The regulation of Rho and Src signaling by integrins
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SUMMARY AND DISCUSSION

Interactions of cells with the extracellular matrix are predominantly mediated through binding of integrin receptors, and these interactions are important for cell adhesion, migration, survival, proliferation, and differentiation. Changes in expression levels of integrins and extracellular matrix proteins often occur during dynamic processes such as angiogenesis, wound healing, development, and cancer as well as several other diseases. In this thesis I describe our recent studies of different integrin-extracellular matrix interactions and how they coordinate cellular signaling pathways and cell behaviour. We mainly address the role of \( \beta 1 \) and \( \beta 3 \) fibronectin-binding integrins. As there are many known connections of integrins with Rho-GTPase and Src family kinase signaling, we test the hypothesis that distinct integrins might differently regulate processes driven by Rho and/or Src.

**Integrins in control of Rho signaling**

Previous results from our group demonstrate that restoration of \( \beta 1 \) integrin expression in \( \beta 1^-/- \) cells supports sustained RhoA GTP-loading and fibronectin matrix assembly (Danen et al., 2002). By contrast, increased expression of \( \beta 3 \) integrins in the same cellular background does not activate RhoA or stimulate fibronectin matrix assembly. Later, we discovered that the differential regulation of Rho-GTPases by \( \beta 1 \) and \( \beta 3 \) integrins also dictates the dynamics of cell-matrix adhesions and controls migratory strategy (Danen et al., 2005). In chapter 2 of this thesis we investigate the mechanisms that could underly the efficient activation of RhoA and fibronectin fibrillogenesis by \( \beta 1 \) integrins. Mesenchymal cells secrete fibronectin molecules as compact soluble dimers, that are assembled into a (insoluble) fibronectin fibrillar matrix upon interactions with integrins and syndecans. The generation of fibronectin fibrils requires remodeling of cell-matrix adhesions and contractility of the actomyosin cytoskeleton, which together mediates the stretching of soluble FN dimers that exposes intermolecular fibronectin-binding sites. Although both \( \alpha 5\beta 1 \) and \( \alpha v\beta 3 \) integrins recognise the common RGD-binding motif in fibronectin, by using several integrin specific knockout cell lines, we unexpectedly find that of these integrins only \( \alpha 5\beta 1 \) efficiently bound soluble fibronectin molecules. The ability of \( \alpha 5\beta 1 \) to bind soluble fibronectin determines not only the efficiency with which it activates RhoA, but is also supports the redistribution of focal adhesions to a few peripheral sites causing a contractile fibroblast-like morphology. Moreover, binding of soluble fibronectin by \( \alpha 5\beta 1 \) is required for fibronectin matrix assembly downstream of the Rho-contractility pathway. The transmembrane proteoglycan Syndecan-4 has been reported to be able to regulate Rho-GTPases (Dovas et al., 2006; Saoncella et al., 1999) and to support fibronectin matrix assembly (Chung and Erickson, 1997) in concert with integrins. However, knockdown studies in our experimental system rule out a role for Syndecan-4 in \( \alpha 5\beta 1 \)-mediated support of RhoA activity, cytoskeletal reorganization, and fibrillogenesis.

During the past two decades the regulation of RhoA activity by integrin-mediated adhesion has been studied by many different groups. It is generally accepted that integrins rapidly suppress RhoA activity during initial cell spreading on fibronectin. At later stages the activity of RhoA gradually increases and supports maturation of focal adhesions and cytoskeletal contractility. Integrins recruit a FAK/Src complex at sites of adhesion and phosphorylate p190RhoGAP, which in turn mediates the initial inhibition of RhoA activity. Intriguingly, it is still unclear which Rho GAPs or GEFs are involved in the activation of RhoA at later stages of cell spreading. It was recently showed that the Rho GEFs Lsc and LARG are activated in cells plated on fibronectin (Dubash et al., 2007). Moreover, a simultaneous knockdown of expression of both GEFs strongly decreases the activity of RhoA, indicating that these two GEFs may specifically link integrins with sustained RhoA activation. Although we still do not know which GEFs are involved in the activation of RhoA by integrins \( \alpha 5\beta 1 \), a simultaneous
knockdown of Lsc and LARG in GEβ1 cells does not affect RhoA activity or the typical fibroblast-like morphology (data not shown), indicating that the regulation of GEFs might be cell type dependent. The data presented in chapter 2 at least indicate that the ability of α5β1 to activate RhoA is not dependent on specific interactions with amino acids at its integrin cytoplasmic domain, as they are replaceable with that of the αvβ3 cytoplasmic domain without interfering with RhoA GDP-loading. Upon binding of soluble fibronectin molecules to the extracellular domain of integrin α5β1, conformational changes and/or clustering at the cytoplasmic domain might affect regulatory proteins upstream of RhoA.

