by an interest in understanding the mechanisms of cell adhesion during development (Franke 2009).
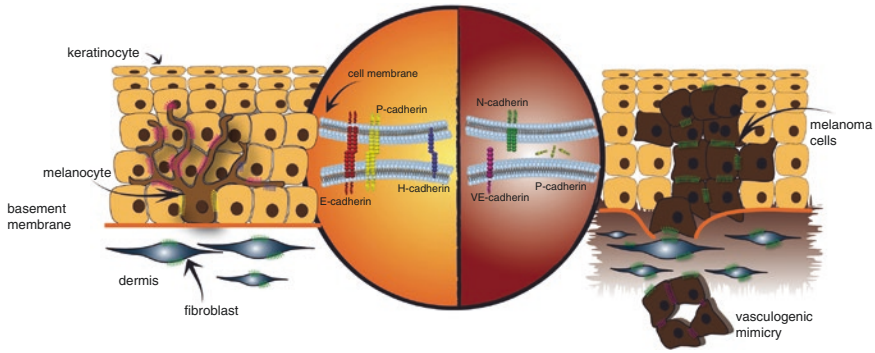
Fig 9.1 Overview of the cadherin repertoire in skin and melanoma (Illustration R.J. Bauer)

Cell–cell as well as cell–matrix adhesions are critical for cells and tissues to respond to mechanical stimuli from their environment. Both cell–cell and cell–matrix adhesions bear intrinsic mechanosensitivity, which allows them to promptly respond to stress and effectively propagate signals controlling cell shape and motility. This mechanosensitive response has been associated with pronounced changes in the size and molecular composition of specific adhesion sites and, consequently, the signals evoked by those adhesion sites. In polarized epithelia of vertebrates, the adherent junction is part of the tripartite junctional complex localized at the juxtaluminal region, which compromises the tight junction (TJ, see below), adherent junction (AJ), and desmosomes (macula adherens).

More than 80 proteins belong to the cadherin superfamily and are separated into the following "adherent junction" (AJ) subgroups in vertebrates:

1. Classical adhesive cadherins of type 1 (6 members) and type 2 (13 members), e.g., E-, N-, P-, R-, and VE-cadherin. The classical cadherin family comprises 19 members that share a common domain organization of five repetitive extracellular calcium-binding subdomains (Overduin et al. 1995). Most of these classical cell–cell adhesion molecules are connected to the actin filaments and microtubules of the cellular cytoskeleton via catenins. The four known catenins, alpha-, beta-, gamma (plakoglobin)-, and delta (p120)-catenin, are important regulatory elements either for sustained cell–cell adhesion or signaling cascades into the cell.
2. The "nonclassical" desmosomal cadherins, transmembrane proteins of desmosomes are, for example, desmocollin 1–3 (Dsc 1–3) and desmoglein 1–4 (Dsg 1–4). They are connected to intermediate filaments.
3. Finally, there are nonclassical cadherins, like the protocadherins (e.g., protocadherin 15, cadherin 23), H-cadherin, and cadherin-like molecules (e.g., Fat, Dachsous, Flamingo, or Ret) belonging to the cadherin superfamily.

The most important classical cell–cell adhesion molecules of the skin and during melanoma development are E (epithelial)-cadherin (CDH-1), N

(neuronal)-cadherin (CDH-2), and P (placental)-cadherin (CDH-3), which belong to the group of calcium-dependent glycoproteins. Certainly, this group of classical adhesion molecules can be extended with atypical VE (vascular endothelial)-cadherin (CDH-5, CD144) and the nonclassical cadherin H (heart)-cadherin (T-cadherin, CDH-13) (Fig. 9.1). In normal epidermis, melanocytes and keratinocytes are mostly connected via E-cadherin, P-cadherin, and H-cadherin (Kuphal et al. 2009; Nishimura et al. 1999; Tang et al. 1994). Whereas melanocytes in the basal layer of the epidermis seem to contain predominantly E-cadherin and H-cadherin, those residing in hair follicles are rich in P-cadherin (Nishimura et al. 1999). In contrast, N-cadherin is expressed on fibroblasts and vascular endothelial cells of normal skin (Hsu et al. 1996).

9.2.1 Loss of E-Cadherin in Tumorigenesis

E-cadherin is the major cadherin in polarized epithelial cells. Furthermore, the crosstalk between melanocytes and keratinocytes mediated by E-cadherin plays an important role in human epidermis. The normal melanocytic phenotype and controlled proliferation of melanocytes are strictly regulated by keratinocytes via E-cadherin. The E-cadherin knockout mouse is lethal in early embryonic stages (Larue et al. 1994) supporting the finding that E-cadherin has an essential role in morpho- and organogenesis. In skin development, there is evidence that E- and P-cadherin play some role in guiding melanocyte precursor cells to their final destination in the epidermis (Nishimura et al. 1999).

Malignant transformation of melanocytes is frequently attended by loss of E-cadherin expression and induction of N-cadherin (Hsu et al. 1996). This leads to the loss of the regulatory dominance of keratinocytes over melanocytes. The degenerated melanocytes/melanoma cells express N-cadherin to get into contact to fibroblasts and vascular endothelial cells during migration and invasion into the tumor stroma, dermis, lymph, and blood vessels (Hsu et al. 2000) (Fig. 9.2). The switch of the cadherin class is an interesting phenomenon of melanoma cells and in epithelial–mesenchymal transition (EMT) in general.

However, immunohistochemical examination of primary melanomas and their metastases has revealed that a proportion of melanoma cells are still E-cadherin-positive and present little, if any, N-cadherin (Danen et al. 1996; Hsu et al. 1996; Sanders et al. 1999; Silye et al. 1998). Therefore, the cadherin switch as an obligatory prerequisite of malignant behavior is still controversial and might depend on the subtype of the melanoma examined. However, immunohistochemistry data could not show whether the expressed E-cadherin is really functionally active regarding adhesion or still possesses signaling function. The general consensus is that E-cadherin is a tumor invasion suppressor.

9.2.1.1 Regulators of E-Cadherin

The mechanism by which E-cadherin expression is lost during malignancy differs between tumor entities. Loss of E-cadherin function can be caused by various genetic or epigenetic mechanisms. In patients with diffuse gastric cancer and breast
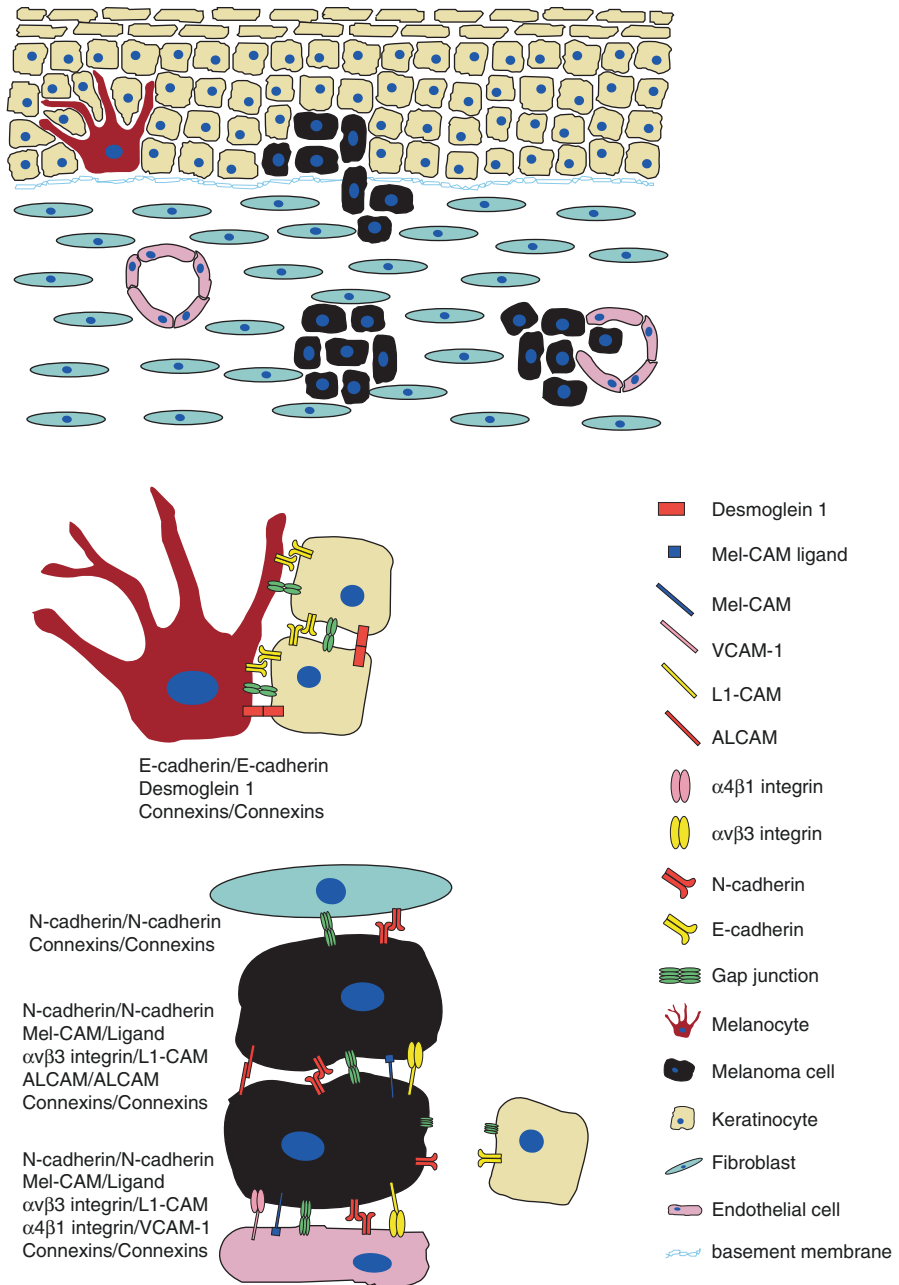
Fig. 9.2 (**a**) Cell–cell adhesion of melanocytes and melanoma cells. Melanocytes adhere to kera-tinocytes via E-cadherin and desmoglein, which enables them to communicate with each other through gap junctions with cells in their environment. (**b**) In melanoma cells, E-cadherin is down-regulated. They interact with each other through N-cadherin, Mel-CAM/Mel-CAM ligand, αvβ3 integrin/L1-CAM, ALCAM, and connexins; with fibroblasts through N-cadherin and connexins; and with endothelial cells through N-cadherin, Mel-CAM/Mel-CAM ligand, αvβ3 integrin/ L1-CAM, α4β1 integrin/VCAM-1, and connexins

cancer, the E-cadherin gene is mutated, leading to the expression of a nonfunctional protein (Strathdee 2002). The consequence is abnormal expression and abnormal subcellular localization of cadherin or the components of the cadherin-containing adhesion complex. Further, the *CDH-1* gene locus can be epigenetically silenced by hypermethylation, leading to downregulation of E-cadherin expression which is known from several cancer entities, e.g., hepatocellular carcinoma (Kanai et al. 1997), squamous cell carcinoma (Saito et al. 1998), and thyroid cancer (Graff et al. 1998).

