The diverse roles of integrin α3β1 in cancer: Lessons learned from skin and breast carcinogenesis

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GENERAL DISCUSSION

The main goal of this thesis was to gain a better understanding of the conditions that are responsible for the different functions of integrin α3β1 in cancer. We aimed to achieve this by investigating the role of α3β1 in few well-defined cancer types and stages: during the initiation of non-melanoma skin cancer and during the development of invasive HER2-driven breast carcinogenesis. These cancer models present distinctive tumor environments, which differ in numerous components: extracellular matrix composition, expression of adhesion molecules, such as integrins and cadherins, expression of α3β1-associated proteins (such as tetraspanin CD151) and the nature of driving oncogenic mutation to just name a few. Furthermore, by investigating the role of α3β1 expressed in hair bulge stem cells (HB SCs) only, we gained an unexpected opportunity to shed light not only on the function of α3β1 in tumor-initiating cells, but also in cells that can regulate tumorigenesis via modulation of tumor environment. Here, we discuss the diversity of α3β1 in cancer in the light of our findings and present some preliminary data that we hope could expand the scope of this research in the future.

PRO-SURVIVAL ROLE OF α3β1 VIA ITS ADHESION TO LAMININ MATRIX

Adhesion to extracellular matrix is one of the main characteristics of integrins expressed by epithelial cells. Ligation by extracellular matrix components leads to integrin clustering and engagement of cytoskeleton, which initiates an outside-in signaling via integrin-associated kinases, such as focal adhesion kinase (FAK) and the proto-oncogene tyrosine-protein kinase Src [1,2]. As discussed in **chapter 2**, such association of integrins with extracellular matrix components can provide essential pro-survival and pro-proliferative signals during early tumorigenesis [3]. Evidently, the extracellular matrix composition of the tumor environment (i.e. the presence of laminin-332 and/or -511 isoforms which are recognized by integrin α3β1) is a primary determinant of whether the adhesion of α3β1 will be essential for tumorigenesis. Tumors, which do not originate from basal cells, such as luminal HER2-enriched cancer that we investigated in **chapter 5**, are not surrounded a by laminin-containing matrix and, therefore, do not rely on the laminin-binding α3β1 for the initial survival and growth. In addition to the presence of laminin, the importance of α3β1-mediated adhesion in promoting early tumorigenesis also depends on redundancy of its function: expression of integrins other than α3β1 that can mediate cell adhesion and thus trigger pro-survival signaling. Such redundant functions have been observed in mouse epidermis, where the deletion of individual integrins had only mild (α3β1) or no detectable effects (α2β1, α9β1 and αvβ5) on the adhesion of basal keratinocyte to the ECM, whereas that of the integrin β1 subunit or the integrin α6β4 resulted in a severe blistering phenotype [11,12]. The activity of these additional integrins could explain why FAK activation is only moderately reduced in K14 *Itgα3* KO mice and why laminin-332 organization, cell adhesion and growth of 3D spheroid culture of transformed keratinocytes, expressing laminin-binding mutant $\alpha3\beta1^{G163A}$ is seemingly unperturbed (**chapter 4**). Thus, it is conceivable that α3β1-mediated adhesion to laminin-containing matrix might not play a decisive role in the initiation of any of the cancer models presented in this thesis (**chapters 3-5**). However, studies performed with the basal mammary tumors and with epithelial bladder carcinomas indicate that α3β1-mediated adhesion of tumor cells to laminin can be crucial for tumor growth and survival in other models [13,14]. In line with this, our preliminary study showed that the growth of 3D organoids from human colorectal adenoma cells strongly depend on α3β1-mediated adhesion to secreted and deposited laminin-332 (**Fig. 1a, b**). Treatment of these organoids with the function-blocking antibody J143, which prevents ligation of α3β1 by laminin [15], resulted in concentration-dependent inhibition of organoid growth over time (**Fig. 1c**), which was not observed in organoids, treated with the function-blocking antibody GoH3 directed against α6β4 [16] (**Fig. 1d**). This preliminary data indicates that initial stages of colorectal cancer could

rely on α3β1 for growth and survival. Further studies should aim to elucidate whether such dependency is a general characteristic of early stages of colorectal cancer by testing a large panel of different adenoma organoids and investigate the mechanism behind it.

