Chapter 11
Tetraspanins in Cancer

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Abstract Tetraspanins play important roles in cancer, especially in metastasis. CD82 and CD9 are frequently down-regulated on progression of epithelial cancers in humans and this has been associated with poor prognosis. In contrast, high levels of CD151 and Tspan 8 are often observed on tumour progression and have also been linked to poor patient outcome. These observations are supported by a large body of evidence from studies in vitro and in animal models. Considerable insights into the mechanisms by which tetraspanins influence tumour behaviour are now emerging. These include effects on cell-matrix and cell-cell interactions which influence migration and invasion of surrounding tissues, as well as angiogenesis. Several tetraspanins influence the function of platelets which can promote metastasis. Tetraspanins are constitutive components of exosomes, which are most important in intercellular communication. This widens the range of tetraspanin activities in physiology and pathology and may well be particularly important during spread and settlement of metastasizing tumor cells. There is hope that the understanding of how tetraspanins contribute to tumour progression indicates novel approaches to therapy.

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11.1 Introduction

Several tetraspanins have been defined as markers of human cancer cells. For example, ME491/CD63, a "founder member" of the tetraspanin family (Wright and Tomlinson 1994) was identified as a melanoma-associated antigen (Hotta et al. 1988). CO-029, a monoclonal antibody that recognised a tumour-associated antigen expressed by gastrointestinal tumours, identified the tetraspanin now known as Tspan8 (Szala et al. 1990). MRP-1 (CD9) was identified as the target of an antibody inhibiting cell migration and the cDNA was subsequently cloned from a breast cancer cell line (Miyake et al. 1991). KAI1, which was isolated as a metastasis suppressor gene located on human chromosome 11p11.2 (Dong et al. 1995), was shown to be identical to the leukocyte antigen, CD82. SAS/Tspan31 was identified as a gene that is amplified in human sarcomas (Jankowski et al. 1995). The target of a monoclonal antibody that suppressed metastasis of a human epidermoid tumour cell line in a chick embryo model was shown to be the CD151 protein (Testa et al. 1999).

Multiple studies have demonstrated the prognostic significance of mRNA or protein expression levels of several of these tetraspanins in human cancers. In general, they affect metastasis rather than primary tumour growth. Paradoxically, while some (notably CD82 and CD9) function as tumour suppressors, others (CD151 and Tspan8) appear to promote metastasis. In this article, we review evidence for their involvement in cancer from clinical studies and animal models, how their expression levels are regulated in cancer cells and how they function to modify cancer cell behaviour. Finally, we discuss tetraspanins as targets for therapeutic intervention.

11.2 Evidence for Altered Expression of Tetraspanin Proteins in Human Cancer and Its Prognostic Significance

11.2.1 CD82

CD82 (Tspan 27; also known as KAI1) is the most clear-cut example of a tetraspanin with altered expression in cancer. Following on from its original characterisation as a tumour suppressor in prostate cancer (Dong et al. 1995, 1996), many studies have been conducted linking CD82 down-regulation at the RNA or protein level with invasive and metastatic potential and/or patient outcome in a variety of epithelial cancers. CD82 is widely expressed in human tissues and reduced levels in tumours as well as an inverse relationship between CD82 expression and invasive or metastatic potential have been reported in many solid tumours including prostate, breast, cervix, gastric, colon, lung, pancreatic, liver, skin and thyroid cancers. These data have been reviewed elsewhere (Liu and Zhang 2006; Miranti 2009).

While some studies reported early and progressive down-regulation during tumorigenesis and metastasis, for example in colorectal cancer (Lombardi et al. 1999), other investigators found a biphasic pattern in colorectal and prostate cancers with increased

CD82 expression in low grade tumours, progressively decreasing with tumour stage or grade (Bouras and Frauman 1999; Maurer et al. 1999). In almost all studies, CD82 has been found to be down-regulated or lost in metastases. However, in breast cancer, CD82 expression was dependent on oestrogen receptor (ER) status. In two series of breast cancer patients, down-regulation of CD82 with respect to normal breast epithelium was found in 76–77% of ER-positive tumour specimens. Notably, ER-negative specimens retained CD82 even in metastases (Huang et al. 2005; Christgen et al. 2008, 2009). These results were surprising in view of the association of ER-negative status with metastasis and poor outcome (Weigelt et al. 2005).

Tonoli and Barrett reviewed 64 studies of CD82 expression in cancer conducted prior to 2005 (Tonoli and Barrett 2005). Of these, 52 studies (83%) reported CD82 down regulation including 12/16 gastrointestinal tract, 6/8 prostate, 7/8 non-small cell lung cancer (NSCLC) and 4/5 pancreatic cancer series. Ten studies (16%), scattered across a range of cancer types, showed upregulation of CD82. Prognostic data (statistically significant differences in survival or the development of metastases) were available from 33 of these studies. CD82 expression indicated favourable prognosis in 28 reports including 7/8 gastrointestinal tract series, 5/5 NSCLC, 2/3 oral carcinoma, 3/4 pancreatic carcinoma and 3/3 prostate cancer studies. In bladder and breast cancers CD82 down-regulation was associated with recurrence after treatment (Huang et al. 1998; Su et al. 2004). Thus, there is strong evidence linking loss of CD82 with tumour progression and poor outcome in many types of cancer.

11.2.2 CD9

CD9 (Tspan 29; also known as MRP-1) is also widely expressed in tissues and, like CD82, a large number of studies have examined the changes in CD9 mRNA and/or protein levels in cancer and its relationship to patient prognosis. These have recently been reviewed (Zöller 2009). CD9 levels were reported as being down-regulated relative to the corresponding normal tissue in breast (two series) and lung cancer, but not in ovarian or gastric cancer. Of the studies reviewed, the presence of CD9 was a positive prognostic factor in lung cancer (4/5 studies), breast cancer (2/3 studies), head and neck cancer (2/3 studies), bladder, and uterus (each one study). In general, in these cancers the extent of CD9 down-regulation was related to tumour grade and/or stage. In prostate cancer, CD9 was down-regulated in a proportion of specimens at all stages of progression with a further significant decrease between localised and advanced disease (Wang et al. 2007a). In contrast to the foregoing cancers, CD9 expression appears to be associated with progression and poor prognosis in gastric cancer (Hori et al. 2004; Soyuer et al. 2010). Furthermore, expression of CD9 in small cell lung cancer specimens and cell lines was associated with chemoresistance. Targeting of CD9 by siRNA or a monoclonal antibody induced apoptosis of chemoresistant cell lines (Kohmo et al. 2010).

An early report linked reduced CD9 expression to attributes associated with metastasis in melanoma specimens (Si and Hersey 1993). More recently, CD9 was identified as a gene expressed at lower levels in each of three pairs of metastatic melanomas compared with the corresponding normal melanocytes (Mischiati et al.

2006). This group also examined a series of specimens representing the stages of melanoma development, CD9 was expressed in all (18/18) naevi, and was lost in most (20/28) melanomas (including radial growth phase lesions which have relatively good prognosis) but only 24/52 metastatic lesions. Fan and co-workers studied CD9 expression and function in six human melanoma cell lines. All six lines displayed reduced CD9 mRNA and protein levels relative to normal melanocytes, however transfection of a line derived from a radial growth phase lesion enhanced invasion through Matrigel (Fan et al. 2010). These authors note that blocking CD9 enhances motility of melanocytes (Garcia-Lopez et al. 2005) and suggest that CD9 may play different roles at different stages of melanoma progression. Specifically, down-regulation of CD9 may facilitate early stages of melanoma development, but subsequent re-expression may promote invasion and metastasis. In view of other evidence indicating a role for CD9 in trans-endothelial invasion during metastasis of multiple myeloma (De Bruyne et al. 2006) and cervical cancer (Sauer et al. 2003), this tetraspanin cannot be considered simply as a tumour suppressor, but rather, may have different functions in different tumours and stages of tumour development.

11.2.3 CD151

The initial reports of the metastasis-promoting action of CD151 (Tspan24) in an in vivo model (Testa et al. 1999) were followed by clinical studies in lung, colon and prostate cancers (Tokuhara et al. 2001; Hashida et al. 2003; Ang et al 2004) which showed that high level expression of CD151 in primary tumours was associated with poor prognosis. Tokuhara et al. studied expression of CD9, CD82 and CD151 in specimens from 145 patients with NSCLC by semi-quantitative PCR and immunohistochemistry (IHC). High level CD151 expression was not correlated with tumour size, lymph node status, histological subtype or grade, but in contrast to CD9 and CD82, it was strongly associated with poor survival (Tokuhara et al. 2001). This group also analysed expression of these three tetraspanins in 146 cases of colon cancer with similar results to the NSCLC study (Hashida et al. 2003). In a series of 76 primary prostate cancer and 30 benign prostate hyperplasia (BPH) specimens studied by quantitative IHC, Ang et al. found significantly elevated CD151 expression in prostate cancer relative to BPH (Ang et al. 2004). CD151 levels were related to histologic differentiation status with the highest levels in poorly differentiated tumours. Increased expression of CD151 was strongly associated with overall survival, especially in patients with well- or moderately-differentiated tumours, and was a better predictor of outcome than the Gleason grade.

