The role of CD151 and integrin 31 in the pathophysiology of kidney and skin
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Chapter 6

General discussion
Introduction

Even though tetraspanins are small transmembrane proteins that lack soluble ligands, they are of considerable physiological importance for multicellular organisms. Several loss-of-function mutations in tetraspanin genes cause disease in fungi, worms, flies, mice, and humans (Hemler, 2005). Tetraspanins directly associate with other lateral transmembrane proteins (e.g. integrins, EWI proteins, CD19) and cluster them into tetraspanin-enriched microdomains through secondary interactions (Hemler, 2005; Yanez-Mo et al., 2009). The main function of tetraspanins seems to be receptor compartmentalization on the plasma membrane (Yanez-Mo et al., 2009). CD151 with its strong affinity for integrins is certainly no exception (Berditchevski, 2001; Yanez-Mo et al., 2009). However, CD151 and the integrin α3β1, which form a stable complex, are co-expressed in certain epithelia of kidney and skin (Sterk, 2003). This complex stands out as being particularly strong and of high stoichiometry (Yauch et al., 1998; Yauch et al., 2000). In this thesis we investigated the functional relevance of both CD151 and integrin α3β1 in specifically these organs using the tools of mouse genetics and cell biology.

(Podocyte-specific) knockout mice for Itga3 and Cd151

The integrin α3β1 has long been suggested to be the major link between podocytes and the GBM, which forms an integral part of the glomerular filtration barrier (chapter 1). Since Itga3 knockout mice soon after birth (Kreidberg et al., 1996) it has been difficult to assess the true significance of α3β1 on podocyte adhesion and glomerular filtration. In chapter 2, we describe the generation of podocyte-specific Itga3-knockout mice which display severe proteinuria due to foot process effacement and GBM abnormalities. We furthermore generated total as well as podocyte-specific Cd151-knockout mice that display a progressive kidney disease similar to (but less severe than) that of podocyte-specific Itga3-knockout mice (chapters 2 and 3). To test whether Cd151 and Itga3 genetically interact we generated compound heterozygote mice (Cd151⁺⁻; Itga3⁶⁶; podocin-Cre⁺). Although these mice are seemingly healthy during much of their adulthood (chapter 2), a fair percentage (3/9) develops albuminuria upon aging (Figure 1). Although promising, these results are preliminary at best due to the heterogeneous genetic background of these mice (FVB/N; C57Bl/6, see below) and lack of several control groups.

Figure 1 Glomerular phenotype of compound heterozygote mice.

Hematoxylin and eosin stained kidney sections of 70 week old mice heterozygous for Cd151 (top) or heterozygous for Cd151 and Itga3 in podocytes (bottom). The compound heterozygous kidney displays glomerulosclerosis (Δ) and proteinaceous casts within tubuli (*) indicative of severe proteinuria whereas none of these abnormalities is observed in the age matched control littermate. Scale bars equal 100 μm (left) and 50 μm (right).
Influences of the genetic background on Cd151-knockout induced nephropathy

Several groups decided to generate mice with a homozygous deletion of Cd151 in order to study its function in vivo. The group of Leonie Ashman at the University of Newcastle, Australia published the first Cd151-knockout mouse strain in 2004 which were essentially healthy (Wright et al., 2004). The only reported defects were increased bleeding, poor keratinocyte outgrowth from explant cultures, and hyperproliferation of T-cells in response to mitogenic stimuli. The unexpected lack of a major abnormal phenotype in e.g. kidney and skin was explained by a possible compensation by the closely related Tspan11 (originally BAB22942). However, in the same month Karamatic Crew et al. described three patients suffering from hereditary nephritis, pretibial epidermolysis bullosa, sensorineural deafness, and β-thalassemia minor caused by homozygous single nucleotide insertions (p.Gly128Aspfs*12) in CD151 (Karamatic Crew et al., 2004). The mutation could theoretically be dominant-negative for compensating tetraspanins since the resulting truncated protein lacks its integrin-binding domain. However, in 2006 we published the independent generation of a second Cd151-knockout mouse strain which suffered from similar kidney malfunctions as human patients confirming the importance of CD151 for kidney homeostasis (Sachs et al., 2006). A third strain of Cd151-knockout mice generated by the group of Martin Hemler at the Dana-Farber Cancer Institute in Boston was healthy apart from few vascular dysfunctions (Takeda et al., 2007). The lack of renal abnormalities in these and previously published Cd151 knockout mice could be explained by the different targeting strategies (deletion of exons 2-7Ash, 2-4Son, or 3-6Hem) or mouse strains used (C57BL/6Ash, 129/ OlaxFVB/NSon, or C57BL/6JHem). Two papers by Ashman’s and our group jointly proved the latter: whereas Cd151−/− (FVB/N) mice developed kidney failure under normal conditions, Cd151−/+ (C57BL/6) mice did not (Baleato et al., 2008; Sachs et al., 2012). The observation that kidney diseases in mouse models are influenced by their respective genetic backgrounds is not without precedent. For example, Alport mice develop renal failure sooner on a 129/Sv background than on C57BL/6 (Andrews et al., 2002) and leptin-receptor deficient mice develop diabetic nephropathy on the FVB/NJ background, but not on C57BL/6J (Chua S Jr et al., 2010). Cd151-knockout mice represent a unique mouse model, because kidney abnormalities can be induced by experimental hypertension on the C57BL/6J background (Sachs et al., 2012). Specific gene products that protect these mice from nephropathy under physiological conditions may thus decrease mechanical stress experienced by podocytes. Since mechanical stress may be a trigger for nephropathy in susceptible humans as well (Freedman and Sedor, 2008), finding these modifying genes could potentially lead to the identification of orthologs influencing this group of kidney diseases in men. Ashman and colleagues generated Cd151−/− mice on the F1 hybrid (C57BL/6 x FVB/N) background which did not show signs of proteinuria up to 6 months of age indicating that recessive traits are responsible for the renal pathology (data mentioned but not shown in Baleato et al., 2008). We confirmed these observations for our F1 hybrids up to 1 year of age. We furthermore intercrossed the F1 generation to identify the percentage of susceptible mice and performed linkage analysis to identify loci segregating with kidney failure. Roughly 12% of the F2 generation developed the same disease as Cd151-knockout mice on the FVB/N background. As shown in Figure 2, quantitative analysis of albuminuria suggests a bimodal frequency distribution.
However, a genome-wide scan for co-segregating haplotypes was not powerful enough to yield statistically significant results. We therefore backcrossed the F1 progeny to the parental FVB background and are currently phenotyping their offspring. Even though preliminary results show an increased ratio of sick vs. healthy mice as expected, at this point a multifactorial determination of the disease cannot be excluded.