In most cases, E-cadherin expression is downregulated at the transcriptional level. The zinc-finger containing transcriptional repressor Snail1, which is a master regulator of neural crest cell specification and melanocyte migration during development in vertebrates, is mainly responsible for the loss of E-cadherin in melanoma (Batlle et al. 2000; Poser et al. 2001). The level of Snail1 expression correlates directly with the loss of E-cadherin expression, and forces overexpression of Snail in primary melanocytes downregulates E-cadherin expression (Poser et al. 2001). Slug (Hajra et al. 2002; Bolós et al. 2003), Snail2, ZEB1 and ZEB2 (Eger et al. 2005; Caramel et al. 2013), and SIP1 (Comijn et al. 2001), also members of the zinc finger transcription factor family of Snail, are further regulators of E-cadherin in melanoma, as well as basic helix–loop–helix transcription factors E12/47 (Perez-Moreno et al. 2001) and Twist (Yang et al. 2004). Additionally, the T-box transcription factor Tbx3 is overexpressed in melanoma, which enhances melanoma invasiveness through prevention of E-cadherin expression (Rodriguez et al. 2008). Furthermore, within human melanoma lesions, GLI-2, a mediator of hedgehog signaling, is associated with loss of E-cadherin (Alexaki et al. 2010).

Proteolytic degradation of E-cadherin by matrix metalloproteinases (MMPs) is another mechanism by which E-cadherin-mediated cell–cell adhesion can be ablated. In this case, cell surface E-cadherin becomes soluble by cleavage of the extracellular domain, a process known as ectodomain shedding. For melanoma, Adam-10 is responsible for E-cadherin shedding (Billion et al. 2006) (see also Chap. 8).

A family of microRNAs, such as miR-200a, miR-200b, miR-200c, and miR-205 was reported to control the expression level of E-cadherin during the epithelial–mesenchymal transition. The microRNA targets the transcriptional repressors ZEB1 and ZEB2 of E-cadherin (Gregory et al. 2008; Hurteau et al. 2007). As one example for cancer, loss of miR-200c expression is significantly correlated with early stage T1 bladder tumor progression (Wiklund et al. 2011). Another miRNA, miR-373, induces expression of genes with complementary promoter sequences. It was found that miR-373 induces E-cadherin expression by recognizing a target site in the promoter of the *cdh-1* gene (Place et al. 2008). Liu et al. 2012 showed that miR-9 is downregulated in metastatic melanomas compared with primary melanomas. A tumor suppressor effect after re-expression of miR-9 in melanoma is mediated through its direct binding to sites within the NF-kB 3′-UTR, resulting in suppression of Snail1 and upregulation of E-cadherin. However, whether microRNAs are responsible for regulating cadherins directly and specifically in melanoma is still not known (see also Chap. 6).

9.2.2   Loss of P-Cadherin During Tumorigenesis

In human skin, P-cadherin is expressed mainly on cells of the epidermal basal layer (Furukawa et al. 1997) and those melanocytes residing in hair follicles (Nishimura et al. 1999). Concerning carcinogenesis, the effective role of P-cadherin remains an object of debate, since it can behave differently depending on the molecular context and tumor cell model studied. In melanoma cells, loss of full-length P-cadherin was reported (Bachmann et al. 2005; Van Marck et al. 2005; Jacobs et al. 2011). Therefore, P-cadherin has a similar tumor-suppressive behavior to E-cadherin. Additionally, a truncated 50 kDa form of the N-terminal part of P-cadherin was found, which appeared to be secreted from the melanoma cells. If this secreted form of P-cadherin is expressed from melanoma cells, it is responsible for cell migration and invasion (Bauer et al. 2005, 2006; Bauer and Bosserhoff 2006).

9.2.3   Loss of T-Cadherin During Tumorigenesis

T-cadherin (truncated-cadherin, cadherin 13, gene name *CDH13*) or H-cadherin, named for its strong expression in the heart, is an atypical member of the cadherin family, lacking the classical HisAlaVal recognition motif at its N-terminus, lacking the typical transmembrane and cytosolic domains and possessing a glycosylphosphatidylinositol moiety that anchors T-cadherin into the outer plasma membrane.

Immunohistochemistry of melanoma tissue samples showed positive T-cadherin staining of the endothelial cells. T-cadherin expression in endothelial cells was demonstrated to be redox sensitive (Joshi et al. 2008). The melanoma cells themselves showed loss of T-cadherin whereas healthy skin showed staining of melanocytes and keratinocytes of the basal layer of the epidermis. Loss of T-cadherin in melanoma is associated with migration and invasion of the cells (Kuphal et al. 2009). In general, the exact functional role and signaling of T-cadherin for melanoma cells itself and for the intratumoral angiogenesis are not clarified, so far. It was only shown that loss of T-cadherin in melanoma regulates AKT signaling and desensitizes for apoptosis (Bosserhoff et al. 2014). Also, a connection of loss of T-cadherin to tumor progression was speculated (Rubina et al. 2013) but not evidenced, until today.

9.2.4   N-Cadherin Expression During Tumorigenesis

N-cadherin plays a pivotal role in cell adhesion between melanoma cells and both dermal fibroblasts and vascular endothelial cells. During the cadherin class switch, loss of E-cadherin expression is accompanied by induced N-cadherin expression, which confers new adhesive properties on the cells (Fig. 9.2). The shift in cadherin profile during melanoma progression has been found not only in vitro but also in vivo (Hsu et al. 1996; Sanders et al. 1999). Experimentally, melanoma cell migration across fibroblasts is impaired upon addition of an N-cadherin neutralizing

antibody (Li et al. 2001). The functional relevance of N-cadherin is to conduct migration and invasion of melanoma cells whereas N-cadherin expression correlates with progression to advanced-stage melanoma. The cell adhesion molecule N-cadherin has been suggested to represent a melanoma progression marker (Watson-Hurst and Becker 2006).

The switch of the cadherin class from E-cadherin to N-cadherin is directly connected. The transcriptional repressor Snail not only regulates E-cadherin repression but also represses the expression of the deubiquitinating enzyme CYLD. Loss of CYLD expression in melanoma in turn led to ubiquitination of Bcl-3 which is a transcriptional regulator of N-cadherin expression (Massoumi et al. 2009).

9.2.5 VE-Cadherin Expression During Tumorigenesis

The term *vasculogenic mimicry* describes the formation of vascular-like tubular structures and patterned networks through the connection of melanoma cells. The vascular structures are essential for the supply of the tumor. Several key molecules are responsible for the formation and maintenance of the tubular networks and these molecules are also often essential in normal blood vessels. One molecule expressed during vasculogenic mimicry of melanoma cells is VE-cadherin, previously considered to be endothelial cell specific. Analyzing VE-cadherin in detail demonstrated an interaction with EphrinA2 (EphA2), a tyrosine kinase. VE-cadherin engages the membrane-bound ligand of EphA2 and becomes phosphorylated on its tyrosines at the cytoplasmic domain. The mutual impact of VE-cadherin and EphA2 results in loosening of cell–cell adhesion and allowing for an increase in cell migration, invasion, and vasculogenic mimicry. Further studies describe the role of VE-cadherin for melanoma transendothelial migration. Here, p38 MAP kinase is necessary for increased VE-cadherin-mediated junction disassembly important for the migration processes of melanoma cells (Hendrix et al. 2001, 2003; Khanna et al. 2010).

9.2.6 FAT Expression During Tumorigenesis

FAT1, FAT2, FAT3, and FAT4 are human homologs of *Drosophila* Fat, which is involved in tumor suppression and planar cell polarity (PCP). FAT molecules belong to the cadherin-like protein family. FAT1 and FAT4 undergo the first proteolytic cleavage by Furin and are predicted to undergo the second cleavage by γ-secretase to release intracellular domain (ICD). Recently, it was shown using Northern blotting that human melanoma cell lines variably but universally express FAT1 and less commonly FAT2, FAT3, and FAT4. Both normal melanocytes and keratinocytes also express comparable FAT1 mRNA relative to melanoma cells. However, in melanoma cells, the non-cleaved proform of FAT1 is also expressed at the cell surface together with the furin-cleaved heterodimer. Moreover, furin-independent processing generates a potentially functional proteolytic product in melanoma cells, a persistent 65-kDa membrane-bound cytoplasmic fragment no longer in association