In the earlier study of primary colon cancer, CD151 levels were compared across tumour specimens of different grade and stage, but were not compared with normal colonic tissue (Hashida et al. 2003). A recent report indicated that CD151 protein levels were reduced in colon cancers relative to the adjacent normal tissue in 137 paired specimens (Chien et al. 2008). Using colon cancer cell lines, this group showed that under hypoxic conditions, CD151 expression was repressed due to binding of hypoxia-inducible factor-1 (HIF-1) to the CD151 promoter. This resulted

in detachment of the cells. They propose that hypoxia induced CD151 down-regulation and detachment might play an important role in metastasis of colon cancer.

CD151 has only recently been studied in breast cancer. In normal breast, CD151 expression is largely confined to the myoepithelial-basement membrane interface in both ducts and lobules (Yang et al. 2008; Novitskaya et al. 2010). In a series of 124 unselected breast cancers, CD151 was found by IHC to be elevated relative to normal breast tissue in 31% of patients and high CD151 expression was positively correlated with tumour grade, ER-negativity and basal-like features. No outcome data were available (Yang et al. 2008). CD151 expression levels have been determined, also by IHC, in two further patient series and have been linked to patient survival in one of these. (Sadej et al. 2009) studied 56 specimens of primary invasive ductal carcinoma. Of these, 30% were scored as having elevated CD151 levels, and this was associated with poor overall survival (estimated 5-year survival 45.8% compared with 79.9% for CD151 low/negative cases). In contrast with the study of (Yang et al. 2008), no correlation was found between CD151 expression and tumour grade or ER status. In a second series, this group studied CD151 levels in 87 specimens of ductal carcinoma in situ (DCIS), including 48 with associated invasive disease (Novitskaya et al. 2010). In this study, elevated CD151 expression was associated with high tumour grade. In related experiments in a xenograft model and in Matrigel cultures, CD151 was shown to promote proliferation of the poorly tumorigenic HB2 breast cell line implying that it acts at the level of the primary tumour, not just to promote invasion and metastasis. Clearly, high expression of CD151 is not restricted to basal cancers and it appears that, in some cases, luminal epithelial cells that normally express little or no CD151 strongly upregulate this protein.

A recent study of specimens from 520 patients with hepatocellular carcinoma (HCC) using IHC (Ke et al. 2009) found that over-expression of CD151 relative to normal hepatocytes was a significant, independent predictor of recurrence and overall survival. High level CD151 expression was correlated with vascular invasion, tumour staging, size and differentiation. The prognostic significance was enhanced by also taking into account expression of the receptor tyrosine kinase c-Met, which was previously shown to form complexes with CD151 (Klosek et al. 2005). In an extension of this work, (Shi et al. 2010) showed that high level expression of CD151 and matrix metalloprotease 9 (MMP9) in tumour tissues was associated with increased microvessel density and together these features strongly predicted poor outcome. CD151 on tumour cells may activate MMP9 (Hong et al. 2006) which in turn may trigger an "angiogenic switch" (Bergers et al. 2000). In the experiments of (Sadej et al. 2009), siRNA down-regulation of CD151 in breast cancer cells resulted in reduced angiogenesis when the cells were grown as xenografts in mice. While these studies demonstrate a role for tumour cell CD151 in promoting neoangiogenesis, CD151 on vascular endothelial cells may also be important. CD151 is known to promote angiogenesis in vitro (Sincock et al. 1999), in animal models of ischemia (Zheng and Liu 2006) and in transplanted tumours growing in CD151-knockout mice (Takeda et al. 2007b). Taken together, these studies indicate that CD151 is a potentially important target for inhibiting tumour angiogenesis.

CD151 expression, determined by IHC, has also been linked to malignant transformation and/or prognosis in other tumour types. Increased CD151 protein relative

to normal tissue was demonstrated in 30 cases of pancreatic cancer largely independent of grade or stage (Gesierich et al. 2005). More recently, a study of 71 patients with pancreatic ductal carcinoma confirmed overexpression of CD151 relative to normal pancreatic tissue and demonstrated association with elevated c-Met levels, tumour stage and poor survival. CD151 and c-Met were independent prognostic factors (Zhu et al. 2010). High level expression of CD151 was associated with tumour stage and poor survival in a series of 489 cases of clear cell renal carcinoma and was an independent prognostic indicator (Yoo et al. 2011). Similarly elevated CD151 expression was found in intrahepatic cholangiocarcinoma (60 patients) and esophageal squamous cell carcinoma (138 patients). In both of these series high CD151 was associated with tumour stage and predicted poor survival (Huang et al. 2010; Suzuki et al. 2011).

Fewer studies have examined *CD151* mRNA in clinical specimens. A gene expression microarray analysis of 50 brain tumours revealed that, together with other potential mediators of invasion such as integrin α3, CD151 was over-expressed in glioblastomas relative to normal brain (Bredel et al. 2005). In a series of 73 cases of gingival squamous carcinoma, expression of tetraspanins CD9, CD63, CD81, CD82, CD151 and NAG-2 (Tspan4) was studied by Q-PCR. Only CD151 and CD9 were significant prognostic factors, with high CD151 being associated with poor survival, while low CD9 was significantly linked to the presence of lymph node metastases (Hirano et al. 2009).

11.2.4 Tspan8

There are fewer studies of Tspan8 (also known as CO-029, TM4SF3 and D6.1A) than CD82, CD9 or CD151 but most available data indicate that it acts as a promoter of tumour progression. Tspan8 was identified as a marker of gastrointestinal tumours (Szala et al. 1990). Differential display mRNA analysis revealed its overexpression in hepatocellular carcinoma relative to normal liver. Using IHC it was shown that the protein was particularly over-expressed in poorly differentiated tumours, especially those showing intrahepatic spread (Kanetaka et al. 2001). A subsequent study in which a human HCC cell line, transfected to overexpress Tspan8, was orthotopically transplanted into immunocompromised mice revealed no change in primary tumour growth relative to the parent line, but an acquired ability to form intrahepatic metastases (Kanetaka et al. 2003).

Tspan8 was found by IHC to be more highly expressed in 24/30 cases of pancreatic cancer compared with normal pancreas, although ducts in chronic pancreatitis also displayed elevated levels. The intensity of staining was largely independent of tumour grade and stage (Gesierich et al. 2005).

Kuhn and coworkers examined expression of Tspan8 in a series of 104 primary colorectal cancers and 66 liver metastases together with normal colon and liver. IHC staining of normal colon was negative or weak with clear upregulation in the majority of primary lesions and metastases. Staining was not related to tumour

stage or grading. Co-expression and complex formation of Tspan8 with claudin, EpCAM and CD44v6 was inversely correlated with disease-free survival and it is proposed that this complex promotes metastasis (Kuhn et al. 2007). Using cell lines derived from primary colorectal cancer and metastases from the same patient, LeNaour et al. (2006a) used proteomic methods to characterise tetraspanin-containing complexes. They found that Tspan8 was strikingly upregulated in the metastatic cell lines. This was followed up by IHC examination of Tspan8 protein levels in matched normal colon, primary tumour and metastases from three patients. In contrast to the findings of Kuhn et al., they reported high expression in normal colon (which was confirmed by western blot), with low expression on both primary tumours and, surprisingly, metastases. The reason for the discrepancy between the two groups in relation to Tspan8 expression in normal colon is not clear although they used different antibodies. In a subsequent study (Greco et al. 2010), Tspan8 expression was examined by IHC using a novel, well validated monoclonal antibody, TS29, in specimens of primary colonic tumours from 52 patients. Intensity of Tspan8 staining was compared between tumorous and adjacent non-tumorous epithelium. Elevated Tspan8 was found to be significantly associated with relapse, especially when combined with cytoplasmic relocalisation of p120 catenin (resulting from E-cadherin down-regulation). This group also demonstrated in vitro that Tspan8 promotes cell migration when E-cadherin is down-regulated, as occurs in aggressive cancers, and propose that it is a potentially important therapeutic target (Greco et al. 2010).

Examination of publicly available gene expression datasets for oesophageal carcinoma indicated upregulation of Tspan8 relative to normal tissue. This was confirmed in 8/14 pairs of normal and cancerous oesophageal specimens by western blotting (Zhou et al. 2008). Transfection of an oesophageal carcinoma cell line with Tspan8 cDNA resulted in acquisition of metastatic ability in a mouse xenograft model.

Overall, these data together with studies of the D6.1A rat tumour model (detailed elsewhere in this Chapter) provide strong evidence for the tumour-promoting, pro-metastatic action of Tspan8.

11.2.5 Other Tetraspanins Implicated in Cancer

11.2.5.1 CD63

Although CD63 (Tspan30) was originally identified as a melanoma antigen (ME491) and has been suggested to be a tumour suppressor, there is a lack of strong evidence from clinical studies to support this. Like the other tetraspanins described above, CD63 is very widely expressed by normal and tumour cells (Pols and Klumperman 2009). In the original reports, monoclonal antibody ME491 was positive on 7/10 melanoma cell lines, 4/4 superficial spreading melanomas and 5/8 melanomas with associated metastases. It was weak or negative on normal melanocytes,

but upregulated in culture (Atkinson et al. 1984; Hotta et al. 1988). However, a more recent study did not support the view that CD63 down-regulation is associated with melanoma progression. Specimens from patients (four benign naevi, two primary tumours and 28 metastatic lesions) were analysed by Q-PCR and CD63 was found to be upregulated in melanoma relative to benign lesions (Lewis et al. 2005). Most evidence for a tumour-suppressive function for CD63, especially in melanoma, comes from studies with cell lines (detailed in Sect. 11.4). Some early publications reporting effects of ectopic expression of CD63 in human melanoma (Radford et al. 1997) were compromised by the subsequent demonstration that the cell line used in the study was in fact of rat origin (Moseley et al. 2003).