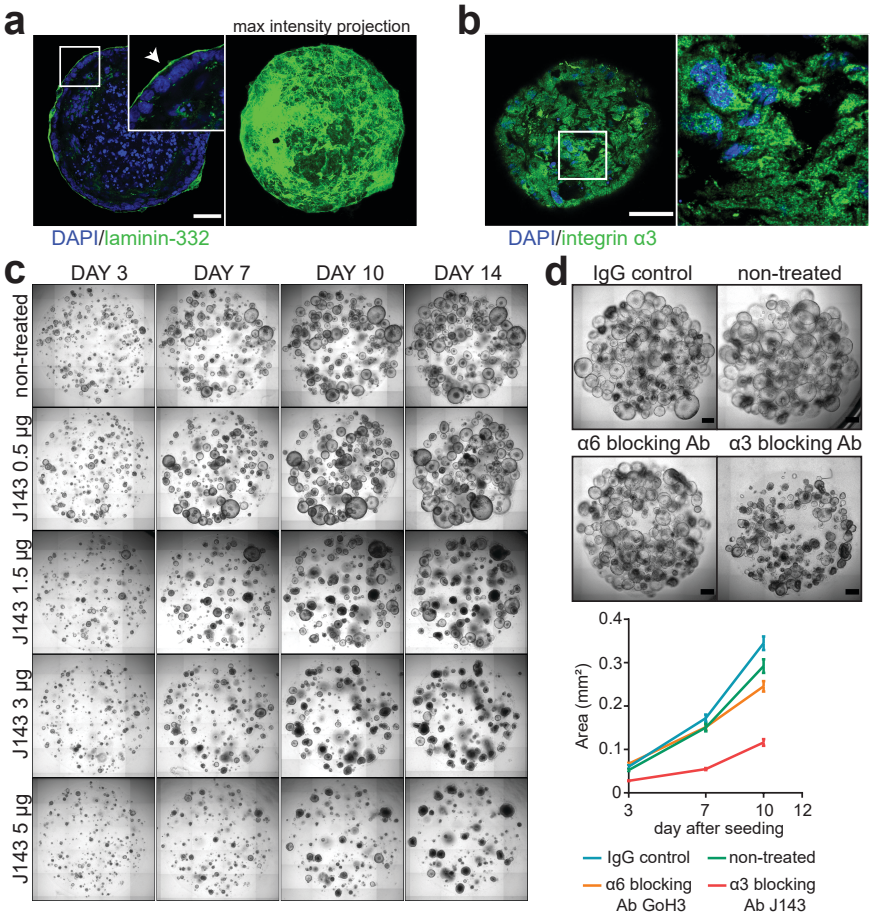

Figure 1: Integrin α3β1-mediated adhesion promotes growth of colorectal adenoma organoids. (**a-b**) Immunofluorescent staining for (**a**) laminin-332 (Ab: R14) and (**b**) integrin α3β1 (Ab: J143). Laminin-332 is deposited by basal cells (arrow) into the extracellular matrix that surrounds the organoids in 3D culture. Integrin α3β1 is expressed by basal cells. Scale bar: 40 µm. (**c**) Concentration-dependent inhibition of the growth of organoids, treated with α3β1 function-blocking antibody J143 and grown for 14 days. (d) Blocking of α3β1, but not α6β4 results in strong inhibition of organoid growth. Organoids were treated with 3 µg of control IgG, J143 or GoH3 antibodies. The area of 60-120 organoids form 3 independent experiments was quantified (mean ±SEM). Colorectal adenoma organoids were obtained and cultured from patient's resected adenoma by Magriet Lemmens, Anne Bolijn, Marianne Tijssen and Pien Delis-van Diemen (research group of dr. G. Meijer). This work was done in collaboration with dr. Beatriz Carvalho and dr. Sanne R Martens-de Kemp from the research group of dr. G. Meijer (the Netherlands Cancer Institute).