with the extracellular fragment. In vitro localization studies of FAT1 showed that melanoma cells display high levels of cytosolic FAT1 protein. Such differences in protein distribution appear to reconcile with the different protein products generated by dual FAT1 processing. It was suggested that the uncleaved FAT1 could promote altered signaling, and the novel products of alternate processing provide a dominant negative function in melanoma (Sadeqzadeh et al. 2011). Among the human *FAT* gene family, *FAT4* gene is recurrently mutated in several types of human cancers, such as melanoma (40 %), pancreatic cancer (8 %), HNSCC (6 %), and gastric cancer (5 %) (Nikolaev et al. 2011).
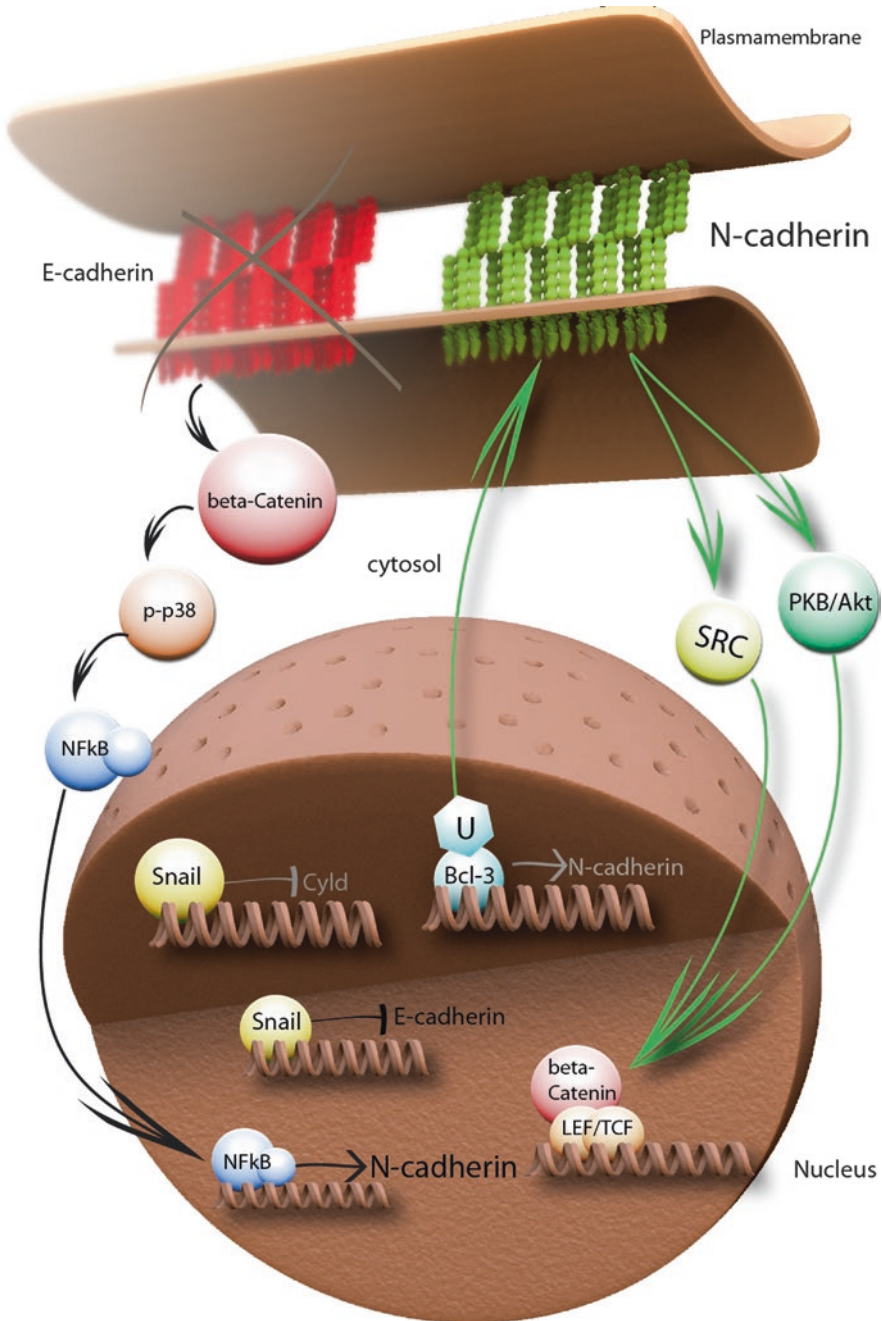
9.2.7   Signaling of Cadherins

In contrast to integrins, evidence for cadherin-induced outside–in signaling came into focus only slowly. Over the last 10 years, a number of studies have appeared to agree that signaling cascades emanating from cadherins play an important role in confluency-dependent growth arrest, migration, invasion, and differentiation. Changes in expression or function of cell adhesion molecules can therefore contribute to tumor progression both by altering the adhesion status and by affecting cell signaling. To date, no enzymatic activity has been attributed to the cytoplasmic tails of adhesion molecules like E-cadherin or N-cadherin. The signaling capability emanates from intracellularly bound kinases and phosphatases that link to the cytoplasmic tail of adhesion receptors (Fig. 9.3).

9.2.7.1 Signaling Cascades of E-Cadherin
Four modes of E-cadherin signaling are known:

1. Modulation of receptor tyrosinase signaling (RTK) (see also Chap. 7)
2. Inhibition of the Wnt signaling pathway (see also Chap. 7)
3. Regulation of cytoplasmic β-catenin signaling
4. Regulation of signaling through Rho GTPases

One way by which E-cadherin transmits growth-inhibiting outside–in signals appears to follow a strikingly similar scheme to that of the integrins. By using an immortalized nontumorigenic keratinocyte cell line, HaCaT, as a model system, Pece and Gutkind (2000) provide evidence that the assembly of calcium-dependent adherens junctions leads to a rapid and remarkable increase in the state of activation of MAPK and that this event is mediated by E-cadherin. Furthermore, it was found in these studies about HaCaTs that E-cadherin stimulates the MAPK pathway through ligand-independent activation of receptor tyrosine kinases, in particular EGF-receptors (Pece and Gutkind 2000). They speculated that upon adherens junction formation, signals emanating as a result of the E-cadherin-EGFR interaction might be involved in maintaining the functional and structural integrity of quiescent epithelia and, as a function of the adhesion status of the cells, possibly in promoting epithelial cell differentiation rather than proliferation. In contrast, another group detected signaling

Fig. 9.3 Schematic depiction of cadherin signaling in melanoma. The transcriptional repressor Snail inactivates E-cadherin expression in melanoma. With the loss of E-cadherin cytosolic beta-catenin activates the MAP kinase p38, which stimulates the transcriptional activity of NFkappaB. NFkappaB has N-cadherin as target gene. Additionally, Snail represses the expression of the tumor suppressor Cyld, which in turn leads to ubiquitination of Bcl-3 which also has N-cadherin as target gene. The overexpression of N-cadherin activates signaling cascades of SRC and PKB/Akt which leads to tumor progression (Illustration R.J. Bauer)

cascade inhibition through EGF-receptor/E-cadherin complex formation in melanoma and breast cancer cells (Qian et al. 2004). Unfortunately, most of the literature on E-cadherin signaling does not cover melanoma. Studies on keratinocytes and other cancer cell types revealed that the E-cadherin complex associates and cooperates with an EGF-receptor family member to activate the PI3K/Akt pathway in a Src-family kinase-dependent manner (Muller et al. 2008; Perrais et al. 2007) (see also Chap. 7).

Some studies showed that homophilic ligation of E-cadherin signals directly through Rho GTPase activity (Braga 2000; Braga et al. 1997). Loss of E-cadherin in melanoma may involve changes in the organization of the cytoskeleton which is exerted by members of the Rho family. They control not only the cytoskeletal organization but also cell motility, migration, and tumor progression to malignancy at the same time. As example, E-cadherin suppresses RhoA activity in melanoma by activating p190RhoGAP (Molina-Ortiz et al. 2009). E-cadherin overexpression led to association of p190RhoGAP and p120[ctn] on the plasma membrane where E-cadherin bounds p120[ctn]. Recently, it was shown that E-cadherin also regulates RhoC GTPase. Here, loss of E-cadherin activates the expression of the RhoC in melanoma through upregulation of the transcription factor ETS-1, which results in increased c-Jun protein stabilization and activation (Spangler et al. 2012).

In addition to its role in adhesion, nuclear β-catenin is involved in Wnt signal transduction, and it interacts with transcription factors of the leukocyte enhancer factor (LEF)/T-cell factor (TCF) family to regulate transcription of target genes implicated in cell growth control such as cyclin D1 and c-myc (van Noort and Clevers 2002). By sequestering β-catenin at the cell surface, E-cadherin has been shown to antagonize nuclear β-catenin signaling pathways and to induce growth inhibition (Gottardi et al. 2001; Shtutman et al. 1999). Furthermore, β-catenin bound to E-cadherin inhibits phosphorylation of p38 and prevents activation of NFkappaB. Unbound cytoplasmic β-catenin activates the signaling pathway ending at transcriptional activation of N-cadherin expression in melanoma cells (Kuphal et al. 2004). In general, it was shown by Onder et al. (2008) that loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. The publication presents ~84 of 617 genes differentially expressed in shE-cadherin human breast epithelial cells (HMLE). They presented, e.g., *twist* and *TCF-8* among other 19 transcription factors as upregulated after loss of E-cadherin.

9.2.7.2 Signaling Cascades of N-Cadherin

N-cadherin-mediated intercellular interactions promote survival and migration of melanoma cells through activation of cytoplasmic signaling cascades. The Src family kinases are involved in the regulation of N-cadherin-mediated cell adhesion and signaling during, e.g., melanoma cell transendothelial migration. Src is localized at the heterotypic contacts of N-cadherin and becomes activated when melanoma cells are transmigrating across the endothelium. Activated Src has the Tyrosine-860 at the cytoplasmic domain of N-cadherin as target site for phosphorylation. The phosphorylation leads to disruption of β-catenin binding followed by nuclear translocation of this molecule to activate gene transcription of genes responsible for proliferation (Qi et al. 2006). N-cadherin mediates cell adhesion-activated antiapoptotic protein Akt/PKB and subsequently increases β-catenin and inactivates pro-apoptotic factor Bad (Li et al. 2001).