Two clinical studies in other cancers have provided some support for a tumour-suppressive function of CD63. One series of 90 lung cancer (NSCLC) patients showed down-regulation of CD63 relative to normal tissue in tumours, especially of those of squamous type, and association with tumour stage. In adenocarcinomas, CD63 expression was more variable, but downregulation was associated with poor survival (Kwon et al. 2007). In ovarian cancer, CD63 mRNA levels were shown to be inversely related to tumour grade (Zhijun et al. 2007). However, CD63 mRNA and protein expression were unchanged in series of pancreatic (Sho et al. 1998) and thyroid cancer specimens (Chen et al. 2004) where significant down-regulation of CD82 associated with progression was observed.

11.2.5.2 Tspan1

Although much less studied experimentally than CD151 and Tspan8, there is growing evidence that Tspan1 (also known as NET-1) is also a tumour promoting tetraspanin. At the mRNA level, *NET-1* was over-expressed in cervical neoplasia compared with normal cervical epithelium. It was strongly expressed in all undifferentiated cervical carcinomas examined (Wollscheid et al. 2002). Expression of NET-1 was studied by IHC in a series of 88 patients with colorectal carcinoma (Chen et al. 2009) and 86 cases of gastric carcinoma (Chen et al. 2008). In both series Tspan1 over-expression was correlated with clinical stage and negatively correlated with survival. Consistent with its role as a tumour promoter, knock-down of Tspan1 with siRNA in the squamous cell skin carcinoma cell line, A431, reduced proliferation, migration and infiltration of cells in vitro (Chen et al. 2010).

11.2.5.3 Tspan13

Emerging data indicate that Tspan13 (also known as NET-6) is a tumour suppressor. Gene expression array experiments comparing HER-2 positive and negative breast cancer cells showed that NET-6 levels are related to HER-2 and ER status and are lowest in HER-2-ER-basal-like tumours (Wilson et al. 2002). Transfection of *NET-6* cDNA into MDA-MB-231 breast cancer cells induced apoptosis and reduced growth in vitro and in a mouse xenograft model (Huang et al. 2005, 2007). A recent

study of NET-6 mRNA and protein levels in prostate cancer specimens showed that it is over-expressed in prostatic intraepithelial neoplasia and the majority of prostate cancers compared with normal tissue. However, in tumour specimens, NET-6 protein levels showed a significant inverse correlation with Gleason grade consistent with down-regulation in high-grade tumours (Arencibia et al. 2009). This is consistent with the findings of Huang et al. in breast cancer indicating that it acts as a suppressor of tumour progression. Thus NET-6 expression appears to be regulated in a biphasic fashion similar to CD82 in prostate and colon cancer (Bouras and Frauman 1999; Maurer et al. 1999).

11.3 Regulation of Tetraspanin Levels in Cancer Cells

From the previous section it can be seen that levels of several tetraspanin proteins and/or mRNA are correlated with progression and prognosis in many human epithelial cancers. While some of these changes have been identified from gene expression array analyses (for example, Tspan8 (Zhou et al. 2008) and Tspan13 (Wilson et al. 2002; Arencibia et al. 2009), it is perhaps surprising that more examples have not emerged from the large amounts of these data that have been generated in recent years. Protein expression can be regulated at many levels and it seems likely that tetraspanin proteins are regulated in several ways encompassing translation and protein turnover as well as transcription. Although several reports have examined both mRNA and protein expression, newer quantitative studies directly comparing tetraspanin mRNA and protein levels in cancers are required. These will guide development of the most appropriate assays for clinical application. Apart from CD82, little is known about how tetraspanin transcription is regulated and more studies of other tetraspanins are needed.

11.3.1 CD82

Regulation of CD82 transcription and silencing are complex processes (Gao et al. 2003; Tonoli and Barrett 2005; Liu and Zhang 2006). There is no evidence for gene mutation or loss of heterozygosity (Tagawa et al. 1999; Liu et al. 2000) and hypermethylation of CpG islands in the CD82 gene has only been seen in patients with multiple myeloma, where combined de-methylation and de-acetylation induced increased expression of CD82 mRNA (Jackson et al. 2000; Drucker et al. 2006). CD82 down-regulation has also been related to the p53 status. Binding motifs for the transcription factor AP2 in the CD82 promoter function synergistically with p53 and junB such that the absence of wild-type p53 and/or loss of junB and AP2 protein expression correlate with CD82 mRNA down-regulation (Marreiros et al. 2003, 2005). There have been some controversial results on the involvement of NFκB in CD82 transcription, which is likely due to the nature of the recruited cofactors.

In non-metastatic cells, IL-1β supports the recruitment of a Tip60 (HIV-1 TAT-interactive protein 60)/Fe65-Pontin complex, which acts as a co-activator together with NFκB p50 and accounts for the displacement of the co-repressor N-Cor/TAB2 (TAK1-binding adaptor protein)/HDAC3 (histone deacetylase 3) complex from NFκB p50. In metastasizing tumour cells, Tip60 is down-regulated and a β-catenin-reptin complex replaces the Tip60-Pontin complex and represses NFκB activity (Telese et al. 2005). Recently it was shown that HIF1α binds directly to the CD82 promoter leading to increased CD82 protein in hypoxia (Kim et al. 2010).

Alternate splicing has also been proposed as a possible mechanism for regulation of CD82 expression and function. A splice variant lacking exon 7 which codes for part of the second extracellular loop and the fourth transmembrane domain was identified in gastric carcinomas and reported to confer increased metastatic ability in a mouse model of colon cancer (Lee et al. 2003). However, a more recent study in bladder cancer found uniformly low levels of mRNA encoding the splice variant, which was not associated with tumour invasion (Jackson et al. 2007).

A role for protein degradation in control of tetraspanin levels has recently emerged. The E3 ubiquitin ligase, gp78, was shown to functionally interact with CD82 leading to its degradation. In an orthotopic mouse model, knockdown of gp78 in the human HT1080 sarcoma had no effect on growth of primary tumour but blocked lung metastasis. This was accompanied by upregulation of CD82. In a tissue microarray of primary sarcomas, an inverse relationship between CD82 and gp78 staining was observed. (Tsai et al. 2007). Inverse expression of gp78 and CD82 was also observed in human mammary carcinoma cells. Ectopic expression of gp78 in the murine mammary gland resulted in decreased CD82 expression and hyperplasia but was insufficient for tumourigenesis (Joshi et al. 2010). CD82 stability and function may also be regulated by palmitoylation (Zhou et al. 2004).

11.3.2 CD9

There is considerable evidence that CD9 expression is regulated epigenetically. While promoter methylation has been reported as a major mechanism in multiple myeloma (Drucker et al. 2006), other reports have indicated that histone acetylation is more important. In another study of multiple myeloma, CD9 levels were inversely correlated with disease activity, with increased CD9 in patients with inactive disease. High CD9 at diagnosis was associated with increased survival. CD9 expression was regulated primarily by histone acetylation (De Bruyne et al. 2008). CD9 expression was also reported to be regulated by histone acetylation in lung cancer (Zhong et al. 2007), melanoma cell lines (Fan et al. 2010) and B lymphomas (Yoon et al. 2010).

One study of CD9 in prostate cancer (Wang et al. 2007a) found point mutations and/or deletions in cDNA from four adenocarcinomas, one case of prostate intraepithelial neoplasia (PIN), and two prostate cancer cell lines. They suggest that

down-regulation of CD9 may result from these mutations. No CD9 mutations were found in cDNA from six normal prostate specimens. However, the generality of these results is uncertain. No mutations were found in CD9 cDNA from six human melanoma lines which had lower levels of CD9 protein than normal melanocytes (Fan et al. 2010).

Recent evidence indicates a role for post-transcriptional regulation of CD9 protein expression. Analysis of the 5'UTR of CD9 cDNA in Merkel cell carcinoma demonstrated two splice variants, the longer of which contained a putative structural pattern that would block translation. There was a shift in favour of this variant in CD9-negative cells suggesting that it may influence CD9 protein expression (Woegerbauer et al. 2010). CUGBP1 is a RNA binding protein that regulates alternate splicing, mRNA stability and translation by binding to the 3'UTR. CUGBP1 binds directly to CD9 mRNA resulting in decreased levels (Le Tonqueze et al. 2010). Another RNA binding protein, HuR, acts by binding to AU-rich sequences in mRNA resulting in stabilisation and enhancement of translation. HuR has been suggested to promote tumour progression, including in breast cancer (Heinonen et al. 2005; Lopez de Silanes et al. 2005). Through co-immunoprecipitation analysis on MCF7 and MDA-MB-231 breast cancer lines, HuR was found to bind CD9 mRNA (Calaluce et al. 2010). Surprisingly, HuR over-expression and knock-down experiments indicated that it decreased CD9 mRNA and protein in MDA-MB-231 cells, but slightly increased their levels in MCF7 cells. Thus, the consequence of HuR binding to CD9 mRNA depends on the cellular context.

CD9 levels may also be regulated post-translationally. CD9 palmitoylation, mediated by the enzyme DHHC2, was shown to protect it from proteasomal and lysosomal degradation (Sharma et al. 2008).

