The role of NCAM signaling and its effector protein, β_1-integrin, in tumor progression

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Summary

Neural Cell Adhesion Molecule (NCAM) is a member of the large family of Ca^{2+}-independent, immunoglobulin (Ig)-like cell adhesion molecules. So far, its function in homophilic and heterophilic interactions has been mainly studied in neuronal cells, where it is implicated in processes such as neurite outgrowth, axon guidance and pathfinding. Apart form its action as a cell adhesion molecule, NCAM contributes to these processes by acting as a modulator of fibroblast growth factor receptor (FGFR) signaling.

NCAM is also expressed in a number of non-neuronal tissues and changes in NCAM expression levels have been correlated with increased malignancy in various tumors. In the Rip1Tag2 mouse model of multistage tumorigenesis, deletion of NCAM expression results in the induction of tissue disaggregation, increased lymphangiogenesis and the formation of metastases. Comparison of cell lines derived from NCAM-expressing and NCAM-deficient tumors showed that NCAM binds to FGFR and thereby activates it, triggering signaling cascades that eventually lead to the activation of β_1-integrin, resulting in the promotion of cell-matrix adhesion. However, it has remained elusive how NCAM loss leads to the induction of lymphangiogenesis and whether the impaired cell matrix adhesion of NCAM-deficient cell lines accounted for the tissue disaggregation and metastasis formation observed *in vivo*.

This study investigates NCAM function on several cellular levels. In *in vitro* co-expression studies NCAM complex formation properties on the cell surface were investigated, showing that NCAM can bind to several growth factor receptors containing Ig domains in their extracellular parts. Studies focusing on the cytoplasmic events and molecular players downstream of the NCAM/FGFR complex formation, revealed a novel, inhibiting function of NCAM in modulating RTK signaling. Finally, in an *in vivo* approach, the role of β_1-integrin, a target protein of NCAM signaling, in tumor progression was analyzed by interfering with β_1-integrin expression in the Rip1Tag2 tumor model. These studies demonstrated that the induction of lymphangiogenesis in NCAM-deficient tumors was not due to the loss of β_1-integrin function and therefore employed an alternative pathway. Yet, loss of β_1-integrin induced tumor cell dissemination but not metastasis formation. Instead, a novel function of β_1-integrin in tumor progression was identified. Upon deletion of β_1-integrin, tumor size was decreased, potentially through the induction of senescence in β_1-integrin-deficient cells.

Taken together, this study has identified novel NCAM-binding proteins and provides insights into NCAM signaling on the molecular level as well as in an *in vivo* context. Furthermore, a so far unrecognized role of β_1-integrin in preventing senescence has been revealed.

Zusammenfassung

Das Neurale Zelladhäsionsmolekül (NCAM) ist ein Mitglied der grossen Familie der aus Immunoglobulin (Ig) Domänen bestehenden, Ca^{2+}-unabhängigen Zelladhäsionsmoleküle. Wie aus seinem Namen schliessen lässt, ist seine Funktion vorwiegend in neuronalen Geweben untersucht worden. Dort ist es in homophile und heterophile Interaktionen involviert und steuert Prozesse wie das Neuritenwachstum oder die Führung von Axonen. In diesen Prozessen spielt eine weitere Funktion von NCAM, nämlich die Bindung zu einem Wachstumsfaktor-Rezeptor (FGFR-1) und die Modulierung dessen Aktivität, eine essentielle Rolle.

NCAM ist auch in anderen, nicht-neuronalen Geweben exprimiert. Interessanterweise korreliert eine Veränderung des Expressionsmusters von NCAM in einigen Tumoren mit erhöhter Malignität. In einem transgenen Mausmodell für Tumorigenese von Insulin-produzierenden β Zellen führt die Deletion von NCAM zu erhöhter Lymphangiogenese, Tumor-Disaggregation und der Bildung von Metastasen. Der Vergleich von Zelllinien, die aus NCAM-exprimierenden bzw. NCAM-deletierten Tumoren gewonnen wurden, ergab dass NCAM auch in diesen Zellen mit einem Wachstumsfaktor-Rezeptor assoziiert ist (FGFR-4). Die Bindung führt zu der Aktivierung des Rezeptors, wodurch Signalkaskaden losgelöst werden, die in der Aktivierung von β_1-Integrin, einem Matrix-bindenden Protein, resultieren. Zellen, die NCAM verloren haben, zeigen daher eine geringere Fähigkeit an extrazelluläre Matritzes zu binden.