9.2.8 Desmosomes/Hemidesmosomes

Desmosomes, composed of desmogleins and desmocollins, are localized spot-like adhesions randomly arranged on the lateral sides of plasma membranes and are also members of the cadherin family. The extracellular domain of the desmosome is called the extracellular core domain (ECD) or the Desmoglea, and is bisected by an electron-dense midline where the desmoglein and desmocollin proteins bind to each other. On the cytoplasmic side of the plasma membrane, there are two dense structures called the outer dense plaque (ODP) and the inner dense plaque (IDP). In the ODP, the cytoplasmic domains of the cadherins desmoglein and desmocollin attach to desmoplakin via plakoglobin and plakophilin, while in the IDP, desmoplakin attaches to the intermediate filaments such as keratine filaments.

A number of melanoma cell lines synthesize, in the absence of desmosomes, the desmosomal cadherin desmoglein 2 (Dsg2) as a frequent plasma membrane glycoprotein that is not assembled into any junction but is dispersed over large parts of the cell surface. Indeed, in tissue microarrays, Dsg2 has been demonstrated in a sizable subset of nevi and primary melanomas (Rickelt et al. 2008). In contrast, Dsg1, Dsg3, and desmocollins 1–3, were absent in the analyzed melanoma cell lines but plakoglobin and plakophilin3 were also expressed in several melanoma cell lines (Schmitt et al. 2007). Future studies will have to clarify the diagnostic and prognostic significance of these different adhesion protein subtypes.

9.3 Integrins

Integrins are transmembrane adhesion receptors localized at cell–matrix contact sites where they link ECM (extracellular matrix) components, e.g., vitronectin, fibronectin, laminin, osteopontin, or collagen, to the actin cytoskeleton and interact with multiple structural and signaling molecules including talin, kindlin, paxillin, vinculin, α-actinin, FAK (focal adhesion kinase), ILK (integrin-linked kinase), Rho GTPases, and SHC (Berrier and Yamada 2007; Papusheva and Heisenberg 2010). The latter are important mediators downstream of integrins by which they interact either directly or indirectly to effect adhesion-dependent responses (Playford and Schaller 2004). The metastatic transformation of melanocytes is associated with altered expression of integrins, which transduce signals upon ligation to ECM proteins that regulate tumor growth and metastasis, apoptosis, differentiation as well as tumor angiogenesis. Integrin receptors are functional dimers of α- and β-integrin subunits, which each have a large ectodomain, a single transmembrane domain, and a generally short cytoplasmic tail (except for β4 integrin). The combination of different α- and β-subunits determines the substrate specificity of the dimer (Danen and Sonnenberg 2003). There are at least 18 known α-chains and 8 β-chains, allowing for at least 24 unique heterodimers.

The pattern of integrins on the cell surface is usually very specific, which makes the cell fit perfectly into its surrounding environment. Importantly, integrin expression patterns differ considerably in vitro versus in vivo. Thus, in vitro studies may not translate into the in vivo situation.

Several publications have shown that the expression levels mainly of αvβ3, α2β1, α3β1, α4β1, and α5β1 appear to increase from primary melanomas to metastatic melanoma tissue sections, whereas there was a significant decrease in α1β1, α2β1, and α6β1 expression levels in metastatic melanoma compared to primary melanoma (Friedl et al. 1998; Natali et al. 1993; Schadendorf et al. 1993). Although many integrins have been implicated in mediating melanoma growth and metastasis, perhaps none have been studied as much as the vitronectin receptor, αvβ3 (Danen et al. 1995; Mortarini and Anichini 1993; Seftor et al. 1999). αvβ3 integrin adheres to vitronectin, fibronectin, laminin, collagen, and osteopontin. Binding fibronectin and vitronectin induces the expression of MMP-2, which is able to degrade the collagen of the basement membrane (Felding-Habermann et al. 2002). Furthermore, osteopontin's RGD-sequence (Arg–Gly–Asp) has high binding affinity and specificity to αvβ3. As the aggressiveness of melanoma has been associated with high osteopontin expression (Sieg et al. 2000), this interaction of αvβ3 and osteopontin is important for melanoma progression. Interaction between αvβ3 and extracellular matrix molecules serves to promote cell attachment, spreading, and migration. αvβ3 integrin also undergoes heterophilic binding with two members of the immunoglobulin superfamily of cell adhesion molecules, PECAM-1 and L1. The αv subunit is widely expressed on melanomas regardless of disease stage. This stands in contrast to the β3 subunit, which is predominantly expressed on melanoma cells in the vertical growth phase. The onset of β3 integrin expression is one of the most specific markers of the transition from radial growth phase to vertical growth phase of melanoma (Albelda et al. 1990; Danen et al. 1995; Natali et al. 1997). Although many studies on human melanoma cell lines have correlated αvβ3 integrin expression with progression and metastasis, in vivo studies are less clear.

9.3.1   Integrin Signaling in Melanoma

Apart from being involved in the attachment of cells to the ECM, integrins are also responsible for signaling between the cells and the environment. Signaling works bidirectionally: "outside–in signaling" can control behavior, proliferation, cell polarity, cell growth, and migration. "Inside–out signaling," on the other hand, changes the integrins from a passive, weak binding state into an active, adhesive state and alters the interaction of the receptors with the extracellular environment. Integrins are receptors for cell movement in response to binding to ECM of the basement membrane or connective tissue or plasma membrane receptors expressed on endothelial cell surfaces. Additionally, integrins bind cytoplasmic adaptor proteins of the actin-myosin filaments and create a plasticity that allows the cell to move. In summary, integrins are bivalent linker proteins, binding simultaneously to extracellular ligands as well as cytoplasmic proteins including intracellular signaling molecules. They influence, for example, tyrosine kinases, serine/threonine kinases, phosphoinositides, and signaling cascades which determine the fate of a cell, letting it grow, proliferate, or die whenever it is necessary in the context of the whole organism. This paragraph introduces some of the most important and best studied proteins which are known to interact with integrins in melanoma.
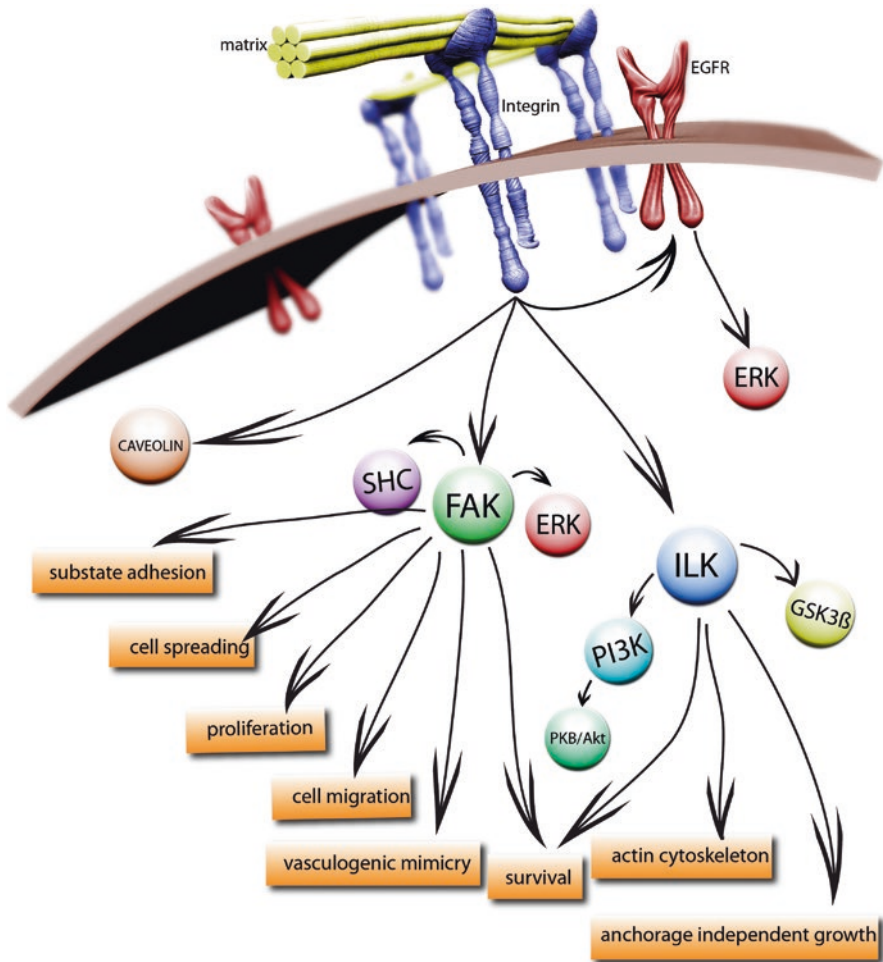
Fig. 9.4 Schematic depiction of the signaling pathways leading from integrins to focal adhesion kinase (*FAK*) and integrin-linked kinase (*ILK*), respectively, and further reactions of the cell (Illustration R.J. Bauer)

There is the non-receptor protein tyrosinase kinase FAK (focal adhesion kinase) (Fig. 9.4) that co-localizes with integrins in focal adhesions. FAK becomes phosphorylated and then controls processes like cell spreading, proliferation, motility, vasculogenic mimicry, and survival (Schaller 2001). Proteins like c-SRC, SHC, CSK, PI3K, and GRB2 are known to interact with FAK to transfer the signaling into the cytoplasm and to link FAK signaling also to MAP kinases (Chakraborty et al. 2002) (see also Chap. 7). FAK expression seems to be required in melanoma cells for substrate adhesion. It has been shown that in melanoma FAK is constitutively active and that it is essential for maintaining adhesiveness in melanoma cells (Hamamura et al. 2008; Kahana et al. 2002).

Furthermore, the integrin-linked kinase (ILK), a serine/threonine kinase, is implicated in connecting cell–extracellular matrix interaction and growth factor signaling to cell survival, cell migration, invasion, anchorage-independent growth, angiogenesis, and epithelial–mesenchymal transition. It has been shown that strong ILK expression was significantly associated with melanoma thickness, migration, and invasion (Wong et al. 2007). Increased expression of integrin-linked kinase is correlated with melanoma progression and poor patient survival (Dai et al. 2003). ILK directly phosphorylates PKB/Akt and glycogen synthase kinase-3 (GSK-3beta), which is inactivated upon phosphorylation (Delcommenne et al. 1998; Troussard et al. 1999). SHC is another protein which is implicated in integrin signaling. It is an adaptor protein capable of binding phosphotyrosine-containing sequences. So far, studies have demonstrated that SHC signaling is involved in pathways, which play a role in the development of malignancies like c-Myc activation (Gotoh et al. 1997), survival signaling (Friedmann et al. 1996; Sakai et al. 2000), cytoskeletal organization, and mitogenic signaling through RAS. It has been proposed that SHC is a substrate for FAK.