11.3.3 CD151

Recent reports have provided some information about the regulation of *CD151* transcription. The SP1 transcription factor was shown to be required for accessibility and function of the *CD151* promoter (Wang et al. 2010). Elevated SP1 is commonly observed in cancer, particularly in advanced disease, and may regulate expression of a number of genes associated with cancer progression (Safe and Abdelrahim 2005) likely including *CD151*. The *CD151* promoter also binds the hypoxia-inducible factor, HIF-1α, leading to down-regulation of CD151 mRNA and protein levels under hypoxic conditions. (Chien et al. 2008). The authors propose a role for reversible CD151 modulation in metastasis.

It is likely that CD151 protein levels are also regulated post-translationally. The membrane-spanning ubiquitin E3 ligase, GRAIL, binds to CD151 and ubiquitylates its N-terminal cytoplasmic domain promoting its removal from the cell surface and lysosomal degradation (Lineberry et al. 2008). Like CD9, CD151 is palmitoylated by DHHC2 blocking its proteasomal and lysosomal degradation (Sharma et al. 2008).

11.4 Tetraspanins and Metastasis

Metastasis formation is the final result of a cascade of events that primary tumour cells pass through by changing their phenotype and their cross-talk with the tumour environment. In epithelial tumours the metastatic cascade may be initiated through a process called epithelial to mesenchymal transition (EMT) of cancer stem cells/cancer initiating cells (Brabletz et al. 2005; Yang and Weinberg 2008), followed by migration from the primary tumour, intravasation, extravasation, settlement and growth in distant organs (Geiger and Peeper 2009). Molecules involved in tumour progression are cell-cell and cell-matrix adhesion molecules, matrix degrading enzymes and their inhibitors. In addition, chemotactic factors released from the degraded matrix and chemokine receptors expressed by the metastasizing tumour cell, apoptosis resistance and angiogenesis inducer genes play an important role (Albini et al. 2008). Finally, several tetraspanins can be involved.

Tetraspanins are proposed to contribute to the metastatic cascade by their involvement in cell motility due to their association with integrins. Although there is some evidence that tetraspanins may modulate the ligand binding activity of associated integrins by stabilizing their activated conformation (Nishiuchi et al. 2005), it is proposed that tetraspanins mostly influence cell migration through integrin compartmentalization, their internalization and recycling or by modulating integrin-mediated signalling (Berditchevski 2001; Stipp et al. 2003; Hemler 2005; Levy and Shoham 2005). Besides integrins, the association with EWI proteins influences cell polarity and migration (Sala-Valdés et al. 2006). Several tetraspanins have been shown to regulate invasiveness, possibly due to their association with peptidases (Le Naour et al. 2006; Rana et al. 2011) ADAMs (A disintegrin and metalloproteinase), particularly ADAM10 (Arduise et al. 2008) and matrix metalloproteinases (MMP) (Lafleur et al. 2009; Yanez-Mo et al. 2008). They may also act by modulating MMP transcription and secretion (Hasegawa et al. 2007). By regulating trafficking and biosynthesis of associated molecules, tetraspanins can also influence cell adhesion events (He et al. 2005; Winterwood et al. 2006), which might mediate their actions in inhibiting or promoting metastasis.

11.4.1 Metastasis Suppressing Tetraspanins

As discussed above, CD82/KAI1 is a prototype of a metastasis suppressor gene. Other tetraspanins like CD9, CD81 and CD63 mostly, but not consistently, hamper tumour progression.

11.4.1.1 CD82, CD81 and CD9 Inhibit Tumour Cell Migration

Studies with human and animal cancer cell lines provide strong evidence that metastasis suppression by CD82 may mostly rely on inhibition of tumour cell migration and

invasion (Jackson et al. 2005; Tonoli and Barrett 2005; Liu and Zhang 2006). Depending on the associating molecules, several mechanisms have been elaborated through which CD82 could inhibit tumour progression (Liu and Zhang 2006; Miranti 2009).

Firstly, co-internalization of the α6 integrin chain with CD82, which is strengthened by concomitant epidermal growth factor receptor (EGFR) activation, is accompanied by impaired laminin adhesion and migration. Adhesion and migration are abolished by mutating the CD82 sorting motif. The authors suggest that the decrease in α6 integrins in CD82 expressing cells might be responsible for reduced adhesiveness and subsequently attenuated α6 integrins promoted motility (He et al. 2005; Odintsova et al. 2000, 2003). Similarly, the L6 antigen associates with CD82 and CD63 in TEM and may facilitate internalization of these tetraspanins (Lekishvili et al. 2008). It also has been reported that high level CD82 expression correlates with low integrin α6β1 and α6β4 expression (He et al. 2005), which in the case of CD151 was shown to affect integrin-mediated cell migration (Winterwood et al. 2006). CD82 expression can also interfere with integrin αvβ3/vitronectin-mediated tumour cell motility (Ruseva et al. 2009).

Secondly, the functional interplay of CD82 with the Ig superfamily member EWI-2 strengthens the motility inhibitory activity of EWI-2 on laminin and fibronectin (Zhang et al. 2003a). EWI-2 associates with ERM (ezrin, radixin, moesin) proteins and prevents their activation (Sala-Valdés et al. 2006), which is required for the linkage with actin (Louvet-Vallée 2000).

Thirdly, tetraspanins can modify the activity of proteases required for invasion. uPAR (urokinase receptor) co-localizes with integrin α5β1 in focal adhesions only in the presence of CD82. In the presence of the tetraspanin, the stable association between uPAR and α5β1, which prevents binding of uPA to its receptor and pericellular proteolysis, a necessary step in invasion, is strikingly reduced (Bass et al. 2005). In multiple myeloma, CD82 and CD81 over-expression affects motility and invasive potential, which is accompanied by reduced MMP9 secretion (Tohami et al. 2007).

Fourthly, CD82 interferes with c-Met signalling such that hepatocyte growth factor (HGF, also known as scatter factor)-induced cell migration is impaired (Takahashi et al. 2007). In a non-small cell lung cancer line over-expressing CD82, phosphorylation of c-Met by HGF stimulation was not affected, but the presence of CD82 interfered with ligand-induced association of c-Met with Grb2, a key molecule in intracellular signal transduction. Interference of CD82 with Grb2 binding is accompanied by inhibition of downstream signalling via phosphoinositide 3-kinase (PI3K) and the Ras → Raf → MAPK signaling axis, activation of rac and Cdc42 GTPases. As a consequence, lamellipodia formation and cell migration is severely impaired. In contrast, in a prostate cancer cell line (PC3), HGF-induced activation of c-Met and src was impaired in the presence of CD82, which inhibited the formation of the FAK (focal adhesion kinase)–p130^{CAS}–Crk complex downstream of Src activation (Sridhar and Miranti 2006). Importantly, the assembly of this complex was linked to increased cell motility. Down-regulation of the p130^{CAS}-Crk complex by CD82/KAI1 also has consequences on integrin-mediated cell migration (Zhang et al. 2003b).

Fifthly, some activities of CD82 rely on the contribution of gangliosides in the organization of TEM (Todeschini and Hakomori 2008; Hakomori 2010). For example, the impact of CD82 on EGFR activation varies depending on the presence of ganglioside GD1a, which facilitates the re-localization of the CD82–EGFR complex in TEM (Odintsova et al. 2006). The CD82–integrin α3β1–Met crosstalk is also regulated by gangliosides. Specifically, formation of the complex of GM2/GM3 with CD82 interferes with c-Met activation and c-Met-dependent downstream signaling. This blockade impairs not only cell motility, but also cell proliferation (Todeschini et al. 2008). It has been proposed that the CD82/GM2/GM3 complex inhibits tumour cell proliferation via a pathway similar to the PKCα-mediated inhibition of EGFR-induced proliferation, whereby GM3 together with CD82 controls translocation and phosphorylation of PKCα, and, consequently, induces EGFR phosphorylation and internalization (Wang et al. 2007b).

CD81 shares several features of motility-inhibiting activity with CD82. In hepatocellular carcinoma, the interaction of CD81 with PI4KII may play an important role in suppressing cell motility by promoting the formation of CD81-enriched vesicles that sequester actinin-4. The association of CD81 with PI4KII is accompanied by redistribution to intracellular vesicles, which might negatively affect actin-bundling activity of actinin (Fraley et al. 2003; Janmey and Lindberg 2004; Mazzocca et al. 2008). GPR56 forms a complex with Gαq and CD81 (Little et al. 2004). In melanoma, GPR56 binds tissue transglutaminase 2 (TG2), a major cross-linking enzyme in the ECM. The binding of the GPR56-Gαq-CD81 complex to TG2 could support adhesion and thereby interfere with tumour cell migration (Xu and Hynes 2007).

CD9 can inhibit or promote metastasis (Ikeyama et al. 1993; Ono et al. 1999; Zheng et al. 2005; Kohmo et al. 2010; Sakakura et al. 2002). The opposing activities are likely to depend on the associating molecules in the tetraspanin web. CD9 homoclustering is promoted by integrins α3β1, α6β4 and by palmitoylation of CD9 and the integrin β4 chain. In contrast, EWI-F- and EWI-2-associated or unpalmitoylated CD9 forms heteroclusters, which particularly are seen on malignant epithelial tumours (Yang et al. 2006).