EXPANDING THE ROLE OF INTEGRIN α3β1 IN CANCER BEYOND CELL-MATRIX ADHESION

Over the last decade, the function of integrins beyond mediating cell-matrix adhesion has become more apparent, bringing new complexity and interest to the integrin field [17,18]. In **chapter 3,** we describe that in HB SCs α3β1 regulates the expression of 15 protein-coding genes during the initiation of DMBA/TPA-mediated tumorigenesis, particularly the expression of matricellular protein connective tissue growth factor (CCN2), which *in vitro* promotes clonogenic potential and 3D growth of transformed keratinocytes. We were not the first to demonstrate that α3β1 can influence cancer progression via changes in gene transcription: in transformed keratinocytes, α3β1 stimulated the expression of fibulin-2 and other genes involved in matrix remodeling and invasion [19], in basal breast carcinoma cells the expression of cyclooxygenase-2 [20] and in mammary carcinoma cells and transformed keratinocytes the expression and activity of matrix metalloproteinase MMP-9 [21–24]. Even though the exact mechanism responsible for these changes in gene expression remains to be determined, several studies demonstrated the ability of α3β1 to promote the stability of mRNA [23–25]. For a comprehensive understanding of how α3β1-regulated gene expression can influence tumor initiation and progression, it is important to note that α3β1-regulated proteins are often part of the cell secretome. The research group of DiPersio has made a notable contribution towards the recognition of α3β1 as a regulator of paracrine signaling, demonstrating that α3β1-regulated secretome can increase angiogenesis during tumor growth [20] and promotes angiogenesis and differentiation of dermal fibroblasts during wound healing [26–28]. Our findings described in **chapter 3** reaffirm the notion that the expression of α3β1 can influence cell secretome, and go a step further by suggesting that α3β1 can in such way influence tumor formation even when it is not expressed by tumor-initiating cells.

The ability of α3β1 to regulate gene expression might still dependent on its signaling function upon ligation by laminin. The fact that the application of function-blocking anti-α3β1 antibody perturbed the expression of MMP-9 in breast carcinoma cells [21] and that paracrine signaling in keratinocytes depended on α3β1-medaited FAK phosphorylation [26] supports this notion. It would be interesting to investigate whether the expression of CCN2 in our model of transformed keratinocytes also depends on association of α3β1 with laminin-containing matrix. This would not be unexpected, as the laminin-rich basement membrane underlies HB SCs. Furthermore, in 3D culture of transformed keratinocytes CCN2 typically localizes to the outer layers of spheroids, where laminin-332 is deposited. However, our observation of an increased angiogenesis in the absence of α3β1 in luminal HER2-driven tumors described in **chapter 5** suggests

that α3β1 can influence tumor stroma also independently of its ligation by laminin. That the function of α3β1 is likely not restricted to its adhesion to laminin is implied already by its localization in the epidermis: apart from the expression at the basal cell surface, α3β1 localizes also to the apical and lateral cell membranes, at the sites of cell-cell contacts. Very little is known about the role of integrins at cell-cell contacts; when associated with CD151, α3β1 is known to form a complex with E-cadherin (23), which can mediate TGFβ signaling [30]. In **chapter 4** we build on this knowledge and demonstrate that when stable cell-cell contacts are intact, i.e. when CD151 is present and E-cadherin function is unperturbed, α3β1 can promote pro-tumorigenic Stat3 and Akt signaling, therefore driving the 3D growth of transformed keratinocytes even without its ligation by laminin. This provides a new insight into the signaling function of α3β1 that is dependent on cell-cell contact integrity rather than the presence of extracellular matrix and as such can be relevant for the survival, growth and collective invasion of tumor cells that are not in contact with laminin.

Even when expressed at the basal cell surface, α3β1 can influence progression of cancer independently of its adhesion to laminin; in **chapter 5** we demonstrate that non-ligated α3β1 suppresses the invasive potential of luminal HER2-driven carcinoma cells. Such laminin-independent role of α3β1 was possible due to the ability of α3β1 to influence the clustering of collagen-binding integrin α2β1, a known suppressor of HER2-driven carcinogenesis [31], which lead to reduced accumulative surface of focal adhesions, reduced cell adhesion to collagen I matrix and increased passive invasion. Therefore, apart from its role in cell-cell contacts, α3β1 can have laminin-independent functions also through the crosstalk, i.e. cross-suppression and transdominant inhibition of other integrins. The notion that integrins in adhesion clusters can assert dominance over each other has been suggested before [26,32,33] and adds another level of complexity to roles of integrins in pathological conditions.