Also, the ERK/MAP kinase cascade is a pathway in which integrin-mediated adhesion is involved. In the ERK pathway, various stimuli of many important integrin signaling molecules like FAK or SHC converge and are able to influence nearly every profound cellular activity (Meier et al. 2005).

Epidermal growth factor receptor (EGFR) is also activated by integrins to generate cellular responses such as adhesion-dependent cell survival and proliferation in response to ECM. Subsequently, integrin-mediated EGFR activation induces ERK/MAP kinase signaling (Howe et al. 2002; Jost et al. 2001). Furthermore, Caveolin-1 (CAV1) is the main structural component of caveolae, which are plasma membrane invaginations that participate in vesicular trafficking and signal transduction events. Following integrin activation, B16F10 cells expressing CAV1 display reduced expression levels and activity of FAK and Src proteins. Furthermore, CAV1 expression markedly reduces the expression of integrin β3 in B16F10 melanoma cells. These findings provide experimental evidence that CAV1 may function as an antimetastatic gene in malignant melanoma (Trimmer et al. 2010).

9.4   Immunoglobulin Gene Superfamily of Cell Adhesion Molecules (CAMs)

Whereas normal melanocytes express few cell–cell adhesion receptors of the immunoglobulin gene superfamily of cell adhesion molecules (CAMs), melanoma cells show an increase in expression of melanoma cell adhesion molecule (MCAM, Mel-CAM, MUC18, CD146), L1 cell adhesion molecule (L1-CAM, CD171), activated leukocyte cell adhesion molecule (ALCAM, CD166), vascular cell adhesion molecule 1 (VCAM-1, CD106), intercellular cell adhesion molecule 1 (ICAM-1, CD54), and carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM-1, CD66a) (reviewed in Haass et al. 2005).

9.4.1   Melanoma Cell Adhesion Molecule (MCAM, Mel-CAM, MUC18, CD146)

Mel-CAM mediates homologous and heterologous interactions between melanoma cells and endothelial cells, respectively, via a heterophilic Ca^{2+}-independent adhesion to its ligand (Shih et al. 1997a, b; Johnson et al. 1997). Recently, Laminin-411 ($\alpha4\beta1\gamma1$ integrin) and Galectin-1 have been identified as Mel-CAM ligands (Flanagan et al. 2012; Jouve et al. 2013; Yazawa et al. 2015). In melanocytic cells, expression of Mel-CAM is first found in nevi, when the cells have separated from the epidermal keratinocytes and have migrated into the dermis (Shih et al. 1994; Kraus et al. 1997). With progression to malignancy, Mel-CAM expression gradually increases and is highest in metastatic melanoma cells (Xie et al. 1997; Johnson et al. 1996; Shih et al. 1994; Lehmann et al. 1987, 1989). In vitro and in vivo data supporting an important role of Mel-CAM in melanoma progression was demonstrated in several experimental studies (reviewed in Haass et al. 2005; Lei et al. 2015). Recently, the zinc finger transcription factor ZBTB7A was found to repress melanoma metastasis by directly binding to the promoter and transcriptionally repressing Mel-CAM (Liu et al. 2015).

An evaluation of tissue arrays of primary and metastatic melanomas revealed that in patients meeting the current criteria for sentinel lymph node dissection, both Mel-CAM expression positivity and intensity were independently predictive of survival and development of lymph node disease in primary melanoma over and above established markers of prognosis, such as Breslow thickness. Mel-CAM-negative patients had a 5-year survival of 92 % compared with 40 % for Mel-CAM-positive patients (Pearl et al. 2008). Recently, a study on 175 patients revealed that sequential molecular detection of Mel-CAM mRNA in the peripheral blood correlated with poor prognosis. The authors suggested to utilize Mel-CAM expression as a "molecular warning of progression" even in early stage patients in otherwise disease-free conditions (Rapanotti et al. 2014). However, larger trials to confirm this finding as a biomarker are still pending.

9.4.2   L1-Cell Adhesion Molecule (L1-CAM, CD171)

L1-CAM, originally described as a neuronal cell adhesion molecule, has also been detected in a number of other non-neuroendocrine tissues and in several malignant tumors, including melanoma (Nolte et al. 1999; Thies et al. 2002b). L1-CAM mediates adhesion both via homophilic (L1-CAM-L1-CAM) and heterophilic (L1-CAM-$\alpha_v\beta_3$ integrin) mechanisms (Hortsch 1996). In melanoma/melanoma cell and in melanoma/endothelial cell interactions, L1-CAM binds to $\alpha_v\beta_3$ integrin (Montgomery et al. 1996). The interaction of L1-CAM and $\alpha_v\beta_3$ integrin plays an important role in transendothelial migration of melanoma cells (Voura et al. 2001) whereas overexpression of L1-CAM promotes conversion from radial to vertical growth phase melanoma without upregulation of $\alpha_v\beta_3$ integrin expression (Meier et al. 2006). There is an increase in L1-CAM immunoreactivity in melanomas and metastases of melanoma compared to acquired melanocytic nevi (Fogel et al. 2003). A study that systematically identified novel melanoma-specific genes confirmed that L1-CAM is not expressed in normal skin and melanocytic nevi, but is highly and differentially

expressed in primary melanoma tissues and melanoma lymph node metastases (Talantov et al. 2005). Evaluation of specimens of nevi, primary melanomas, sentinel lymph nodes, and distant metastases showed that L1-CAM can serve as a highly sensitive and specific diagnostic marker for melanoma (Thies et al. 2007). A 10-year retrospective biomarker study, evaluating 100 melanoma specimens, showed that the expression of L1-CAM in human primary cutaneous melanoma is significantly associated with metastatic spread and that L1-CAM expression is an independent predictor for the risk of metastasis (Thies et al. 2002b). A recent study revealed that the CE7 epitope of L1-CAM on a variety of tumors (however, melanoma was not included in the study) may be amenable to targeting by CE7R+ T cells, making it a promising target for adoptive immunotherapy (Hong et al. 2014).

9.4.3   Activated Leukocyte Cell Adhesion Molecule (ALCAM, CD166)

ALCAM is involved in homophilic (ALCAM-ALCAM) (Degen et al. 1998) and heterophilic (ALCAM-CD6) (Patel et al. 1995) cell–cell adhesion interactions. ALCAM is expressed in metastatic human melanoma cells, whereas it is absent in non-metastatic cells (Degen et al. 1998). Immunohistochemistry on a series of common nevi, primary melanomas, and melanoma metastases revealed that ALCAM expression correlates with melanoma progression (van Kempen et al. 2000). ALCAM is therefore proposed to be a molecular melanoma progression marker. Intact cell adhesion function of ALCAM favored primary tumor growth and represented a rate-limiting step for tissue invasion, which supported the view that dynamic control of ALCAM plays an important role in progression (van Kempen et al. 2004). An immunohistochemical biomarker study, evaluating tissue microarrays showed that a significantly greater percentage of melanomas (combined primary and metastatic) than nevi contained cells that expressed ALCAM (Klein et al. 2007). Interestingly, a recent study evaluating ALCAM expression and long-term survival in melanoma patients suggested that, in primary melanomas, high ALCAM expression was a marker of negative outcome, but in regional lymph node melanoma metastases low expression of ALCAM was a feature associated with unfavorable prognosis (Donizy et al. 2015). ALCAM upregulation in metastatic melanoma cells is driven by miR-214 and depends on transcriptional mechanisms mediated by TFAP2 and posttranscriptional mechanisms mediated by miR-148b, which itself is controlled by TFAP2. Therefore, miR-214 and miR-148b have opposite effects on melanoma cell dissemination and are part of a regulatory loop (Penna et al. 2013).

9.4.4   Intercellular Adhesion Molecule-1 (ICAM-1, CD54)

ICAM-1 can be induced in a cell-specific manner by several cytokines, e.g., TNF-α (tumor necrosis factor-alpha), IL-1 (interleukin-1), and IFN-γ (interferon-gamma). The ligands of ICAM-1 are $\alpha_L\beta_2$ (lymphocyte function-associated antigen 1, LFA-1) and Mac1 on lymphocytes (van de Stolpe and van der Saag 1996). ICAM-1

correlates with melanoma progression and increased risk of metastasis (Johnson et al. 1989). Its expression in melanoma is stronger than in common nevi and increases with the Breslow index in primary melanomas (Natali et al. 1990, 1997; Schadendorf et al. 1993, 1995). The observation that stage I patients with ICAM-1-positive melanomas had a significantly shorter disease-free interval and overall survival than those with ICAM-1-negative tumors (Natali et al. 1997) and that the suppression of ICAM-1 in an animal model reduced the metastatic capacity (Miele et al. 1994), supported the role of ICAM-1 in melanoma progression and metastasis. However, the specific role of ICAM-1 in melanoma progression remains to be determined. Expression of ICAM-1 may promote aggregate formation with leucocytes, which can enhance survival in the vascular system and encourage extravasation (Aeed et al. 1988). On the other hand, ICAM-1 is shed from melanoma cells (Giavazzi et al. 1992) – possibly in a form that inhibits lymphocyte–tumor cell interaction and thus contributes to tumor survival (Becker et al. 1993). A recent study has unraveled a mechanism by which shear flow-regulated melanoma cell adhesion to the endothelium can upregulate endothelial ICAM-1 expression (Zhang et al. 2014). Elevated ICAM-1 levels may serve as receptors to recruit neutrophils and bind fibrin, which assists melanoma cell adhesion and migration. An increase of ICAM-1 expression on endothelial cells could be a result of direct ligation of tumor CD44 and endothelial E-selectin, through the PKCα-p38-SP-1 pathway. This suggests a new mechano-signaling cascade triggered by stretching E-selectin to induce ICAM-1 expression (Zhang et al. 2014).