In dieser Studie untersuchten wir die zellulären Funktionen von NCAM auf verschiedenen Ebenen. In *in vitro* Studien erforschten wir, ob NCAM mit zusätzlichen Wachstumsfaktor-Rezeptoren an der Zelloberfläche interagieren kann und fanden in der Tat mehrere Rezeptoren die an NCAM binden. Ausserdem untersuchten wir auch Vorgänge innerhalb der Zelle. In Experimenten, die auf die Identifizierung der einzelnen Moleküle in der Signalkaskade abzielten, fanden wir eine neue, den Rezeptor inhibierende Funktion von NCAM.

Schlussendlich untersuchten wir, ob die Deletion des Zielproteins der NCAM-induzierten Signalkaskade, β_1-Integrin, ähnliche Auswirkungen auf die Tumorigenese hat wie die Deletion von NCAM selbst. Im speziellen waren wir daran interessiert, ob der Verlust dieser Signalkaskade zu erhöhter Lymphangiogenese führt und diese Signalkaskade somit einen Regulationsmechanimus für Lymphangiogenese darstellt. Weiters interessierte uns, ob der Verlust von β_1-Integrin zu Tumorzell-Disaggregation und dies wiederum zur Bildung von Metastasen führt. Wir fanden heraus, dass die Regulierung von Lymphangiogenese unabhängig von der NCAM induzierten Signalkaskade zu β_1-Integrin ist. Deletion von β_1-Integrin resultiert jedoch in der Loslösung von Tumorzellgruppen in lymphatische Gefässe, nicht aber in der Bildung von Metastasen. Interessanterweise sind β_1-Integrin-negative Tumoren kleiner, vermutlich, weil der Verlust von β_1-Integrin zur Zell-Seneszenz führt. Hiermit haben wir eine bis dato unbekannte Funktion von β_1-Integrin identifiziert.

1. GENERAL INTRODUCTION

1.1. Cancer

Cancer, from the Greek word carcinos (crab, crayfish) was already described and studied by the ancient Greek physician Hippocrates around 400 years BC. Today, cancer is one of the leading causes of death in the developed world. Cancer arises when cells acquire fundamental changes in their genome, leading to disturbances in their behavior within the context of a multi-cellular organism. To understand and eventually treat cancer, experimental and clinical research from the last decades has accumulated a huge and complex amount of knowledge, focusing on oncogenes with dominant gain of function mutations and tumor suppressor genes with recessive loss of function mutations as well as other molecular players of cancer development.

An increasing body of evidence suggests that tumorigenesis is a multistep process, a succession of genetic changes, leading to the progressive conversion of normal cells into cancer cells. Despite the fact that the actual molecules or changes important in tumor initiation and progression might differ among the hundreds of cancer types, it has becomes more apparent that most, perhaps all solid cancers have acquired and share a small number of common, principle properties. It has been suggested that six essential alterations in cell physiology together instruct malignant growth (Hanahan and Weinberg, 2000)

These alterations include, in the initial tumor stages, the insensitivity of cells to anti-proliferative signals, evasion of apoptosis and acquired self-sufficiency in growth signals. For further progression from benign to malignant cancers, the genetic changes lead to the gain of limitless replicative potential, sustained angiogenesis and finally the capability to invade tissues and to form metastases (Figure1).