Activation of RhoA by integrin α5β1 regulates several important processes: cytoskeletal contractility, focal adhesion dynamics, fibronectin matrix assembly, and it promotes a random mode of migration. In complete agreement with our findings, it has recently been demonstrated in fibroblasts expressing both α5β1 and αvβ3, that interfering with αvβ3 recycling enhances α5β1 recycling to the cell surface causing increased ROCK activity and a more random mode of migration (White et al., 2007). Just like fibronectin, integrin α5β1 is essential for embryonic development. Our findings demonstrate that the binding of soluble fibronectin to α5β1 regulates a mesenchymal morphology by redistributing focal adhesions and Rho-mediated cytoskeletal contractility and this might actually explain why α5β1 is so important for development, a process during which epithelial-to-mesenchymal and mesenchymal-to-epithelial transitions frequently occur. The support of Rho-mediated contractility by α5β1 in activated endothelial cells may also play an important role in angiogenesis (Hoang et al., 2004; Kim et al., 2000). Moreover, it was recently found that invasion of carcinoma cells is preceded by invading fibroblasts using α5β1 adhesions (Gaggioli et al., 2007). To invas and remodel the extracellular matrix, these leading fibroblasts require adhesion through integrin α5β1 and Rho-mediated actomyosin contractility. These results together with our own suggest that the selective interaction of α5β1 and soluble fibronectin might be a very valuable target for therapeutic intervention to prevent cancer progression.

**Integrins in control of Src signaling**

Increased activity of c-Src is frequently found in melanoma’s and multiple carcinoma’s, and correlates with increased tumor growth and/or progression. Although in rare cases mutations may induce activation of c-Src, it remains largely unknown what mechanism is responsible for activation of c-Src in human cancer. Interestingly, elevated expression levels of integrin αvβ3 are also observed in similar cancer types as which increased c-Src activity has been reported. Moreover, knockout studies indicate that a functional connection of αvβ3 and c-Src may exist, as the phenotypes of Src-/- and Itgb3-/- knockout mice are partially overlapping. In chapter 3 we test the hypothesis that integrin αvβ3 and c-Src may cooperate during tumor development. To investigate the role of integrins in tumor formation driven by c-Src, we express a Src^{Y530F} mutant, which is no longer inactive but instead adopts a primed conformation, in β1-/- cells. Subsequently, we analyze Src-mediated oncogenic transformation in presence of reconstituted levels of β1 integrins or in the absence of β1 and increased expression levels β3 integrins. Colony formation in soft-agar and subcutaneous tumor formation are not supported by β1 integrins, however, integrin αvβ3 appears a strong enhancer of primed c-Src-mediated oncogenic transformation in these experiments. Oncogenic transformation driven by the activated Ras oncogene is unaffected by integrin expression levels, indicating that a specific cooperation exists between αvβ3 and primed c-Src during tumor formation. We subsequently find that the cytoplasmic domain of β3 is required and sufficient for promoting Src^{Y530F}-driven tumor formation, because expression of the β3 cytoplasmic domain stimulates full kinase activity of Src^{Y530F}, phosphorylation of established Src substrates, and enhances survival and proliferation. At that time, Arias-Salgado and colleagues reported a direct
interaction of the last four amino acids of the β3 cytoplasmic domain with the c-Src SH3 domain that supports activation of c-Src in platelets (Arias-Salgado et al., 2003). Our results actually suggest that this interaction can also support Src-mediated oncogenic transformation and may be highly relevant for tumorigenesis. Expression of mutated β3 or Src variants, that interfere with the putative binding interface of β3 and c-Src indicate that this direct interaction is probably responsible for full kinase activity of primed c-Src and for tumor formation. We propose that integrin αvβ3 clusters primed c-Src molecules, thereby supporting autophosphorylation in Src’s kinase domain. Alternatively, clustered αvβ3 integrins may already induce conformational changes in the kinase domain of c-Src and they may recruit additional downstream signaling intermediates that contribute to oncogenic signaling.