9.4.5 Carcinoembryonic Antigen-Related Cell Adhesion Molecule 1 (CEACAM-1, CD66a)

CEACAM1 is involved in intercellular adhesion and subsequent signal transduction events in a number of epithelia. In epithelial cells, CEACAM1 is believed to act as a growth suppressor, since its expression was shown to be lost or significantly down- or dysregulated in carcinomas of liver, prostate, endometrium, breast, and colon (reviewed in Haass et al. 2005). On the other hand, CEACAM1 is upregulated in non-small cell lung cancer (Sienel et al. 2003). CEACAM1 interacts with the β_3 integrin subunit via the CEACAM1 cytoplasmic domain. CEACAM1 and the β_3 integrin subunit co-localize at the tumor–stroma interface of invading melanoma masses, suggesting that CEACAM1–integrin β_3 interaction plays a role in melanoma cell migration and invasion (Brummer et al. 2001). The expression of CEACAM1 in primary melanomas is associated with the subsequent development of metastatic disease (Thies et al. 2002a). Furthermore, the overexpression of CEACAM1 in CEACAM1-negative melanocytic cells and melanoma cell lines increases the migratory and invasive growth potentials in vitro (Ebrahimnejad et al. 2004) supporting the role of CEACAM1 in melanoma progression and metastasis. Evaluation of specimens of nevi, primary melanomas, sentinel lymph nodes, and distant metastases showed that CEACAM1 can serve as a highly sensitive

and specific diagnostic marker for melanoma (Thies et al. 2007). Indeed, CEACAM1 was shown to be one of the seven plasma markers best able to identify metastatic melanoma patients (Kluger et al. 2011).

9.5   Gap Junctions/Connexins

Connexins belong to a family of transmembrane proteins that form gap junctions (GJs), cell–cell junctions that are essential for intercellular communication. Gap junctional intercellular communication (GJIC) in the skin is involved in maintenance of homeostasis, regulation of proliferation, differentiation, barrier function, and recruitment of inflammatory cells. GJIC is thus a critical factor in the life and death balance of cells (Djalilian et al. 2006; Langlois et al. 2007; Maass et al. 2004; Man et al. 2007) (reviewed in Kretz et al. 2004; Mese et al. 2007). Furthermore, GJIC is critical in keratinocyte–melanocyte interaction (Hsu et al. 2000; Satyamoorthy et al. 2001). Alternatively, connexins can form hemichannels, which allow release (e.g., ATP, NAD$^+$) or putative uptake of molecules and ions to and from the cellular environment (Barr et al. 2013; Chandrasekhar and Bera 2012). Finally, connexins, especially Cx43, interact with structural and signaling molecules, which may add further functions to these molecules (Herve et al. 2007).

GJs form channels between adjacent cells allowing the intercellular transport of small metabolites, second messengers, and ions (Loewenstein 1981; Spray 1994). In addition to molecular weight and size, the ability of a solute to transverse these channels depends on its net charge, shape, and interactions with specific connexins that constitute gap junctions in particular cells (Goldberg et al. 2004). Each GJ channel consists of two hemichannels called connexons, each formed by six connexins (reviewed in Richard 2000). Twenty-one connexins have been identified, 11 of which are in the skin (Di et al. 2001; Willecke et al. 2002; Zucker et al. 2013). GJs can be homotypic, heterotypic, homomeric, and heteromeric (reviewed in Richard 2000). A connexon is homomeric if it is composed of six identical connexin subunits (e.g., Cx32 only), or heteromeric if it is composed of more than one connexin species (e.g., Cx32 and Cx43 and/or others). Channels are homotypic if both connexons are homomeric of the same type, heterotypic if homomeric connexons are of different types, and heteromeric if both connexons are heteromeric. Not all connexins are equally compatible at forming a connexon – even though they may co-exist in the same cell (reviewed in Haass et al. 2004). The type of connexin-forming GJ channels influences their selectivity and thereby controls the specificity of GJIC. For example, channels formed by Cx26 prefer cations, while those formed by Cx32 prefer anions (Brissette et al. 1994; Elfgang et al. 1995; Veenstra 1996). Thus, the up- or down-regulation of a certain connexin in a tissue may change its GJIC considerably. In addition, connexins can also form hemichannels, which have been shown to be able to exchange molecules with the extracellular microenvironment. These hemichannels are relevant for signal propagation and especially for calcium homeostasis (reviewed in Evans et al. 2006).

9.5.1 Connexins Are Conditional Tumor Suppressors

Loss of gap junctional activity and/or downregulation of connexins have been reported both in cell lines as well as in tissues of many tumor types, such as hepatocellular carcinoma, gastric carcinoma, prostate cancer, lung cancer, glioma, mammary carcinoma, basal cell carcinoma, squamous cell carcinoma, and melanoma. This phenomenon was first observed half a century ago (Loewenstein and Kanno 1966) and summarized in a number of review articles (Cronier et al. 2009; Mesnil et al. 2005; Naus and Laird 2010). The type of connexins lost during tumor progression varies according to tumor type. In the 1980s and 1990s, a series of studies were published showing that reagents and/or oncogenes that promote tumor onset or progression frequently inhibit GJIC or downregulate connexin expression (Lampe 1994; Trosko et al. 1990; Atkinson et al. 1981). The role of connexins as potential tumor suppressors was also shown in gene knockdown studies (Shao et al. 2005). Correspondingly, ectopic expression of connexins in tumors restored functional communication and reduced tumor proliferation and growth both in vitro and in vivo (reviewed in Naus and Laird 2010). Importantly, ectopic expression of connexins partially differentiated transformed cells (Zhu et al. 1991; McLachlan et al. 2006; Hellmann et al. 1999; Hirschi et al. 1996). Moreover, functional abrogation of connexins, using antisense or dominant-negative mutant approaches, have demonstrated an enhancement of the malignant phenotype in several tumor types, such as Cx26 in HeLa cells (Duflot-Dancer et al. 1997), Cx32 in hepatocellular carcinoma (Dagli et al. 2004), Cx43 in lung cancer (Avanzo et al. 2004), Cx43 in glioma (Omori and Yamasaki 1998), and Cx43 in bladder carcinoma (Krutovskikh et al. 1998). Finally, Cx32 knock-out mice have an increased incidence of tumor onset when challenged with carcinogens (Temme et al. 1997; King and Lampe 2004a, b; Moennikes et al. 2000).

This may lead to the assumption that connexins are general tumor suppressors, but it appears that this is only the case in the earlier steps of cancerogenesis. In fact, the role of connexins in invasion and metastasis is very complex, and connexins might facilitate invasion, intravasation, extravasation, and metastasis (Krutovskikh et al. 1994; el-Sabban and Pauli 1991, 1994; Ito et al. 2000; Saunders et al. 2001; Lin et al. 2002; Miekus et al. 2005; Pollmann et al. 2005; Kanczuga-Koda et al. 2006; Bates et al. 2007; Li et al. 2007; Dobrowolski et al. 2008; Cotrina et al. 2008; Elzarrad et al. 2008; Ezumi et al. 2008). The following model supports both the tumor suppressor and the tumor driver theories (Cronier et al. 2009): for the step from primary to invasive tumors, there is a need for disruption of intercellular junctions including GJs, consistent with the model that connexins are tumor suppressors. In contrast, for the tumor cell dissemination and metastasis steps, increased cell contacts and communication are needed in order to enable interaction with the tumor stroma – especially between cancer cells and endothelial cells. Therefore, connexins might be better classified as conditional tumor suppressors that modulate cell proliferation as well as adhesion and migration (Naus and Laird 2010).

9.5.2   Cx43 in Cancer

Cx43 is decreased in prostate cancer (Tsai et al. 1996), mammary cancer (Hirschi et al. 1996), glioma (Huang et al. 1999), lung cancer (Jinn et al. 1998; Zhang et al. 1998), bladder carcinoma (Krutovskikh et al. 2000), cervical carcinoma (King et al. 2000), and various skin cancers including melanoma (Haass et al. 2006; Tada and Hashimoto 1997; Wilgenbus et al. 1992). Electron microscopy investigations have shown that basal and squamous cell carcinomas do not have fully developed GJs, and that Cx43 is not restricted to these poorly developed GJs but is present in the cytoplasm (Tada and Hashimoto 1997). In several cancers, Cx43 acts as a tumor suppressor gene with loss of Cx43 contributing to metastasis (Czyz 2008; Gershon et al. 2008; Shen et al. 2007). Functional abrogation of Cx43 enhances the malignant phenotype in lung cancer (Avanzo et al. 2004), glioma (Omori and Yamasaki 1998), and bladder carcinoma (Krutovskikh et al. 1998).

In contrast to other cancers, hepatocellular carcinoma is associated with an induction of Cx43, which is, however, localized in the cytoplasm, and thus is not involved in GJIC (Krutovskikh et al. 1994). The loss of GJIC might help the tumor cells to survive, as GJIC has been shown to spread cell-killing signals, most likely Ca^{2+} ions (Krutovskikh et al. 2002). In addition, downregulation of Cx43 expression or function resulted in increased proliferation and migration in primary keratinocytes, implying a contribution of Cx43 to controlling early stages of tumorigenesis (Mori et al. 2006; Wright et al. 2009; Pollok et al. 2011). Finally, increased opening of hemichannels formed by connexins resulted in cell death in cochlear supporting cells of the ear and in keratinocytes of the epidermis (Xu and Nicholson 2013).

Conversely, expression of Cx43 has also been shown to increase tumor metastasis in breast cancer, glioma as well as in melanoma through increased attachment and communication with the vascular endothelium (Bates et al. 2007; Kanczuga-Koda et al. 2006; Cotrina et al. 2008; Lin et al. 2002; el-Sabban and Pauli 1991, 1994; Pollmann et al. 2005; Elzarrad et al. 2008).