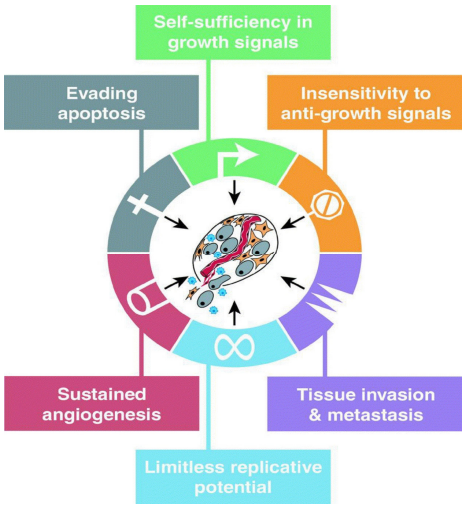


Figure 1: The Hallmarks of Cancer

Common functional traits acquired by cells during the formation of tumors and the progression to malignant cancers. Adapted from (Hanahan and Weinberg, 2000)

1.2. The Rip1Tag2 mouse model of multistep tumorigenesis

In order to study genetic alterations and the molecular mechanisms of tumorigenesis *in vivo*, various mouse models of tumorigenesis have proven to be invaluable tools. Several approaches to induce carcinogenesis have been developed over the last thirty to forty years. For example, chemical mutagens such as 7,12-dimethylbenzanthracene (DMBA) or ethyl-nitrosourea (ENU) have been used to induce skin cancers (Quintanilla et al., 1986). However, this method is currently used preferentially in combination with genetically modified mouse lines (Balmain, 2002). In another approach, tumor cell suspensions are orthotopically, subcutaneously or intravenously injected into immune-deficient mice, allowing investigators to assay potential effects of different treatments on tumors as well as to monitor capabilities of cells to metastasize to specific organs (Kubota, 1994).

However, the use of these two model systems is limiting in respect to the investigation of type and localization of primary tumor formation, and because of the absence of an intact immune system. This limitation can be overcome by the use of genetically modified mice, in which oncogenes are introduced into the mouse germ line or gene functions are ablated by homologous recombination. Further improvement can be gained through the development of techniques to induce or delete gene function in specific tissues and/or at certain time points (tissue specific, inducible transgenic mice or tissue specific, conditional knock out mice). These models therefore allow more controlled reproduction of sporadic tumor onset and progression and have been used for many proof-of-concept studies, demonstrating the causal function of a particular gene in tumor development.

The introduction of highly oncogenic viral proteins, such as for example the large T antigen (Tag) of simian virus 40 (SV40) into the mouse genome is frequently used to generate tumor-bearing transgenic mice. Tag expression disrupts cell cycle control by binding to and inactivating the tumor suppressor gene products p53 and pRb, leading to cell transformation and tumor development.

The Rip1Tag2 mouse model was established about twenty years ago (Hanahan, 1985). In these mice, Tag expression is targeted specifically to the pancreatic islets of Langerhans using the β cell specific Rat insulin promoter (Rip). In the mouse, around 400 islets of Langerhans (endocrine tissue of the pancreas) can be found embedded in the exocrine pancreas. Each islet is composed of a set of secretory cells, namely glucagon-producing α cells, somatostatin-producing γ cells, pancreatic polypeptide (PP) cells and, most abundant in islets, the insulin-producing β cells. In the Rip1Tag2 transgenic mice, T antigen expression starts at embryonic day 8.5, but it is not until the age of 4 weeks that hyperplastic islets begin to appear. Even though all islets express Tag, only 50% develop into hyperplastic lesions at the age of 10 weeks. The onset of an angiogenic switch in a subset of hyperplastic islets triggers the formation of new blood vessels, resulting in the progression to angiogenic islets at 6 weeks and solid tumors at 9 to 10 weeks of age. At 12 to 14 weeks, 2%-4% of all islets have developed into well-encapsulated, benign tumors (adenomas) and only 0.5% of all islets advance into de-differentiated, invasive carcinomas

(Figure 2). Importantly, these mice never develop metastases, probably because they succumb to hypoglycemia around the age of 14 weeks. Thus, the Rip1Tag2 model is a model for multistage tumorigenesis in which tumors of all different stages can be reproducibly found and investigated, making it a very powerful tool to study distinct molecular events that may influence tumor growth and progression as well as tumor angiogenesis, lymphangiogenesis and metastasis.