**Treating C1d51-knockout induced nephropathy**

C1d51-knockout mice on the FVB/N background develop progressive nephropathy inevitably causing end-stage renal disease. Since we hypothesized the molecular cause of the disease to be a reduction in podocyte adhesion to the GBM (see below), we aimed at pharmacologically reducing mechanical stress imposed onto podocytes (chapter 3). When treated with enalapril, which as an angiotensin-converting enzyme inhibitor lowers systemic as well as intraglomerular blood pressure (Dworkin et al., 1993), C1d51 knockout mice survived significantly longer than untreated control mice. When treating these mice with verapamil instead, which as a calcium-channel inhibitor decreases systemic but not intraglomerular blood pressure (Dworkin et al., 1993; Harris et al., 1987), we did not detect amelioration of the disease. We consequently propose that lowering intraglomerular blood pressure is beneficial for the C1d51-knockout mice (chapter 3). There are, however, several caveats: Firstly, we did not measure intraglomerular blood pressure in the treated cohorts due to technical limitations. Secondly, we ignored direct possible effects of angiotensin converting enzyme inhibitors on podocytes. Evidence for direct effects of the angiotensin on podocytes comes from transgenic rats that develop glomerulosclerosis upon overexpression of the angiotensin II type 1 receptor specifically in podocytes (Hoffmann et al., 2004). Lastly, we treated mice from birth onwards which affects not only renal hemodynamics but may also cause abnormal kidney morphology and function (Guron, 2005). In the attempt to overcome at least the latter limitation, we fed C1d51-knockout mice from six weeks of age 20 mg enalapril per liter drinking water and again found survival to be significantly increased as compared to untreated littermates (Figure 3).

**Figure 2 Frequency distribution of albuminuria in a cohort of 134 C1d51-/- mice on a mixed C57BI/6 x FVB/N background.**

Mice were assigned to 21 groups according to their albuminuria values over 24 h. Upper group boundaries were defined as \( f(x) \). Continuous probability distributions of these groups were calculated as \( f(x) \) for all mice (n=134; \( \mu=7.5; \sigma=3.78 \)), ‘healthy’ mice (n=118; \( \mu=6.4; \sigma=2.35 \)), and ‘sick’ mice (n=16; \( \mu=15.6; \sigma=1.86 \)). The data is best described by a bimodal distribution using the means of ‘healthy’ and ‘sick’.
An alternative treatment regimen, independent of reducing intraglomerular pressure, may be to target Rho-associate kinases (ROCKs) since they are activated upon hypertensive tissue damage and their pharmacological inhibition has proven beneficial for a variety of nephropathies (Wakino et al., 2005). In addition, CD151 has been shown to influence RhoA activation (Johnson et al., 2009) and ROCK inhibition rescued mechanically induced cell-cell junction defects of \textit{Cd151} deficient podocytes \textit{in vitro} (see below).