MECHANISMS UNDERLYING THE TUMOR-PROMOTING ROLE OF INTEGRIN α3β1 AND TETRASPANIN CD151 IN DMBA/TPA-INDUCED SKIN CARCINOGENESIS

Integrin α3β1 as a mediator of pro-tumorigenic signaling

Previous studies from our group showed that while the deletion of *Itga3* in mouse epidermis results in a strong reduction of tumorigenesis, knockout of *Cd151* (*Cd151* KO mice) has a relatively moderate effect on the formation of tumors in two stage skin carcinogenesis model [34,35]. These findings indicate that the function of α3β1, but not that of CD151 is critical for the initiation stage of DMBA/TPA-driven tumorigenesis. Similarly, we found that the growth of *Hras1* transformed keratinocytes into spheroids

depends on α3β1 but not on CD151 (**chapter 4**). Integrin α3β1 promotes the spheroid growth of these cells through its ability to activate and/or support the activation of FAK/Src, PI3K/Akt and Stat3 signaling. In line with these findings, both FAK and Stat3 have been shown to play a critical role in the initiation of DMBA/TPA-driven tumorigenesis [36,37]. Whether Akt is also needed for the formation of papillomas is not clear from the existing *in vivo* studies [38]. However, the fact that papillomas have high levels of phosphorylated Akt [39] and that active Akt is indispensable for the 3D growth of transformed keratinocytes (**chapter 4**) strongly suggest that Akt activation is needed for tumor outgrowth. Taking into consideration also our observation of reduced pro-oncogenic signaling in DMBA/TPA-primed K14 *Itgα3* KO epidermis (**chapter 4**), it can be concluded that DMBA-initiated basal keratinocytes depend on α3β1 for activation of signaling that enables their clonal outgrowth into papillomas.

However, further tumor growth and the expansion of suprabasal transformed keratinocytes depends on both, α3β1 and its binding partner CD151. Despite their initial outgrowth, papillomas lacking CD151 are smaller and display decreased keratinocyte proliferation. Interestingly, in the CD151-deficient papillomas, proliferation of keratinocytes is restricted to the laminin-adherent basal cell layer, whereas in WT papillomas scattered Ki67-positive keratinocytes can be observed also in suprabasal layers [34]. These observations can be explained by our finding that the survival and expansion of suprabasal differentiating transformed keratinocytes depends on the ability of α3β1-CD151 complex to promote Stat3 and Akt signaling. Remarkably, this function can be supported by α3β1 that is not ligated by laminin (**chapter 4**). Whether these findings translate *in vivo* should be investigated by assessing the activation of FAK/Src, Stat3 and Akt in *Cd151* KO papillomas. If CD151 is not needed for α3β1-mediated FAK/Src signaling during the initiation stage of tumorigenesis, but together with α3β1 mediates tumor expansion by promoting Stat3 and Akt-mediated survival of differentiating suprabasal keratinocytes, we would expect to detect a marked reduction in Stat3 and Akt, but not FAK/Src phosphorylation in the absence of CD151.

The role of α3β1 in maintenance of tumor-initiating cell population

The outgrowth of papillomas from DMBA-initiated keratinocytes depends on the ability of these cells to persist long enough in the epidermis during the initiation stage of chemically induced skin carcinogenesis to acquire the necessary genetic alterations. Slow-cycling basal keratinocytes have long been recognized as the tumor-initiating cells [40]. Even though some of the previous studies, including a study by our group, indicated that slow-cycling HB SCs represent such reservoir of tumor-initiating cells [35,41,42], our investigations described in **chapter 3** contests this hypothesis. Instead, several epidermal stem cell populations, including those in the interfollicular epidermis