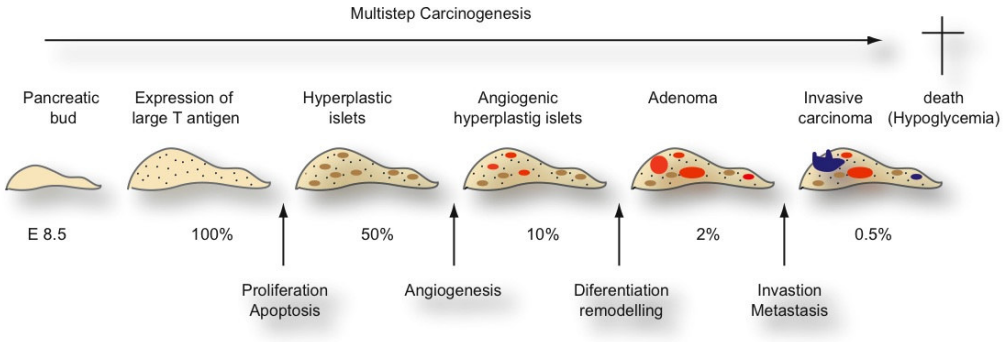


Figure 2: Multistep β cell carcinogenesis in Rip1Tag2 transgenic mice

As indicated, islets (black dots) sequentially progress into hyperplastic islets (large brown ellipses), angiogenic islets (red ellipses), benign adenoma (large red shapes), and malignant carcinoma (blue shape). Percentages indicate the subset of initial islets that have developed into a specific tumor grade at 12-14 weeks of age. The exocrine pancreatic tissue is drawn in light brown. E8.5, embryonic day 8.5 (Modified from G. Christofori, Mol Endocrinol, 1995).

1.3. Mechanisms contributing to tumor progression and metastasis

Most cancer deaths result from the formation of metastases at distant sites rather than from the primary tumors themselves. Just like primary tumor initiation, it is now believed that tumor progression and metastasis are processes that involve several rate-limiting steps. Importantly, these processes do not only involve changes in the tumor cells per se, but also require additional concerted, "pro-metastatic" contributions from the tumor microenvironment, (Kopfstein and Christofori, 2006).

After the initial transformation and growth of cells, and when tumors exceed a mass of 1 mm, tumors have to induce neo-vascularisation or angiogenesis in order to survive and further proceed. The synthesis and secretion of several pro-angiogenic factors by tumor and host cells and the absence of anti-angiogenic factors play a key role in establishing a capillary network from the surrounding host tissues. The "angiogenic switch" allows not only the tumors to have access to required nutrients but also eventually provides a route for haematogenic spread of cancer cells (Fidler, 2002; Ahmad and Hart, 1997). Blood vessel endothelial cells (BVECs) also secrete growth factors (such as PDGFs, EGFs and FGFs) that stimulate tumor cell growth and facilitate tumor lymphangiogenesis (Cao, 2005).

In order to disseminate from the primary tumor, polarized epithelial cells have to convert into motile cells, gaining a fibroblastoid, migratory and invasive phenotype. Cell adhesion molecules have been shown to be important players in this process (Cavallaro and Christofori, 2004) and will be discussed in more detail in the next section and in Chapter 1.5. Next, malignant tumor cells have to further invade the local ECM, intravasate into tumor-associated lymphatics or vasculatures, avoid immunological attacks as well as survive and proliferate in the circulation and the secondary organ tissues after extravasation. However, in these processes, tumor cells get supported by their surrounding environment. Among others, tumor associated macrophages (TAMs) and carcinoma associated fibroblasts secrete several factors, as for instance growth factors, matrix metalloproteinases (MMPs) or cytokines, supporting remodeling of the ECM, cell survival, invasion and immune tolerance (reviewed in Bogenrieder and Herlyn, 2003; Fidler, 2002; Ahmad and Hart, 1997, Kopfstein and Christofori, 2006).