Though CD9 can interfere with tumour progression at several steps of the metastatic cascade, migration inhibiting pathways are so far best described. In ovarian carcinoma cells, expression levels of CD9 and β1, α2, α3, α5 and α6 integrin chains are correlated, and downregulation of CD9 is accompanied by weaker matrix adhesion and dispersed growth in vitro (Ikeyama et al. 1993; Furuya et al. 2005). In addition, CD9 can associate with gangliosides, which can have distinct effects on the cell fate depending on the expression level. A non-invasive bladder cancer line expresses the GM3–CD9 complex at a high level. This correlates with a strong association with α3β1 and low cell motility. The reverse is true for an invasive bladder cancer line. When GM3 is expressed at a low level, it activates Src, whereas a high level GM3 causes Csk (C-terminal Src kinase), an endogenous inhibitor of the Src-family protein tyrosine kinases, translocation into TEM microdomains with subsequent inhibition of Src phosphorylation (Mitsuzuka et al. 2005).

CD9 also can hamper the migration of the isolated metastasizing cells. CD9 associates with the EGFR such that CD9 antibody cross-linking or EGF stimulation promotes EGFR internalization, which results in reduced EGFR autophosphorylation and reduced SHC phosphorylation and recruitment of Grb2. CD9 antibody cross-linking was noted to activate JNK and p38 MAPK and, after 24–48 h, caspase 3. The authors propose that this was due to tyrosine phosphorylation selectively of the p46 Shc isoform and speculate that CD9 might regulate apoptosis in tumor cells through initiating specialized signal transduction pathways (Murayama et al. 2008). In addition, CD9 is associated with the transmembrane form of transforming growth factor (TGF)α and, therefore, may affect the autocrine and juxtacrine activity of the protein (i.e., EGFR-dependent signalling) (Shi et al. 2000). Ectopic overexpression of CD9 in human fibrosarcoma cells correlated with transcriptional down-regulation of WAVE2 (Huang et al. 2006), a member of the WASP (Wiskott-Aldrich syndrome proteins) family of proteins, which act upon actin cytoskeleton and play a critical role in lamellipodium and filipodium formation. CD9 can also affect tumour cell motility through down-regulation of WISP-1 and MMP26, downstream targets of the Wnt signalling pathway associated with aggressive tumour growth (Yamamoto et al. 2004).

Finally, CD9 may affect the transendothelial migration of tumour cells. CD9, CD81 and CD151 co-localize at the tumour cell—endothelial cell contact area, where CD9 promotes strong adhesion via β1 integrins, which hampers transendothelial migration of the tumour cell (Longo et al. 2001). On the other hand, although down-regulated in metastases, high level CD9 expression at tumour cones can support transendothelial migration in cervical carcinoma and recovery of these cone-localized CD9 "hot spots" is a highly significant indicator of lymphangiogenesis (Sauer et al. 2003). Strong CD9 expression is also observed on myeloma cells in close contact to bone marrow endothelial cells (De Bruyne et al. 2006). The reason(s) for these opposing observations likely rely on differences in the CD9–containing "web" of individual tumour cells.

11.4.1.2 Tumour-Related, Migration-Independent Activities of CD82, CD81, CD9 and CD63

For CD82 two additional, migration-independent mechanisms have been described, whereby CD82 interferes with tumour progression. The first of these involves KITENIN, an unrelated four-transmembrane domain protein. Over-expression of KITENIN in a murine colon carcinoma line promotes adhesion to ECM ligands, tumour cell migration and metastasis (Rowe and Jackson 2006). By a not yet fully defined mechanism, binding of KITENIN to the C-terminal tail of CD82 appears to interfere with its metastasis-promoting activity (Lee et al. 2004). Secondly, CD82 interacts in trans with DARC (Duffy antigen receptor for chemokines) on vascular endothelial cells. This induces tumour cell senescence via reduced expression of the senescence related transcription factor TBX2 (T-box 2) gene and up-regulation of

the cyclin-dependent kinase inhibitor $p21^{WAF1}$, which is repressed by TBX2 (Prince et al. 2004). Accordingly, the metastasis-suppressor activity of CD82 is significantly reduced in $DARC^{-/-}$ mice (Bandyopadhyay et al. 2006).

While activities of CD82 in tumour cells are largely restricted to the metastatic process, CD9 can have an impact on tumourigenicity. Transformation of chicken or mouse fibroblasts with v-Jun suppresses transcription of GM3 synthase (Miura et al. 2004). Consequently, Jun-induced oncogenic transformation is accompanied by loss of the CD9-GM3 association. This leads to integrin activation, enhanced cell motility, and increased capacity for soft agar colony formation. Transfection with the GM3 synthetase gene, which reverts the oncogenic phenotype, is accompanied by re-establishment of the CD9-GM3 association. Tumour growth inhibition by CD9 may also rely on CD9-dependent regulation of expression of tumour necrosis factor (TNF)α whose production is delayed in $CD9^{-/-}$ mice (Yamane et al. 2005). A similar phenomenon has been described in the hepatic carcinoma cell line H22 (Li et al. 2006). How CD9 influences transmembrane TNFα activity has not been clarified. However, it has been shown that CD9 and ADAM17 can associate and that CD9 negatively regulates ADAM17 sheddase activity on TNFα (Gutiérrez-López et al. 2011; Moss and Bartsch 2004).

CD9 might also interfere with EMT the initiating step of the metastatic cascade. CD9 expression in HT1080 and A549 cells was shown to induce down-regulation of several Wnt family genes, such as *Wnt1*, *Wnt2b1* and *Wnt5a* and their targets including WISP-1, WISP-3, c-Myc, VEGF-A and MMP26. Wnt proteins are a large family of secreted glycoproteins that activate signal transduction pathways to control a wide variety of cellular processes such as determination of cell fate, proliferation, migration, and polarity (Coombs et al. 2008). There is evidence that CD9 is involved in the downregulation of several Wnt family genes, as well as of the rac GTPase regulated WAVE-2, which results in suppression of transformation and EMT (Huang et al. 2004, 2006). More recently it has been shown that by the association of glycoprotein 90 K with CD9 and CD82 the Wnt/β-catenin pathway becomes suppressed via a novel proteasomal-ubiquitination pathway (Lee et al. 2010).

11.4.2 Tumour Progression Promoting Activities of CD151 and Tspan8

In contrast to CD82, two tetraspanins, CD151 and Tspan8, have consistently been reported to promote tumour progression, where the main activity, particularly of CD151 is linked to tumour cell motility and invasiveness.

The first evidence for CD151 as a metastasis promoting molecule derived from a study in which an anti-CD151 antibody inhibited metastasis of a human epidermoid carcinoma line in a chick embryo model. The antibody inhibited cell migration without having any effect on cell adhesion or cell growth (Testa et al. 1999). Subsequently, an association between high CD151 expression and a poor prognosis has been described for many cancers (Sect. 11.2).

The metastasis promoting activity of CD151 mostly relies on its effect on tumour cell migration. Several lines of evidence point towards a link between MMPs and CD151. CD151 contributes to pericellular activation of MMPs by associating with proMMP7. This results in activation of MMP7, a phenomenon which can be prevented by anti-CD151 antibodies (Shiomi et al. 2005). In addition, CD151 has a positive effect on MMP9 expression through the mechanisms involving FAK, Src, p38 and JNK kinases. Signalling is initiated via CD151-associated integrin α3β1 or α6β1 and is stimulated by CD151 homophilic interactions (Hong et al. 2006; Yang et al. 2008). Reduced expression of MMP2, MMP7 and MMP9 in a CD151-knockdown carcinoma line confirmed the involvement of CD151 in MMP expression, complex formation and co-localization at the leading edge of lamellipodia (Shiomi et al 2005; Hasegawa et al. 2007).

Transfection of FAK competent and deficient fibroblasts with CD151 cDNA provided evidence that FAK is needed for CD151 mediated increased migration, Matrigel invasion and metastasis (Kohno et al. 2002). Further studies confirmed that CD151 is important for proper localization of laminin5-binding integrins during tumour cell–stromal cell interactions. Upon EGFR stimulation CD151 and α3β1 become internalized in HSC5 epidermal carcinoma cells. Furthermore, in HSC5-CD151-knockdown cells, α3β1 is partially internalized, α6β4 is redistributed and MMP2, MMP7 and MMMP9 expression is downregulated (Hasegawa et al. 2007). The authors speculate that CD151 might contribute to cell migration by inducing integrin re-localization and MMP production. In line with this is the finding that CD151-knockdown A431 epidermoid carcinoma cells display impaired motility, anomalously persistent adhesive contacts and impaired integrin α3β1 internalization (Winterwood et al. 2006). Notably, too, CD151 regulates glycosylation of α3β1. CD151 knockdown cells with reduced α3β1 glycosylation show strongly impaired migration towards laminin (Baldwin et al. 2008). Confirming the importance of CD151 for integrin traffic, expression of a CD151 molecule with a mutation of the sorting motif in the C-terminal domain markedly attenuates endocytosis of CD151-associated integrins such as α3β1, α5β1 and α6β1 (Liu et al. 2007). Thus, CD151 plays a critical role in integrin recycling as a mechanism to regulate tumour cell migration.

CD151 is also an important regulator of collective tumour cell migration. Monolayers of CD151 knockdown A431 cells display strikingly increased remodelling rates and junctional instability, which is caused by excessive RhoA activation and loss of actin organization at cell-cell junctions. There is evidence that CD151 regulates the stability of tumour cell-cell interaction through its association with integrin α3β1 (Johnson et al. 2009).

Quantitative in vivo assays and intravital imaging using the chicken chorioallantoic membrane model confirmed the impact of CD151 on tumor cell migration. A CD151-specific antibody inhibits matrix-mediated migration, but has no impact on extravasation. Migration inhibition is due to a failure to detach at the rear end. As migration of CD151-knockout cells was not affected, the authors suggested that—when present—CD151 might recruit partner molecules that control de-adhesion, but this process is suppressed in the presence of the CD151 antibody (Zijlstra et al. 2008).