9.5.3   Cx32 in Cancer

Cx32 is downregulated in gastric carcinoma (Uchida et al. 1995), lung cancer (Jinn et al. 1998), and hepatocellular carcinoma (Eghbali et al. 1991; Loewenstein and Rose 1992; Krutovskikh et al. 1994; Yamaoka et al. 1995). In the latter case, the remaining Cx32 is localized in the cytoplasm or in the plasma membrane free from contact with other cells. In addition, it was found that there was no mutation in the coding sequence of Cx32 in hepatocellular carcinoma; instead, it appears that the aberrant localization of Cx32 is a consequence of the disruption of Cx32 gap junction plaque formation (Krutovskikh et al. 1994). Functional abrogation of Cx32 enhances the malignant phenotype in hepatocellular carcinoma (Dagli et al. 2004). Cx32 knock-out mice have an increased incidence of tumor onset when challenged with carcinogens (Temme et al. 1997; King and Lampe 2004a, b; Moennikes et al. 2000). In contrast to most other tumors, Cx32 is upregulated in some breast cancer cells (Saunders et al. 2001).

9.5.4 Cx26 in Cancer

Whereas in mammary carcinoma cells, there is a downregulation of both Cx43 and Cx26 (Hirschi et al. 1996); in human basal cell carcinoma, Cx43 is downregulated but there is an induction of Cx26 (Haass et al. 2006; Wilgenbus et al. 1992). Cx26 is also highly expressed in HeLa cells, where its functional abrogation enhances the malignant phenotype (Duflot-Dancer et al. 1997).

9.5.5 Connexins in Melanoma

Reflecting the situation in many other cancer types as discussed above, the role of connexins and GJIC is still highly controversial also in melanoma and its tumor microenvironment.

Cx43 is the most-studied connexin in melanoma. Western blotting revealed Cx43 protein expression in foreskin-derived melanocytes and several melanoma cell lines (Hsu et al. 2000). This was confirmed by immunofluorescence detecting Cx43 expression in human melanoma cell lines (Lin et al. 2010). While neither study quantified the Cx43 protein expression levels, a qRT-PCR and immunofluorescence study demonstrated lower Cx43 expression levels in human melanoma cell lines compared to human melanocytes (Schiffner et al. 2011). Also, a microarray study revealed that Cx43 was expressed at low levels in human melanoma cell lines and, importantly, that its overexpression suppressed anchorage-independent growth in colony-forming efficiency assays, suggesting a tumor-suppressor role of Cx43 in melanoma (Su et al. 2000). By qRT-PCR, no expression for Cx26, Cx30, Cx31.1, Cx36, and Cx37; low expression for Cx30.3 and Cx31; and higher expression levels for Cx32, Cx40, Cx43, and Cx45 were detected in human melanoma cell lines (Zucker et al. 2013). Surprisingly, Western blotting showed much higher Cx43 expression levels in migrating than in non-migrating cells (Zucker et al. 2013). Consistently, high levels of Cx43 protein expression were found in human metastatic melanoma cell lines (Villares et al. 2009). Loss of protease-activated receptor-1 (PAR-1) expression resulted in the loss of Cx43 and, correspondingly, overexpression of PAR-1 contributed to melanoma metastasis via upregulation of Cx43 (Villares et al. 2009, 2011). Interestingly, while initial levels of Cx43 were low in B16 mouse melanoma cells, Cx43 protein levels increased after infection with bacteria or treatment with interferon-γ (Saccheri et al. 2010). This was followed by the transfer of preprocessed antigenic peptides from melanoma cells to dendritic cells, which then presented those peptides on their surface and activated cytotoxic T cells against the tumor antigen. Correspondingly, melanoma cells in which Cx43 had been silenced, failed to elicit a cytotoxic antitumor response after infection with bacteria (Saccheri et al. 2010).

In addition to the discussed in vitro data, there are also a number of studies on human melanoma tissue. Using immunofluorescence on human tissue samples, we did not detect Cx43 (nor Cx26 and Cx30) in nevi, primary melanomas, or cutaneous melanoma metastases, while the internal controls (adjacent epidermis) were positive

in the expected layers (Haass et al. 2006, 2010). In contrast, using immunohisto-chemistry, other groups reported Cx43 expression in human melanoma tissue, higher than in human nevi (Rezze et al. 2011; Sargen et al. 2013). However, neither of these studies provided high magnification images to confirm the subcellular localization nor did they show appropriate positive and negative controls. Indeed, in both studies, Cx43 expression in melanoma cells appeared to be cytoplasmic and hence would argue for a cell–cell or cell–matrix communication-independent role of these con-nexins. This would not support the mechanism for melanoma survival in brain metas-tasis proposed by Lin and colleagues, who showed that reactive astrocytes protect metastatic melanoma cells in the brain from chemotherapy by sequestering intracel-lular calcium through direct cell–cell communication (Lin et al. 2010). Moreover, in the Rezze and Sargen studies, the expression pattern of Cx43 in nevi and different melanoma stages appeared very variable and the typical Cx43 staining in the epider-mis was missing (Rezze et al. 2011; Sargen et al. 2013). An Oncomine analysis of human tissue showed that increased Cx43 (and Cx26) gene expression in primary lesions correlated with metastasis and poor patient survival (Stoletov et al. 2013).

Cx26 and Cx30 are much less studied. Cx26 was found to be upregulated in the highly aggressive BL6 sub-line of B16 mouse melanoma cells compared to the less aggressive F10 sub-line (Ito et al. 2000). F10 cells transfected with wild-type Cx26 exhibited similar metastatic behavior to the BL6 cells. Correspondingly, BL6 cells transfected with a dominant-negative Cx26 mutant showed the less aggressive behavior characteristic of F10 cells. Cx26 was not found to be expressed in human melanoma in situ but was upregulated in invasive melanomas (Ito et al. 2000). However, in this study, Cx26 staining in both melanoma cells and epidermal kerati-nocytes was cytoplasmic. Moreover, the study did not distinguish between Cx26 and Cx30. In contrast, we showed in immunofluorescence studies on human mela-noma tissue samples, that all areas of melanocytic nevi, primary melanomas, and cutaneous melanoma metastases lacked Cx26 and Cx30 expression (Haass et al. 2006, 2010) – similar to our findings in Merkel cell carcinoma (Haass et al. 2003a). This was confirmed by other groups who did not detect Cx26 in melanoma using immunohistochemistry on human tissue samples (Sargen et al. 2013) or did not find Cx26 and Cx30 expression in human melanoma cell lines using qRT-PCR (Zucker et al. 2013). Contrastingly, a positive correlation between Cx26 expression and metastatic potential was reported using Cx26 shRNA in B16 mouse melanoma cells (Stoletov et al. 2013). This was supported by an Oncomine analysis of human tis-sue, which showed that increased Cx26 expression in primary lesions correlated with metastasis and poor patient survival (Stoletov et al. 2013).

Interestingly, loss of Pannexin 1, a channel-forming glycoprotein remotely related to connexins, attenuated melanoma progression by reversion to a melanocyte-like phenotype (Penuela et al. 2013).

The Oncomine data (Stoletov et al. 2013) do not seem to match the data on pri-mary melanomas in other studies; however, it would be interesting to re-analyze these data more in detail. As there appears to be a correlation to tumor thickness, is there no or little expression on thin tumors and a differential expression pattern in different areas of thick melanomas?

The discrepancies between the different studies in Cx43, Cx26, and Cx30 in melanoma may be due to the following reasons:

1. Several studies investigated the molecules on mRNA level only. The presence of mRNA does not necessarily mean that the respective protein is present.
2. In tissues, it is difficult to separate between connexins present in melanoma cells and those present in epidermal, mesenchymal, or endothelial tissues enclosed by the tumor.
3. Immunohistochemistry is often dependent on staining conditions and can result in false-positive and false-negative results. Appropriate positive and negative controls showing the sensitivity and specificity of the antibody are indispensable for the interpretation of these results. For example, the Cx26 antibody used in some of the discussed studies shows cross-reactivity with Cx30.

Importantly, most of the apparent discrepancies in this paragraph can be explained by a model, which implies that connexins are tumor suppressors during early melanomagenesis but tumor drivers during metastasis (Cronier et al. 2009). During early melanomagenesis, the respective connexins are typically located in the cell membranes indicating that they are functioning through GJIC. In contrast, in advanced stages, connexins are typically located in the cytoplasm indicating a different function – possibly through interaction with signaling molecules.

9.5.6   Connexins in the Epidermal Tumor Environment of Melanoma

Keratinocytes communicate with melanocytes but not with melanoma cells via GJIC; instead, melanoma cells communicate among themselves and with fibroblasts and endothelial cells (Hsu et al. 2000). This switch in communication partners coincides with the E- to N-cadherin switch, suggesting that the gain of N-cadherin with the concurrent loss of E-cadherin facilitates GJ formation with fibroblasts and endothelial cells (Hsu et al. 2000). Additionally, GJ formation in human melanoma cell lines appears to require MCAM (Satyamoorthy et al. 2001). This switch will allow melanoma cells to de-couple from the epidermal microenvironment and to communicate with cell types important for their metastatic spread. Several studies have suggested that connexins may promote metastasis in melanoma and other tumors by forming intercellular connections between cancer cells and vascular endothelium that are able to initiate tumor cell diapedesis (Hsu et al. 2000; Villares et al. 2009; el-Sabban and Pauli 1991, 1994; Saito-Katsuragi et al. 2007; Pollmann et al. 2005). Melanoma cells expressing higher levels of Cx43 show increased coupling to vascular endothelial cells (el-Sabban and Pauli 1991) and the ability of tumor cells to metastasize appears to correlate with the ability of tumor cells to communicate with endothelial cells (Pollmann et al. 2005). Also, Cx26 may contribute to the metastasis of melanoma by facilitating communication between melanoma cells and their surrounding endothelial cells (Saito-Katsuragi et al. 2007).