1.3.1. Cell adhesion molecules in tumor progression and metastasis

The "prototype" and probably best-studied cell adhesion molecule (CAM) is E-cadherin, a member of the classical cadherin family of CAMs. Classical cadherins are single-span transmembrane domain glycoproteins, localized in adherens junctions and desmosomes. They mediate cell-cell adhesion by homophilic protein-protein interaction at the cell surface in a Ca^{2+}-dependent way.

At their cytoplasmic tails, Cadherins interact with various proteins, termed catenins, to assemble a cytoplasmic cell adhesion complex (CCC). β-catenin and γ-catenin (also named

plakoglobin) bind to the same conserved site at the C-terminus of E-cadherin in a mutually exclusive way (Ozawa et al., 1989; Nathke et al., 1994), whereas $p120^{ctn}$ interacts with multiple sites on the cytoplasmic tail of E-cadherin, including the juxtamembrane region. Direct binding of β-catenin and γ-catenin to α-catenin links the CCC to the actin cytoskeleton. Formation of the CCC is dependent on cell-cell adhesion, and conversely, disturbance of the CCC compromises cadherin-mediated cell-cell adhesion.

E-cadherin is expressed in epithelial cells and is a key player in the maintenance of cell polarity and epithelial organization (Gumbiner, 2005). In many tumors of epithelial origin, loss of E-cadherin-mediated cell-cell adhesion was observed to coincide with progression towards malignancy, and reduced E-cadherin levels were correlated with poor prognosis, suggesting a critical role of this CAM in tumor progression. In fact, forced expression of E-cadherin in cultured tumor cells lead to the re-establishment of a functional E-cadherin-catenin complex and resulted in the reversion from an invasive, mesenchymal phenotype to a benign, epithelial phenotype *in vitro* (Vleminckx et al., 1991).

Using the above described Rip1Tag2 tumor model, our group demonstrated previously that loss of E-cadherin mediated cell-cell adhesion is causally involved in the progression from adenoma to carcinoma *in vivo* and is one rate limiting step in the conversion from adenoma to carcinoma as well as the subsequent formation of metastases. Intercrossing Rip1Tag2 mice with transgenic mice that maintain E-cadherin expression in the β cells of pancreatic islets lead to arrest of tumor progression at the adenoma stage. In contrast, expression of a dominant-negative form of E-cadherin in the same tumor model induced early invasion and metastasis (Perl et al., 1998).

The mechanisms by which E-cadherin down-regulation in tumors leads to a more invasive phenotype might be similar to the mechanisms of a phenomenon that normally occurs during embryonic development, inflammation, tissue remodeling and wound healing, namely the epithelial to mesenchymal transition (EMT; Grunert et al., 2003). During EMT, cells down-regulate epithelial markers such as E-cadherin and up-regulate the expression of various mesenchymal markers, like N-Cadherin and vimentin (Thiery, 2002). Recently, our lab revealed that E-cadherin down-regulation also induced the expression of the neuronal cell adhesion molecule, NCAM (Lehembre et al., submitted). E-cadherin loss leads to the disassembly of adhesion junctions between neighboring cells, reduced cell polarity and increased migratory and invasive-growth properties. Several potential signaling pathways are thought to have an active part in this process and only two of them are discussed hereafter (Cavallaro and Christofori, 2004).

Components of the CCC, namely β-catenin and γ-catenin do not only play crucial roles in the assembly of the complex but also have important functions in the canonical WNT-signaling pathway (Bienz and Clevers, 2000; Polakis, 2000). If they are not engaged in the CCC, free cytosolic β-catenin and γ-catenin are phosphorylated by glycogen synthase 3β (GSK3β) in a complex also involving the proteins adenomatous polyposis coli (APC) and axin. Following phosphorylation, β-catenin and γ-catenin are degraded. If the tumor suppressor APC is non-

functional (as observed in many colon cancer cells), or GSK3β activity is blocked by the activated WNT-signaling pathway, β-catenin is not degraded, therefore accumulates in the cytoplasm and further translocates to the nucleus, where it binds to members of the TCF/LEF1 family of transcription factors. Thereby it modulates the expression of several target genes that are implicated in cell proliferation and progression.