Taken together, CD151 regulates cell migration, mostly through its association with integrins α3β1, α6β4 and MMPs. The TEM location, which facilitates the recruitment of integrins, additional transmembrane and cytosolic proteins in multimolecular complexes, contributes to this dominating theme (Hemler 2005).

Far less is known about the engagement of Tspan8 in tumour cell motility. Tspan8 associates with CD9, CD81, CD151 and several integrins including α3β1 and α6β4, but the integrin associations are probably indirect. Known non-integrin Tspan8-associated molecules are EWI-F, EpCAM, CD13, CD44, PKC and PI4KII (Claas et al. 2005; Zöller 2009).

Tspan8 over-expression in tumours correlates with poor differentiation and metastasis (Sect. 11.2). Tspan8 can support tumour cell proliferation, protection from apoptosis, and induction of angiogenesis and can enhance tumour cell motility. Tspan8-promoted tumour cell motility and liver metastasis may involve its association with integrin α6β4, as it is only seen in tumour cell lines that over-express both Tspan8 and α6β4 (Herlevsen et al. 2003; Gesierich et al. 2005). Tspan8 associates with integrin α6β4 only after PMA stimulation and disassembly of hemidesmosomes, which is accompanied by transient internalization of the Tspan8-α6β4 complex and increased motility (Huerta et al. 2003; Herlevsen et al. 2003). This continuing internalization to the endosomal compartment and rapid recycling back to the cell surface via a short loop recycling machinery under the control of rab4 has been described for several integrins (Caswell and Norman 2008). It may well account for the motility promoting activity of Tspan8.

11.5 CD151 and Tspan8, Tumour Growth and Angiogenesis

11.5.1 The Impact of CD151 and Tspan8 on Tumour Cell Proliferation and Apoptosis Protection

In a cellular model for mammary ductal carcinoma in situ, CD151 was found to support proliferation in a process that does not require direct contact with α3β1 integrin. Depletion of CD151 is accompanied by partial restoration of cell polarity and reduced ERK1/2 and Akt phosphorylation (Novitskaya et al. 2010).

Increased Tspan8 expression in a dedifferentiated rat hepatoma cell line promotes proliferation (Tanaka et al. 2002). Furthermore, interactions with platelets were suggested to provide tumour cells with a shield, which could provide a survival advantage in the hostile environment encountered during metastatic spread (Kanetaka et al. 2003). High Tspan8 expression may be also associated with increased apoptosis resistance (Huerta et al. 2003; Kuhn et al. 2007), which is likely to occur via a Tspan8-associated EpCAM-claudin-7 complex. In human and rat cancer lines, a striking decrease in drug resistance was observed upon knockdown of EpCAM or claudin-7. This was accompanied by reduced PI3K activation and loss of phosphorylation of Akt and downstream anti-apoptotic proteins. Signals are

initiated by the recruitment of the EpCAM-claudin-7 complex into TEM, which is accompanied by claudin-7 phosphorylation, possibly via Tspan8-associated PKC (Nübel et al. 2009).

11.5.2 Tetraspanins and Angiogenesis

Angiogenesis defines the process of new capillary formation from a pre-existing vasculature, which is crucial to supply a growing organism with oxygen. Accordingly, the rapid growth of tumour cells essentially requires blood supply (Folkman 2004). Tumour angiogenesis proceeds through several sequential steps. The process is believed to be initiated by angiogenic factors, angiogenin, epidermal growth factor, IL8, TNFα, TGFα, TGFß and VEGF (Hillen and Griffioen 2007), that are produced by tumour cells and bind to endothelial cell (EC) receptors including VEGFR-1, -2, -3 and neuropilins. Stimulated EC grow and secrete matrix degrading enzymes that digest the basement membrane surrounding the vessel. The junctions between EC become altered and EC migrate towards the source of the angiogenic stimulus, e.g., towards the tumour mass. At this stage, sprouting EC are reorganized to form tubes and assemble a new basement membrane. The formation of a lumen is driven by interactions between EC and the extracellular matrix. Molecules involved in this process are, among others, galectin-2, CD31 (PECAM-1) and VE-cadherin (Holderfield and Hughes 2008). An increasing body of recent evidence suggests that tetraspanins may directly regulate the development and functions of the vascular system and the pathogenesis of vascular diseases (Zhang et al 2009).

Several studies have reported that CD151 is important in angiogenesis induction (Dumartin et al. 2010; Takeda et al. 2007b; Zhang et al. 2002, 2009). Though patients with mutations in the CD151 gene and CD151 knockout mice showed no obvious defects in vasculogenesis (Karamatic Crew et al. 2004; Wright et al. 2004; Sachs et al. 2006), defects are seen in angiogenesis. Thus, CD151 expression by the tumour-bearing host facilitates tumour growth due to angiogenesis induction. CD151 supports EC invasiveness, migration, cable formation, matrigel contraction, tube formation and sprouting, activities which are all impaired in CD151 knockout mice (Takeda et al. 2007b). Selective defects in activation on laminin substrates of adhesion-dependent signalling molecules including PKB/c-Akt, e-NOS, Rac and Cdc42 contribute to impaired angiogenesis induction (Takeda et al. 2007b; Zheng and Liu 2007). Also, over-expression of CD151 promotes revascularization and improves blood perfusion in an ischemia model (Lan et al. 2005). Importantly, as in tumour cells, CD151 seems to support functional activity of endothelial cells via the associated integrins, particularly laminin-binding integrins (Liu et al. 2011; Zhang et al. 2009).

In addition to a direct involvement of endothelial CD151, expression of this protein (and other tetraspanins) in tumour cells and tumour-derived exosomes can also play an important role in tumour angiogenesis. In fact, Tspan8 is a strong angiogenesis inducer that contributes to a systemic angiogenic switch by Tspan8

over-expressing tumour cells as well as by exosomes derived thereof (Gesierich et al. 2006). The precise mode of activity of exosomal tetraspanins has not yet been explored. However, we will propose our hypothesis in the following section.

11.5.3 Tetraspanins and Thrombosis

Tumour vessels frequently have thin walls, an incomplete basement membrane and decreased numbers of pericytes, cells that are associated with microvasculature. As a consequence, tumour vessels are leaky, which allows for the extravasation of plasma proteins that form a scaffold for newly migrating EC. The leakiness of the EC layer also facilitates initiation of thrombus formation. Spontaneously occurring focal haemorrhages are a common feature of tumour vessels (Franchini et al. 2007) and a prothrombotic state that can culminate in disseminated intravascular coagulation is frequent in cancer patients, where tumour-initiated angiogenesis and the leakiness of tumour vessels are considered to be important (De Cicco 2004). Knowledge of factors regulating angiogenesis and coagulation has strengthened the expectation that these two systems are closely interconnected. The coagulation cascade is initiated when tissue factor, the principal initiator of coagulation, which is provided by many tumour cells, becomes exposed to plasma components. The cascade ends with platelet bound prothrombin becoming converted to thrombin that initiates clot formation by catalysing fibrinogen cleavage and fibrin polymerization. Tumour angiogenesis facilitates blood clotting through the hyperpermeability of tumour endothelium and the leakage of fibrinogen and other clotting agents. Activated platelets in turn support angiogenesis by releasing pro-angiogenic factors like VEGF and angiopoietin-1. Thrombin also supports angiogenesis by cleaving PAR-1 on EC thereby inducing activation and secretion of proteases including MMPs and uPA (Tsopanoglou and Maragoudakis 2007). Taken together, the particular features of tumour vessels support thrombus formation and the coagulation cascade provides a feedback for angiogenesis induction (Ruf and Mueller 2006), which is supported by platelet-derived tetraspanins.

CD63, CD9 and CD151 are abundantly expressed on platelets (Griffith et al. 1991; Fitter et al. 1995; Schröder et al. 2009). Whereas CD151 is required for efficient platelet activation/aggregation (Lau et al. 2004; Orlowski et al. 2009), $CD9^{-/-}$ mice show alteration in blood coagulation, where CD9 appears to prevent excessive thrombus growth, but does not appear to play a critical role in primary hemostasis (Mangin et al. 2009). From the viewpoint of tetraspanin engagement in tumour cell dissemination, the more interesting aspect relies on the isolated tumour cell within the blood stream taking advantage of CD9 down-regulation. CD9 associates with the platelet aggregation-inducing factor podoplanin. Ectopic expression of CD9 in podoplanin-expressing tumour cells leads to reduced lung metastasis formation accompanied by impaired tumour-induced platelet aggregation (Nakazawa et al. 2008). Platelets bind via CLEC-2 (C-type lectin-like receptor-2) to podoplanin, which induces platelet degranulation (Suzuki-Inoue et al. 2006). Because CLEC-2

is unable to recognize CD9-associated podoplanin (Nakazawa et al. 2008), platelet aggregation will be impaired upon contact with tumour cells expressing both CD9 and podoplanin. Consequently, formation of tumour cell platelet aggregates, which facilitates embolization of the microvasculature and metastasis formation, will also be suppressed. Decrease in the formation of these aggregates will also make tumour cells more susceptible to a host anti-tumour immune attack (Sierko and Wojtukiewicz 2007).