Activated c-Src not only induces oncogenic transformation, it also triggers dramatic morphological alterations that lead to an inhibition of cell-matrix and cell-cell adhesions and suppresses cell spreading. The actin cytoskeleton completely changes as stress fibers and focal contacts disappear and are replaced by invasive podosome structures. In chapter 4 we investigate to what extent the signaling pathways triggered by activated c-Src during oncogenic transformation overlap with those involved in morphological transformation. Expression of SrcY530F in β1/- cells readily inhibits cell adhesion and spreading, and efficiently induces the formation of podosomes. When we analyze these morphological alterations in presence of β1 or β3 integrins, we observe that αvβ3 in fact protects against Src-induced loss of adhesion and cell spreading whereas podosome formation still occurs. Expression of chimeric integrin subunits of which the cytoplasmic domains of β1 and β3 integrins are swapped further demonstrates that the protection against loss of adhesion and spreading by αvβ3 is mediated by the integrin extracellular domain. These results illustrate two major novel findings regarding signaling by activated Src: because the cooperation of αvβ3 and SrcY530F during tumor formation is dependent on the β3 cytoplasmic domain, whereas morphological alterations are dependent on the extracellular domain of αvβ3 we are able to show that oncogenic and morphological transformation by SrcY530F are regulated by separate pathways. Secondly, since αvβ3 protects against the inhibition of adhesion and spreading induced by SrcY530F, but podosomes are still formed, we conclude that these morphological alterations are independent aspects. Lastly, the introduction of non-phosphorylatable β1 or β3 mutants in SrcY530F expressing cells demonstrate that phosphorylation of β3 by SrcY530F is not involved in any of the aspects of oncogenic or morphological transformation. Instead, phosphorylation of β1 by SrcY530F is required for podosome assembly, probably to suppress Rho-mediated contractility.

When taking the results of chapters 3 and 4 together we propose a following working model for the cross-talk between Src transformation and integrins (see figure 8 of chapter 4). Clustering of integrin αvβ3 increases activation of primed c-Src (perhaps αvβ3 also binds to inactive c-Src in tumor cells) by interacting with the SH3 domain of c-Src. Once fully activated c-Src phosphorylates its substrates, including Stat3 and FAK, that will contribute to increased proliferation and survival signaling in tumor cells and supports tumor growth. Secondly, activated c-Src will change the morphology of tumor cells. Although in normal cells α5β1 would induce strong activation of RhoA and cytoskeletal contractility, phosphorylation of β1 by activated c-Src inhibits RhoA-mediated contractility enabling the formation of invasive podosomes. When expressing high levels of αvβ3 these tumor cells will be protected against Src-induced rounding (inhibition of adhesion and spreading), which may also promote survival of these cells. In presence of high levels of β1 integrins no such protection occurs. Because of differences in controlling adhesive and spreading properties, the distribution of podosomes will be different in cells expressing β3 or β1 integrins. Therefore changes in relative expression levels of β3
and β1 integrins may contribute to different invasive strategies during Src-driven tumor progression. Future studies should clarify how integrin interactions contribute to Src-driven tumorigenesis in vivo.

In this regard, the fact that mutations in the Src gene occur only rarely in cancer, argues against a role for c-Src in tumor initiation. Nevertheless, we do find that elevated c-Src activity promotes tumor growth in a cell autonomous fashion in vitro. We have attempted to set up an in vivo model to study the role of primed c-Src in mammary tumorigenesis. Transgenic expression of SrcY530F under the MMTV-promotor in mice already interfered with early mammary gland development, and subsequently female mice expressing MMTV-SrcY530F have problems with lactation. However, we did not observe an induction of mammary epithelial hyperplasias as was reported earlier by Webster and colleagues (Webster et al., 1995). This could be due to the low levels of expression of the transgene and, in fact, may allow us to address the cooperation between αvβ3 and SrcY530F in breast cancer in vivo in future studies. For this purpose we have also initiated a transplantation model for breast cancer using primary mouse mammary epithelial cells (MECs). These MECs were isolated from the mammary gland of adult female mice and expanded in vitro during which we retrovirally introduced SrcY530F. Transplantation of MECs into cleared mammary fat pads enables us to investigate in vivo tumor development. The studies for SrcY530F are still ongoing, but preliminary results indicate that SrcY530F can in fact induce hyperplasias and tumor formation in the mammary glands of transplanted mice. This promising in vivo model can thus be employed to investigate the cross-talk between αvβ3 and SrcY530F in breast cancer focusing on tumor growth and progression.

Integrins and Src in control of sensitivity to chemotherapeutics
The sensitivity of tumor cells to treatment with chemotherapeutic agents is modulated by interactions with the tumor microenvironment. Lastly, in chapter 5 we describe how β1 and β3 integrins affect the induction of apoptosis by DNA-damaging agents of cells expressing oncogenes. Expression of SrcY530F in the context of β1 integrins strongly increases sensitivity to DNA-damaging agents through eliciting a p53-independent apoptosis program. Cells expressing β3 integrins do not display such an increase in sensitivity, and the sensitizing effect of integrin β1 does not occur in the absence of oncogenes, or in the presence of oncogenic Ras. Our data suggest that expression of SrcY530F in the context of β1 integrins involves an elevated endoplasmic-reticulum-stress response and caspase-3 cleavage. We propose that profiling of integrin expression levels together with analyzing c-Src activity might be a valuable predictor of drug sensitivity in cancer.

In conclusion, we have discovered that interactions of cells with the extracellular matrix through distinct integrins leads to very different patterns of signaling through Rho-GTPases and Src family kinases. Thus, switching between different types of integrins can control various aspects of cellular behaviour and may play important roles during development and cancer.
REFERENCES