**Mechanism underlying \textit{Cd151}-knockout induced nephropathy**

We find CD151 and integrin \(\alpha 3\beta 1\) to interact at the base of podocyte foot processes which contact the glomerular basement membrane and form an integral part of the glomerular filtration barrier (\textit{chapter 3}). Using genetically modified podocytes we show \textit{in vitro} that CD151 strengthens \(\alpha 3\beta 1\)-mediated adhesion on laminin confirming binding assays of liposomes reconstituted with \(\alpha 3\beta 1\) with or without CD151 (Nishiuchi et al., 2005). We furthermore show that CD151 modifies the lateral distribution of \(\alpha 3\beta 1\) on the basal membrane and suggest that the resulting absence of \(\alpha 3\beta 1\) from focal adhesions is responsible for adhesion strengthening through a change in avidity (\textit{chapter 3}). We base our suggestions on the fact that CD151 is beneficial for podocyte adhesion with disrupted cell-cell-junctions, a depolymerized actin cytoskeleton, blocked \(\alpha 3\beta 1/\alpha 6\beta 4\) integrins as well as fully activated integrins. At this point however, the relationship between \(\alpha 3\beta 1\)-distribution and adhesion strengthening is merely correlative. To prove a causal relationship one could theoretically construct a model to show whether a certain distribution of \(\alpha 3\beta 1\) is favorable or experimentally manipulate \(\alpha 3\beta 1\) molecules to adopt a certain distribution on nanopatterned substrates (Huang et al., 2009).

Importantly, CD151 has been implicated not only in cell-matrix, but also cell-cell adhesions (Chattopadhyay et al., 2003; Johnson et al., 2009; Zhang et al., 2011). Given the delicate structure of the podocyte slit-diaphragm and its central role during glomerular ultrafiltration, weakening of this specialized cell-cell junction in the absence of \textit{Cd151} might very well...
contribute to disease progression. Although normal podocytes in vitro formed normal cell-cell junctions independent of the presence of Cd151 (chapter 3), mechanical stress, which did not cause cell detachment caused partial rupture of cell-cell junctions (Figure 4). Inhibition of Rho associated kinases or non-muscle myosin II rescued the defects indicating that disconnection of cell-cell junction requires active contractility. Further experimentation in podocytes ± Cd151 should aim at quantifying RhoA-activation following mechanical stimuli as well as determining cell-cell adhesion strength.

Figure 4 CD151 strengthens cell-cell junctions in glomerular epithelial cells (GECs).
(A) Cell-cell junctions of podocytes lacking Cd151 (GEC-) disconnect after 5 min exposure to moderate shear stress (200 dyne/cm²), whereas cell-cell junctions of wild-type parental cells (GEC+) are barely affected as judged by apical ZO-1 stainings. (B) Pretreating the cells for 30 min with 50 μM blebbistatin or 50 μM Y-27632 rescues the defect.

CD151 and integrin α3β1 in skin tumorigenesis
CD151 as well as integrin α3β1 are strongly expressed in basal keratinocytes of the skin (Sincock et al., 1997). However genetic deletion of Cd151 or Itga3, respectively, causes no obvious (chapter 2) or only minor abnormalities in the homeostatic skin (Margadant et al., 2009). Since expression of CD151 as well as ITGA3 is altered during the progression of epidermal neoplasms (Li et al., 2012; Suzuki et al., 2011; Van Waes et al., 1995) we investigated whether either protein plays a causal role in the development of skin tumors. The results are described in chapters 4 and 5. We induced DMBA-mediated skin carcinogenesis in epidermis-specific Cd151 or Itga3 knockout mice and found tumor numbers and size to be significantly reduced. Mechanistically, loss of integrin α3β1 increases keratinocyte migration and epidermal turnover leading to a loss of long-lived label retaining cells (LRCs) which fuel the constant keratinocyte flow. Since LRCs are thought to be the primary source of chemically induced skin tumors (Morris et al., 1985; Morris et al., 1986; Stenback et al., 1981), we suggest their continuous loss to be responsible for
the decreased number of tumors (chapter 4). We do not, however, provide direct evidence for a loss of DMBA-initiated cells through increased epidermal turnover. To do so, one could apply tritium-labeled DMBA to the skin of wild-type and epidermis-specific Itga3-knockout mice and compare disappearance rates of radioactive DMBA-DNA-adducts from several epidermal compartments using chromatography (DiGiovanni et al., 1986). Antibodies against DMBA-DNA adducts would be extremely useful to detect initiated cells in the Itga3-null epidermis using immunohistochemistry. We observe a similar phenotype in mice lacking epidermal Cd151 (chapter 5). Tumor numbers as well as size following DMBA/TPA carcinogenesis are decreased in the absence of Cd151 and drop further when one Itga3 allele is also absent. However, in contrast to Itga3, the influence of Cd151 on tumorigenesis is dependent on TPA since an equal number of tumors are formed in epidermis-specific Cd151-knockout and wild-type mice following DMBA only carcinogenesis. Furthermore, only upon TPA treatment is turnover of the Cd151-null epidermis significantly faster than that in the wild-type epidermis. Surprisingly, CD151 positively influences proliferation of untransformed keratinocytes independent of integrin association. It would be of interest to identify which proteins CD151 interacts with when it not bound to an integrin.

Conclusions

We show that CD151 and the integrin α3β1 form a physiologically relevant complex in the epithelia of kidney and skin. Our studies highlight the importance of cell adhesion to the extracellular matrix and modifications thereof. Future studies will hopefully be aimed at manipulating cell-matrix adhesion or mechanical stress in order to ameliorate disease.

References


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