Finally, platelet-derived exosomes constitute about 70–90% of circulating exosomes in the plasma (Berckmans et al. 2001) with a life span of about 30 min (Flaumenhaft 2006). The procoagulant activity of platelet-derived exosomes is well known. Specifically, it has been suggested that exosomes provide negatively charged phospholipids, which are required for factor IXa and Xa activation (Shet et al. 2003). Though still controversial, the therapeutic efficacy of anti-glycoprotein IIb/IIIa could be a consequence of altered platelet exosome formation (Morel et al. 2004; Razmara et al. 2007). The abundance of platelet-derived exosomes and their functional activity in coagulation implies that they may contribute to the prothrombotic state frequently seen in cancer patients. It remains to be explored whether platelet-derived exosomal CD151, CD9, Tspan32 and CD63 contribute to the procoagulant activity. On the other hand, tumour-derived exosomes may also be of utmost importance for platelet activation. Thus, rats transplanted with a Tspan8 over-expressing tumour line develop disseminated intravascular coagulation, which could be prevented by a Tspan8-specific antibody (Claas et al. 1998). Though the underlying mechanism remains to be elaborated, it is tempting to speculate that exosomal Tspan8 contributes to platelet activation.

Taken together, the engagement of tetraspanins in angiogenesis and thrombosis has only recently received attention and work so far covers only few members of the tetraspanin family. Nonetheless, data gathered so far hold promise for a wealth of information in the near future. Since angiogenesis and thrombosis are important parameters in oncology, this knowledge may well lead to new therapeutic options.

11.6 Perspective: Tetraspanins and Exosomes

One feature of tetraspanins, though well known, has received little attention so far. Tetraspanins are enriched in exosomes and we consider it very likely that exosomal tetraspanins play a major role in exosomal message delivery.

11.6.1 Exosomes

Exosomes, small 30–100 nm vesicles, which are believed to derive from fusion of the intraluminal vesicles of multivesicular bodies (MVB) with the plasma membrane (Fevrier and Raposo 2004; de Gassart et al. 2004; Lakkaraju and Rodriguez-Boulan

2008). The molecular composition of exosomes reflects their origin from intraluminal vesicles (Johnstone 2006). Besides a common set of membrane and cytosolic molecules, which includes several tetraspanins, including CD9, CD37, CD53, CD63, CD81, CD82, CD151 and Tspan8, exosomes harbor subsets of proteins, such as adhesion molecules, molecules associated with vesicle transport, cytoskeletal proteins, signal transduction molecules, enzymes and others that are linked to cell type-specific functions (Schorey and Bhatnagar 2008; Mathivanan et al. 2010). Importantly, exosomal proteins maintain their functional activity, including antigen presentation, peptide and protein cleavage (Potolicchio et al. 2005; Stoeck et al. 2006). Another notable feature is the presence of phosphatidylserine at the exosomes' outer membrane leaflet which can trigger exosome uptake by cells expressing phosphatidylserine-binding proteins (scavenger receptors, integrins, complement receptors) (Zakharova et al. 2007). Exosomes contain mRNA and miRNA (so called shuttle RNAs) which are transferred to the target cell, where they can be translated or mediate RNA silencing (Ratajczak et al. 2006; Deregibus et al. 2007; Valadi et al. 2007; Burghoff et al. 2008). Exosome-mediated transfer of DNA to their target cells is specific, so that RNA is transcribed in one, but not another type of cells (Simons and Raposo 2009). In addition, the relative abundance of proteins, mRNA and miRNAs differs between exosomes and the cells from which they are derived. This implies active sorting into MVB (Lakkaraju and Rodriguez-Boulan 2008), which for proteins can be achieved by mono-ubiquitination, localization in cholesterol-rich membrane microdomains, or higher order oligomerization (Gruenberg and Stenmark 2004; Hurley and Emr 2006; Fang et al. 2007; Smalheiser 2007). The mechanisms underlying selective sorting of mRNA and miRNA into exosomes are unknown (Subra et al 2007). Thus, exosomes constitute a most potent mode of intercellular communication that has become appreciated as important in immunity (André et al. 2002), cell-to-cell spread of infectious agents (Johnstone 2006; Schorey and Bhatnagar 2008) and tumour progression (Zöller 2006). Accordingly, therapeutic exploitation of exosomes appears very promising and is already in clinical use as a vaccine strategy (Iero et al. 2008). Exosomes may also be the most potent gene delivery system (Belting and Wittrup 2008; Simpson et al. 2009; Pap et al. 2009; Seow and Wood 2009; Xiao et al. 2009).

11.6.2 Exosomal Tetraspanins

Tetraspanins are abundantly recovered in intracellular vesicles and exosomes (Escola et al. 1998; Sincock et al. 1999; Hemler 2003; Berditchevski and Odintsova 2007; Pols and Klumperman 2009; Zöller 2009). Some tetraspanins possess a tyrosine-based sorting motif, a sequence of Tyr-Xaa-Xaa-ϕ where ϕ stands for an AA with a bulky hydrophobic side chain, in the C-terminal cytoplasmic domain (Marks et al. 1997). By this sorting motif, these tetraspanins are predisposed for delivery to intracellular compartments (Marks et al. 1997; Berditchevski and Odintsova 2007). However, some tetraspanins enriched in exosomes do not possess a sorting motif

(CD9) or have an inappropriately located sorting motif (Tspan8) (Berditchevski and Odintsova 2007). This partial independence of a sorting motif indicates that individual tetraspanins likely follow different routes of internalization. Molecular mechanisms controlling trafficking routes of tetraspanins and associated proteins are reviewed in detail in another chapter of this volume.

Irrespective of the donor cell type, tetraspanins are enriched in exosomes and the tetraspanin web is mostly maintained in them (Abache et al. 2007). Whether tetraspanins are involved in sorting of proteins, mRNA or miRNA to exosomes is currently unknown (Gibbings et al. 2009; Simons and Raposo 2009).

11.6.3 Exosomal Tetraspanins, the Premetastatic Niche and Angiogenesis

Evidence has started to emerge that tetraspanins are important in target cell selection during premetastatic niche formation as well as tumour-angiogenesis. Lodgement of metastasizing tumour cells is facilitated by the establishment of special niches in (pre)metastatic organs (Bissell and Labarge 2005). Niche preparation involves stimulation of local fibroblasts by tumour-derived factors and chemokines that attract tumour cells and hematopoietic progenitors (Kaplan et al. 2006). Nonetheless, information on long-distance communication between a tumour and host organs is still limited and exosomes have been suggested to contribute to premetastatic niche formation as well as tumour-associated angiogenesis and thrombosis (Aharon and Brenner 2009; Al-Nedawi et al. 2009). Notably, under hypoxia tumour cells have been described to secrete exosomes enriched in Tspan15, CD9 and CD81, which have a major impact on the tumour microenvironment such that angiogenesis and metastatic potential becomes increased (Park et al. 2010).

An involvement of exosomes in metastasis was first described for platelet-derived exosomes. These exosomes transferred the αIIb integrin chain to lung cancer cells, stimulated the MAPK pathway and increased expression of MT1-MMP, cyclin D2 and angiogenic factors as well as enhancing adhesion to fibrinogen and human umbilical vein endothelial cells (Janowska-Wieczorek et al. 2005). A direct transfer of metastatic capacity by exosomes was demonstrated for B16 melanoma cells. Exosomes derived from a highly metastatic variant transferred metastatic capacity to low metastatic B16F1 cells. Lung metastasis formation by B16F1 was accompanied by protein uptake from exosomes of the metastasizing subclone (Hao et al. 2006). Tspan8 and/or CD151-containing exosomes also contribute to premetastatic niche formation. After subcutaneous application of CD151- and Tspan8-enriched exosomes together with a soluble tumour matrix, exosomes supported recruitment of hematopoietic progenitors from the bone marrow as well as activation of stroma cells and leukocytes in premetastatic lymph nodes such that a non-metastatic tumour line settled and formed metastases (Jung et al. 2009). Ongoing work aims to define the contribution of exosomal CD151, Tspan8 and associated integrins in target cell selection and binding.

While the question of target cell selection and the contribution of tetraspanins remains to be defined in premetastatic niche preparation, initiation of tumour-angiogenesis was shown to require Tspan8 in a rat adenocarcinoma model. Only Tspan8-expressing exosomes interact with endothelial cells. Furthermore, binding to and uptake by endothelial cells is dependent on the formation of the integrin α4β1-Tspan8 complex. The uptake of Tspan8-bearing exosomes by EC is accompanied by transient recovery of mRNA selectively enriched in the exosomes and initiates transcription of several angiogenesis-related genes, proliferation, migration and sprouting of endothelial cells. Importantly, Tspan8-positive exosomes also bind to endothelial cell progenitors and promote endothelial cell progenitor maturation (Nazarenko et al. 2010).

Exosomes are easy to manipulate and provide a powerful means of protein and gene transfer. Thus, it becomes crucial to further explore the engagement of tetraspanins in target cell selection. This would offer a powerful means to interfere with pathological angiogenesis and metastasis, two major targets in cancer therapy (Pap et al. 2009; Zöller 2009).

11.7 Tetraspanin Based Therapeutic Options

Taking into account the importance of some tetraspanins in tumour progression and angiogenesis, it is important to consider these molecules as therapeutic targets. Due to their mode of activity as molecular facilitators in a wide range of cell types (Maecker et al. 1997), this will not be an easy task. As some tetraspanins function as metastasis suppressors, while others promote metastasis, we will discuss these two aspects of tetraspanin-based therapies.