Cx26 expression is associated with lymphatic vessel invasion and poor prognosis in human breast cancer (Naoi et al. 2007).

Melanoma brain metastases are surrounded and infiltrated by astrocytes, and these astrocytes can play a role similar to their established ability to protect neurons from apoptosis (Lin et al. 2010). In co-culture experiments, astrocytes reduced apoptosis in human melanoma cells treated with various chemotherapeutic drugs. This chemoprotective effect was dependent on physical contact and GJIC between astrocytes, which express high levels of Cx43, and tumor cells. Moreover, the protective effect of astrocytes resulted from their sequestering calcium from the cytoplasm of tumor cells. These data suggest that brain metastases can harness the neuroprotective effects of reactive astrocytes for their own survival (Lin et al. 2010). In a chick embryo model, B16 mouse melanoma cells, which express Cx26 but not Cx43, colonized the chicken brain forming numerous microtumors invading along the preexisting vasculature (Stoletov et al. 2013). In contrast, Cx26 knockdown B16 cells formed significantly fewer and less invasive tumors, suggesting that in metastatic melanoma cells Cx26 expression enhances microtumor formation in the brain in association with the existing vasculature (Stoletov et al. 2013).

While these studies demonstrate the interaction of melanoma cells with the stroma and the role of connexins and/or GJIC in the early and late steps of melanomagenesis, interactions between melanoma and the epidermal tumor microenvironment (ETM) – the multilayered epithelium of the skin – are poorly understood. In this regard, we have demonstrated the induction of Cx26 and Cx30 in the epidermis adjacent to malignant tumors (e.g., melanoma and Merkel cell carcinoma), but not in the epidermis adjacent to benign tumors (e.g., melanocytic nevi and angiomas) (Haass et al. 2003a, 2006). Subsequently, we found correlation between (a) tumor thickness (Breslow index) and vertical Cx26 and Cx30 expression in the ETM, (b) tumor thickness and horizontal Cx26 dissemination in the ETM, (c) metastasis and horizontal Cx26 expression in the ETM, and (d) vertical epidermal expression patterns of Cx26 and Cx30 and the proliferative index in the ETM. We thus provided evidence for the association of ETM alteration with tumor malignancy and progression (Haass et al. 2010). The results of this study, which included dysplastic nevi as well as thin melanomas which are often difficult to distinguish (reviewed in Haass and Smalley 2009), suggest that membrane expression of Cx26 and Cx30 in the epidermal tumor microenvironment may be a useful diagnostic aid for the distinction of melanomas and melanocytic nevi (Haass et al. 2010). As neither Cx26 nor Cx30 are expressed in the melanoma itself, but both are induced in its tumor microenvironment, they may be useful complementary melanoma markers.

Cx26 and Cx30 upregulation in the epidermal tumor microenvironment did not correlate with the proliferative index of the melanoma cells, but correlated significantly with the proliferative index in the epidermis. In transgenic mice expressing Cx26 ectopically, proliferation was increased in the epidermis (Djalilian et al. 2006), suggesting that Cx26 influences keratinocyte proliferation and not vice versa. Interestingly, Cx26 overexpressing mice showed a delay in wound healing, which needs to be explored with regards to ulceration, a biomarker associated with very poor prognosis for melanoma patients (Balch et al. 2001). In our study, all

melanomas with ulceration showed Cx26 (and Cx30) expression in all layers of the epidermal tumor microenvironment (Haass et al. 2010). Induction of angiogenesis by the hyperplastic epithelium could stimulate growth and progression of melanoma (McCarty et al. 2003). This suggests a positive feedback mechanism: tumor cells induce alterations in keratinocytes, which results in the production of growth factors which, in turn, stimulate tumor survival via endothelial cells. The induction of Cx26 and Cx30 in the epidermis adjacent to melanoma putatively leading to GJIC or signaling via hemichannels may play a role in this feedback mechanism by inducing proliferation and other functions. An interruption of this vicious circle may provide a novel therapeutic approach.

9.6   Tight Junctions

In simple epithelia and endothelia, tight junctions (TJs) are responsible for the formation and maintenance of the tissue barrier between distinct compartments by controlling the paracellular pathway ("barrier function") (reviewed in Stevenson and Keon 1998; Tsukita et al. 2001). Subsequently, the involvement of TJs in the barrier function of a complex epithelium, the epidermis, was shown (Pummi et al. 2001; Brandner et al. 2002, 2003; Furuse et al. 2002; Langbein et al. 2002). In addition, TJs prevent the diffusion of membrane proteins and lipids from the apical to the basolateral side of an epithelial cell sheet, helping to maintain cell polarity ("fence function") (reviewed in Mitic and Anderson 1998; Tsukita et al. 2001). Therefore, TJs are crucial for the epithelium to generate chemical and electrical gradients that is necessary for vectorial transport processes such as absorption and secretion (reviewed in Martin and Jiang 2009). Moreover, TJ molecules act as intermediates and transducers in cell signaling, thus playing a role in the processes of polarity, cell differentiation, cell growth, and differentiation. Finally, TJs act as cell–cell adhesion molecules and as a barrier to cell migration (reviewed in Martin and Jiang 2009).

TJs are composed of integral transmembrane proteins (claudin 1–24, occludin, and junctional adhesion molecules A-C, 4 (JAMs)), peripheral plaque proteins (zonula occludens (ZO) proteins 1–3, MAGI 1–3, MUPP-1, PAR-3, PAR-6, AF-6, CASK, and CAROM), and associated proteins (symplekin, ZONAB, cingulin, Rab-13, Rab-3B, c-src, α-catenin, PKA, ZAK, and Rho GTPases). The molecular composition of TJs is highly complex and varies according to the cell type and degree of differentiation. TJ molecules from neighboring cells associate and form paired strands which seal the paracellular pathway and which contain aqueous pores or paracellular channels, explaining the ion and size selectivity for passaging molecules of TJ (Tsukita and Furuse 2000).

In cancer, disruption of TJs should occur in three critical steps: (1) detachment of the tumor cell from the primary tumor, (2) intravasation of the tumor through the endothelium, and (3) extravastion of the circulating tumor cell (reviewed in Martin and Jiang 2009). Early studies have shown a correlation between lack of TJs and tumor differentiation and there is evidence that TJs need to be overcome by cancer

cells in order to metastasize (reviewed in Martin and Jiang 2001, 2009). Cancer cells frequently exhibit deficiencies in TJ function, as well as decreased differentiation and cell polarity (Weinstein et al. 1976; Soler et al. 1999). Loss of TJ integrity may be particularly important in allowing the diffusion of nutrients and other factors necessary for the survival and growth of the tumor cells (Mullin et al. 1997). In addition, decreased polarity and differentiation may be important for the metastatic phenotype, where individual cells must leave the primary site and enter the blood vessels to reach distant sites (Ren et al. 1990).

Electron microscopy studies in human thyroid tumors showed that TJs decrease in number and are attenuated during carcinogenesis, which is associated with loss of tumor differentiation (Kerjaschki et al. 1979). Expression of TJ proteins is decreased in some cancer types, e.g., ZO-1 and occludin in gastrointestinal adenocarcinoma (Kimura et al. 1997), occludin in epithelial-derived tumors (Li and Mrsny 2000), claudin 3 in glioblastoma multiforme (Wolburg et al. 2003), claudin 1 in sporadic and hereditary breast cancer (Kramer et al. 2000), and claudin 7 in ductal carcinoma of the breast (Kominsky et al. 2003). On the other hand, some TJ molecules appear to be upregulated in some cancers. We found protein expression of claudins 3, 4, and 5, occludin, and ZO-1 in Merkel cell carcinoma cells (Haass et al. 2003b). Strikingly, expression of some claudin family members is highly elevated in various human cancers, e.g., claudin 7 in two breast cancer cell lines (Nacht et al. 1999), claudin 1 in colorectal cancer (Miwa et al. 2000), and claudins 3 and 4 in ovarian (Hough et al. 2001; Rangel et al. 2003) and prostate cancer (Long et al. 2001).

The expression of TJ proteins in melanoma tissues and cultured melanoma cells was described on RNA and on protein level (Cohn et al. 2005; Smalley et al. 2005; Leotlela et al. 2007; Schmitt et al. 2007; Morita et al. 2008). In a tissue array study, Claudin-1 was found to be significantly reduced in metastatic melanoma (Cohn et al. 2005). These data were, however, directly contradicted by another study (Leotlela et al. 2007). In this study Claudin-1 appeared to contribute to melanoma cell invasion, as transient transfection of melanoma cells with Claudin-1 increased metalloproteinase 2 (MMP-2) secretion and activation, and subsequently, motility of melanoma cells as demonstrated by wound-healing assays. Conversely, knockdown of CLDN1 by siRNA resulted in the inhibition of motility, as well as decreases in MMP-2 secretion and activation (Leotlela et al. 2007).

In contrast to most cancers, where levels of ZO-1 are typically downregulated, leading to increased motility, we found that ZO-1 expression is upregulated in melanoma cells and is located at adherens junctions between melanoma cells and fibroblasts (Smalley et al. 2005). Immunofluorescence and co-immunoprecipitation studies showed co-localization of ZO-1 with N-cadherin. Downregulation of ZO-1 in melanoma cells through RNA interference produced marked changes in cell morphology – leading to a less dendritic, more rounded phenotype. Consistent with a role in N-cadherin-based adhesion, RNAi-treated melanoma cells were less adherent and invasive when grown in a collagen gel. These data provided the first evidence that increased ZO-1 expression in melanoma contributes to the oncogenic behavior of this tumor and further illustrated that protein products of genes, such as

ZO-1, can function in either a pro- or anti-oncogenic manner when expressed in different cellular contexts (Smalley et al. 2005).

In summary, while it appears that functional TJs may be tumor suppressors, the upregulation of certain TJ proteins can contribute to oncogenic behavior. The relationship between TJ protein overexpression and cancer initiation or progression is thus unclear at present, but may be explained by the lack of functional TJs and that the upregulated TJ proteins therefore likely function through TJ-independent mechanisms.

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