and isthmus in hair follicles likely exert this function [43]. The genetic deletion of *Itga3* causes an increased epidermal turnover and loss of slow-cycling basal keratinocytes, which is further increased upon TPA-treatment. Thus, the tumor-promoting role of α3β1 has been previously attributed to its ability to retain HB SCs in their niche and protect them from premature loss through terminal differentiation [35]. Putting aside the assumption that HB SCs crucially contribute to the formation of papillomas, the loss of slow-cycling tumor-initiating cells in hair follicles and interfollicular epidermis remains the mechanism underlying the absence of tumor formation upon the deletion of α3β1. This is likely caused by a combination of a reduced adhesion strength of *Itga3* KO basal keratinocytes, reflected in reduced FAK activation (4,28, **chapter 4**) and an increased rate of terminal differentiation, promoted by decreased Akt and Stat3 activation in α3β1-depleted suprabasal keratinocytes (**chapters 3 and 4**).

In contrast, no clear defects could be observed in CD151-depleted epidermis and epidermal turnover was only slightly increased upon TPA-treatment (i.e. to a lesser extend compared to K14 *Itga3* KO mice) [44]. Similarly, the loss of slow-cycling basal keratinocytes was more prominent upon the deletion of *Itga3,* compared to that of *Cd151* [34,35]. Therefore, contrary to the loss of α3β1, tumor-initiating cells lacking CD151 persist for longer periods of time in their niches, which results in only moderate decrease in tumor formation upon DMBA/TPA-treatment [34]. It is thus likely that the TPA-driven increase in epidermal turnover of *Cd151* KO mice depends exclusively on deregulated differentiation of the TPA-induced hyperplastic skin. This is in line with our findings that CD151 together with α3β1 promotes Akt and Stat3 activation and regulates the survival of differentiating keratinocytes (**chapter 4**). Inspection of short-term DMBA/TPA-treated *Cd151* KO epidermis for differentiation markers, active Akt and nuclear Stat3 could provide a firmer ground for this hypothesis.

Integrin α3β1 as a promoter of pro-tumorigenic cell environment

In addition to its crucial role in promoting pro-tumorigenic signaling in tumor-initiating cells and retaining them in their niches, α3β1 affects tumor formation and tumor growth also when expressed by keratinocytes that do not (significantly) contribute to the tumor cell mass, such as HB SCs (K19 *Itga3* KO and WT mice) (**chapter 3**). We were not the first to report that gene modification of HB SCs can affect two-stage carcinogenesis model, although other studies attributed this effect to the now disputed role of HB SCs as tumor-initiating population [41,42]. During the submission of our manuscript, a research paper was published by the group of Valentina Greco, in which they describe that HB SCs show tolerance to *Hras* mutation, as well as that they affect neighboring epithelial and stromal cells [45]. As far as we are aware, this is the first study (apart from ours) that demonstrates that transformed keratinocytes in HBs can

directly affect their environment. We were not able to elucidate whether α3β1 in HB SCs affects tumor formation through changes in neighboring epithelial cells or stromal cells, such as fibroblasts, endothelial cells or immune cells, which are known to play an important part in development and progression of DMBA/TPA-initiated tumors [46,47]. Immunohistochemical staining for markers of macrophages, T-cells and myofibroblasts showed no obvious differences between their expression or localization in the skin of short-term DMBA/TPA-treated K19 *Itgα3* KO and WT skin (data not shown), however, a more detailed study of several markers using flow cytometry would be needed to properly asses this.