11.7.1 Rescuing Metastasis Suppressor Genes

CD82 inhibits migration and invasion by associating directly or via bridging integrins with a multitude of different molecules as well as by the recruitment of the partner molecules in TEM. Some of the CD82-based interactions involve transmembrane domains. CD82 inhibits formation of microprotrusions and the release of microvesicles. Mutations of three polar residues in the transmembrane domains of CD82 disrupt these inhibitions (Bari et al. 2009). The authors provide evidence that the transmembrane interactions mediated by these polar residues determine a conformation either in or near the transmembrane regions and that this conformation is needed for the intrinsic activity of CD82. They speculate that a therapeutic perturbation of CD82 transmembrane interactions may open a new avenue to prevent cancer invasion.

Rescuing CD82 gene expression also should prevent tumour progression, which, however, requires an awareness of the regulation of CD82 gene transcription as well

as of the mechanisms that down-regulate CD82 expression in tumour cells (Tonoli and Barrett 2005; Liu and Zhang 2006) (Sect. 11.3).

Besides reviving CD82 expression at the transcriptional level, CD82 expression may also be rescued by proteasome inhibitors or by targeting specific components of the ubiquitin system, as ubiquitin ligase gp78 which regulates CD82 expression (Tsai et al. 2007).

The potential therapeutic efficacy of CD82 has already been demonstrated. Thus, nerve growth factor has been shown to rescue CD82 expression, which was accompanied by abrogation of tumourigenicity of prostate cancer cell lines (Sigala et al. 1999). Furthermore, CD82 transfected murine Lewis Lung carcinoma cells lose the capacity to form lymph node metastasis. Even more strikingly, intratracheal administration of adenovirus encoding CD82 or CD9 cDNA in mice orthotopically preimplanted with LLC cells dramatically reduced metastases without affecting growth of the primary tumor (Takeda et al. 2007a).

11.7.2 Interfering with Metastasis and Angiogenesis Promoting Activities of Tetraspanins

Therapeutic approaches aimed at interference with metastasis promoting activities of tetraspanins are mostly based on antibodies, recombinant soluble ECL2 or post-transcriptional gene silencing via siRNA (Hemler 2008; Stipp 2010).

Some tetraspanin-specific antibodies have been shown in several instances to be of potential clinical relevance. Intratumoural application of anti-CD9 inhibited colon carcinoma growth and intravenous application of anti-CD9 inhibited the subcutaneous growth of gastric cancer cell lines (Ovalle et al. 2007; Nakamoto et al. 2009), anti-CD37 improved the survival of B-CLL xenografted mice (Levy et al. 1998) and anti-CD151 interfered with metastasis formation (Testa et al. 1999; Kohno et al. 2002; Zijlstra et al. 2008). Though the underlying mechanisms have not been fully elucidated, it has been suggested that antibodies may interfere with the lateral associations of tetraspanins or promote clustering of tetraspanins and tetraspanin-associated molecules in TEM and thereby interfere with the activity not only of the targeted tetraspanin, but also of associated molecules including cytoplasmic partners. In line with this suggestion, tetraspanin antibodies have in some instances been shown to exert stronger effects than the knockout of an individual tetraspanin, e.g. anti-CD81 has been shown to interfere, besides others, with T and B cell activities, but only the B cell response was impaired in CD81 knockout mice (Oren et al. 1990; Boismenu et al. 1996; Miyazaki et al. 1997; Tsitsikov et al. 1997; Levy et al. 1998). Taking this into account, one has to be aware that the activity of tetraspanin-specific antibodies may vary depending on the recognized epitope (Serru et al. 1999; Yauch et al. 2000; Geary et al. 2001), which may enhance or block the effect of a tetraspanin as demonstrated for anti-CD151 promoting adhesion (Zijlstra et al. 2008) and for anti-CD9 that can amplify the tumour suppressor function (Ovalle et al. 2007).

Besides their blocking or enhancing activity, tetraspanin-specific antibodies repeatedly have been described to induce apoptosis (Murayama et al. 2004), for example in a SCID mouse model, where anti-CD9 interferes with gastric cancer growth by exerting anti-proliferative, pro-apoptotic and anti-angiogenic activity (Nakamoto et al. 2009). Anti-tetraspanins also can support complement and antibody-dependent cellular cytotoxicity (Zhao et al. 2007).

Finally, antibodies can be used as drug transporters as reported for ^{131}I-labelled anti-CD37 (Press et al. 1989) or for transporting nanoparticles with siRNA (Peer et al. 2008), which has not yet been explored for tetraspanins.

Taken together, antibodies have proven in many instances to be a powerful adjuvant cancer therapy (Boyiadzis and Foon 2008). Nonetheless, abundant expression of a molecule, like most tetraspanins, in non-transformed cells can provide a major obstacle (Grünwald et al. 2009). We consider the use of bispecific antibodies that target with both arms the tumour cell as a most promising solution. Such an approach has been used by the group of Hollander for targeting CD44, which is abundantly expressed on many cells. Yet, using anti-CD44/anti-idiotype bispecific antibodies, side effects were avoided and the anti-tumour efficacy was strengthened (Avin et al. 2004). As tetraspanins act as molecular facilitators, this kind of bispecific antibodies can be expected to be highly efficient.

Besides antibodies, the soluble form of the large extracellular domain (ECL2) of tetraspanins as a competitor has mainly been tested with respect to leukocyte endothelial cell interaction via CD9 and CD151 (Barreiro et al. 2005), egg-sperm fusion (Zhu et al. 2002) and virus infectivity, where the ECL2 may be superior to antibodies, as it does not only compete for binding, but additionally exerts functional activity (Molina et al. 2008).

Another therapeutic approach is based on silencing tetraspanins via siRNA. CD9 silencing resulted in pronounced ovarian cancer dissemination (Furuya et al. 2005) and CD151 silencing interfered with integrin-dependent adhesion and migration (Winterwood et al. 2006). Feasibility of this approach has been demonstrated in experiments describing successful lentiviral CD81 shRNA delivery into the nucleus accumbens or the ventral tegmental area of the mesolimbic dopamine system which resulted in a significant decrease in locomotory activity (Bahi et al. 2005).

Therapeutic settings currently being discussed include modulation of amino acids important for transmembrane folding (Tarasova et al. 1999). The authors argue that a therapeutic perturbation of TM interactions may open a new avenue to prevent cancer invasion, which could be far easier approached than a blockade of individual signalling pathways. Modulation of the PDZ domain (Dev 2004; Latysheva et al. 2006), of key interaction sites in the ECL2 (Yauch et al. 2000; Seigneuret 2006), of palmitoylation sites (Berditchevski et al. 2002; Charrin et al. 2002; Yang et al. 2002, 2004; Kovalenko et al. 2005) including targeting of the responsible acyltransferase (Sharma et al. 2008) are additional therapeutic approaches to be discussed. Recently convincing evidence has been provided for different requirements of the CD151-α3β1 and the CD151-α6β4 interaction, which would allow to selectively interfere with CD151-α3β1 adhesion and migration on laminin5 and CD151-α6β4-mediated stable attachment (Zevian et al. 2011).

Finally, taking into account the increasingly appreciated role of exosomes as intercellular communicators and the strong presence of tetraspanins in exosome membranes, it is tempting to speculate that tetraspanins could be used as an exosome delivery system. This requires further elaboration of the engagement of tetraspanins and the associated molecules that together bind to and become internalized by selective targets (Zöller 2009; Nazarenko et al. 2010). Knowledge of exosome binding and uptake of tetraspanin complexes by selective target cells could enable generation of competitive exosomes carrying desired siRNAs or other drugs that interfere with exosome initiated premetastatic niche preparation, angiogenesis and thrombosis.

In summary, though there are promising concepts, one should be aware that tetraspanin-based therapeutic protocols require sophisticated controls as the composition of TEM may well determine the balance between opposing activities.

11.8 Conclusion

Tetraspanins function as molecular facilitators that assemble a web including many distinct families of transmembrane proteins in specialized membrane microdomains that serve as a scaffold for localised signal transduction and regulation of cytoskeletal dynamics. The reversibility of TEM and their composition, which depends on the cell's activation state, the abundance of associating molecules and their ligands, adds a major constraint in defining tetraspanin functions. Nonetheless, modulation of cell motility, cell fusion and intercellular communication via exosomes may well cover the essential activities of tetraspanins in cancer. The involvement of tetraspanins in these actions basically can follow five routes: (1) Tetraspanins may act as receptors for defined ligands; (2) Tetraspanins are known to directly influence adhesion, signal transduction and/or gene transcription via associated molecules; (3) Tetraspanins indirectly initiate activities via the recruitment of different molecules into TEM, a process that frequently involves gangliosides; (4) Tetraspanins initiate internalization and relocation of associated molecules in distinct membrane regions; (5) Tetraspanins initiate recruitment into MVB and release of TEMs in exosomes, where exosomal tetraspanin and associated molecules may be of major importance in target cell selection and in exosome fusion with the target cell.

Through these different activities, tetraspanins contribute to metastasis inhibition and promotion, to premetastatic niche formation, to angiogenesis and the tumour-associated prothrombotic state. Nonetheless, one of the key questions, why some tetraspanins suppress (CD82) or promote (CD151, Tspan8) tumour progression remains unanswered. In addition, application of tetraspanins as tumour biomarkers and therapeutic targets in human cancer will also require a better understanding of their association with different tumour subsets (e.g., CD82 in ER-positive and negative breast cancer) and the pattern of modulation of their levels at different stages of disease. Answering these questions may provide a solid ground for therapeutic interference with tetraspanin activities in tumour progression.

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