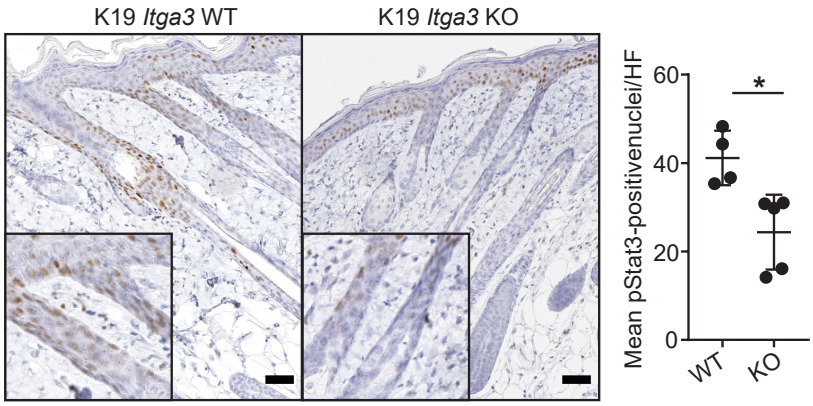

Figure 2: Reduction of nuclear phosphorylated Stat3 in the hair follicles of K19 *Itgα3* KO mice. IHC images stained for pStat3 (Y705, Ab D3A7 Cell Signaling) were quantified manually. Each dot on the graph represents a mouse and is an average of quantification of 30-50 hair follicles, longitudinally cut and in anagen hair cycle stage. (mean ± SD, student t test, $p<0.05$). Scale bars: 50 μm.

In **chapter 3** we provide evidence that suggests that α3β1 might affect the tumor environment through the secretion of the matricellular protein CCN2. We show that the tumorigenic potential of transformed keratinocytes can be enhanced by extracellular CCN2 *in vitro* when α3β1 is present. However, we were not able to determine the mechanism behind this process. Several options seem likely: Firstly, because CCN2 is known to bind different cell surface receptors, such as receptor tyrosine kinases and integrins, it may impact the tumorigenic potential of cells by altering the function of these receptors. Furthermore, CCN2 can directly bind cytokines, some of which are known to play a crucial role during two stage carcinogenesis (e.g. TGFβ), and thus regulates their availability and activity [48]. How α3β1 regulates CCN2 expression remains to be investigated. α3β1 regulates activation of several known promoters of CCN2 expression, such as FAK, Smad2/3 and Stat3 [48]. Since we observed IL6-mediated upregulation of CCN2 in 2D culture of transformed keratinocytes, the possibility that α3β1 promotes CCN2 expression via Stat3 activation is particularly attractive (**chapter 3**). This hypothesis

is additionally supported by the finding that deletion of Stat3 in HB SCs strongly reduced tumorigenesis [41] and our observations of reduced active Stat3 in the hair follicles of K19 *Itgα3* KO mice (**Fig. 2**). However, α3β1 also promotes CCN2 expression in the absence of IL6 stimulation, which in 2D culture of transformed keratinocytes is needed for Stat3 activation (data not shown). Therefore, it is likely that α3β1-mediated expression of CCN2 is not exclusively regulated by α3β1-mediated activation of Stat3.

Despite a strong reduction of tumorigenesis, K14 *Itgα3* KO mice still developed some sporadic, small papillomas of 3 mm in diameter or less [35]. Since the tumor-promoting role of α3β1 is well established and we know that α3β1 is not absent from all keratinocytes in K14 *Itgα3* KO mice (**chapter 3**), it seems likely that these papillomas originated from the cells that have escaped Cre-mediated recombination. During the studies described in this thesis, we mostly worked with short-term DMBA/TPA treated skin of K14 *Itgα3* KO mice and thus did not verify this hypothesis. However, provided it withstands scrutiny, it would offer an additional substantial evidence for the role of α3β1 in the promotion of pro-tumorigenic environment, as it would confirm that α3β1-expressing tumors still depend on the expression of α3β1 by their neighboring keratinocytes in order to grow efficiently.

Role of integrin α3β1 in human non-melanoma skin cancer

Whether our findings in mice would hold true in human patients should be an interesting next step for future research endeavors. Our preliminary data on the expression of α3β1 in biopsies of different stages of the disease looks promising (**Fig. 3**). For the presented pilot experiment, we collected biopsies of five benign non-melanoma lesions (keratoacanthoma and Bowen's disease) and five progressed tumors (invasive squamous cell carcinomas) with matching normal skin and assessed them for the expression of α3β1. 60% of the squamous carcinomas displayed lower levels of α3β1 compared to normal skin. This is in line with findings of the two-stage carcinogenesis model, in which α3β1 integrin is crucial for the initial stages of tumorigenesis as discussed above, but suppresses progression of tumors towards invasive phenotype [35]. The analysis of the expression of α3β1 in biopsies of Bowen's disease also nicely correspond with the role that α3β1 has in early skincarcinogenesis: out of four analyzed samples, 80% had higher α3β1 expression compared to normal skin. Similarly, the only sample of keratoacanthoma that we obtained revealed a much higher level of expression of α3β1 compared to normal skin. Of course, larger sample groups are needed to properly assess the expression levels of α3β1 at different stages of non-melanoma skin cancer in patients. Furthermore, inspecting the benign tumor samples for activation of Akt, Stat3 or FAK, and the expression of CCN2, which we all found to be controlled by α3β1 during two-stage skin carcinogenesis in mice should be of high interest.

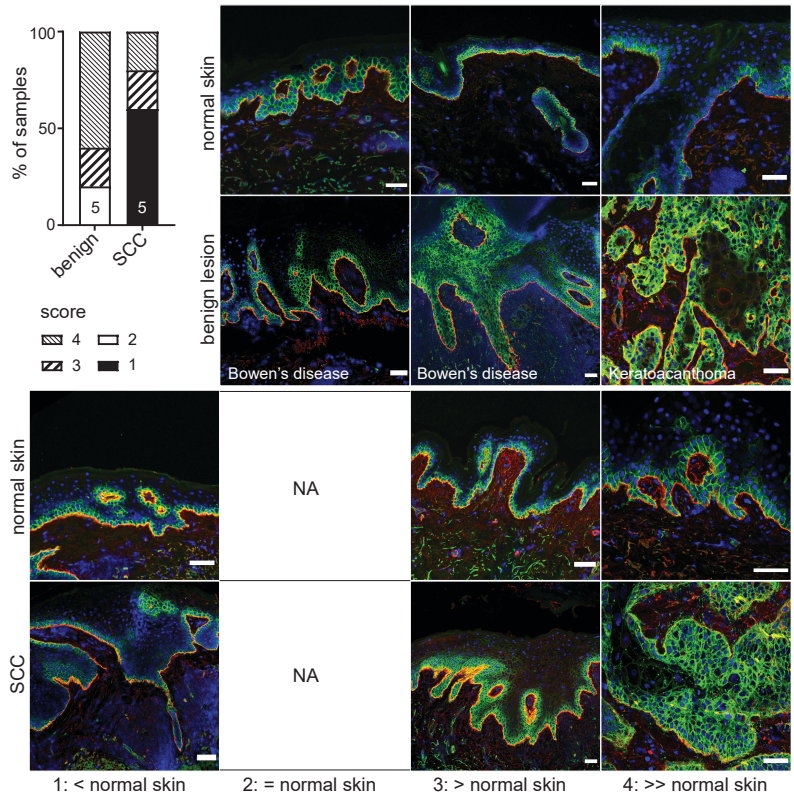

Figure 3: An increased expression of α3β1 in benign, but not progressed human skin lesions. Biopsies of five patients with benign Bowen's disease and keratoacanthoma and five patients with invasive squamous cell carcinomas (SCC) with matching normal skin were stained for integrin α3β1 (Ab: J143) and laminin-332 (Ab: R14) and the surface of α3β1 staining in lesional skin was scored based on its relative expression compared to normal skin (1: less than normal skin, 2: comparable to normal skin, 3: more than normal skin, 4: much more than normal skin). Scale bars: 50 μm.

CONCLUSIONS

The work presented in this thesis focuses on the role of α3β1 in HER2-driven breast carcinogenesis and non-melanoma skin cancer. Presented studies showed that integrin α3β1 can take on diverse roles, depending on the type and stage of cancer. We show that α3β1 can regulate pro-tumorigenic signaling pathways and promote formation of pro-tumorigenic tumor environment during the initiation phase of two-stage skin carcinogenesis, and can promote clustering and adhesion strength of other integrins to suppress invasive potential of HER2-overexpressing mammary carcinoma cells. We hope that demonstrating such complexity will prevent an oversimplistic and generalistic view on the function of integrins in cancer, which is essential for their applicational potential in the treatment of this disease.

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