Other signals induced through the loss of E-cadherin might affect the actin cytoskeleton and thereby modulate the migratory properties of cells. The family of RHO GTPases, including RhoA, Rac1 and Cdc42 are implicated in the formation of actin stress fibers, lamellipodia and filopodia (see also section 1.5.3.). E-cadherin, when engaged in cell-cell adhesion, can suppress RhoA activity. Furthermore, free (not engaged in the CCC) cytosolic $p120^{ctn}$ is able to recruit and activate Rac1 and Cdc42, thereby promoting cell migration (Cavallaro and Christofori, 2004).

In most cases, E-cadherin is down-regulated on the transcriptional level: the transcriptional repressors Snail (Batlle et al., 2000), Slug (Hajra et al., 2002) and Sip1 (Comijn et al., 2001) as well as E12/E47 (Perez-Moreno et al., 2001) bind to the promoter of the E-cadherin gene and actively repress its expression (see also section 1.5.4.). Furthermore, a negative correlation between E-cadherin levels and the expression of the transcription factor Twist have been reported (Yang et al., 2004). It is not clear, though, if Twist binds directly to the E-cadherin promoter or modulates E-cadherin levels in an indirect way. In many cancers, such as thyroid carcinomas, further down-regulation is achieved epigenetically by the subsequent silencing of the E-cadherin promoter through hypermethylation (Di Croce and Pelicci, 2003). Moreover, mutations in the E-cadherin gene that lead to the expression of a non-functional protein have been reported in patients with diffuse gastric cancer, lobular breast cancer, thyroid, bladder and gynecological cancers (Strathdee, 2002).

Finally, tyrosine phosphorylation of the CCC has been implicated in the regulation of cadherin function. RTKs such as epidermal growth factor receptor (EGFR), hepatocyte growth factor receptor (c-Met) and fibroblast growth factor receptor (FGFR), as well as Src phosphorylate E-cadherin, β- and γ-catenin and $p120^{ctn}$, resulting in the disassembly of the CCC (Behrens et al., 1993). One mechanism by which RTKs can disrupt the CCC is by targeting E-cadherin for degradation: recently, a E3 ligase named Hakai has been identified. Hakai specifically binds and ubiquitylates tyrosine-phosphorylated E-cadherin, resulting in endocytosis and proteasomal degradation of E-cadherin (Fujita et al., 2002). Interestingly, this process seems to be, at least under certain circumstances, dependent on β_1-integrin (see section 1.5.4.).

1.3.2. Tumor lymphangiogenesis

Angiogenesis, or formation of new blood vessels from pre-existing ones, is essential for normal development and wound healing. Abnormal regulation of angiogenesis has been implicated in the pathogenesis of several disorders, including cancer. Newly formed tumor-associated blood vessels do not only support tumor progression by supplying growing tumors

with oxygen but, also contribute to the metastatic process by providing a route for haematogenic metastasis of tumor cells. The molecular mechanisms underlying tumor angiogenesis have been studied to a great extent, leading to the discovery of potential targets for drug development. For example, the vascular endothelial growth factor A (VEGF-A) has been identified in playing a key role in angiogenesis. Early clinical experience with the anti-VEGF-A monoclonal antibody Avastin (Genentech) support the hypothesis that its inhibition may represent a novel approach for cancer treatment (Ferrara, 2002; Carmeliet and Jain, 2000).

Attention to tumor-lymphangiogenesis, the formation of new tumor-associated lymphatic vessels was drawn to researchers only recently. The identification of lymphatic specific markers such as the homeobox transcription factor Prox-1 (Wigle and Oliver, 1999) or the lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) (Banerji et al., 1999), as well as the development of new molecular tools advanced research in this area (Pepper and Skobe, 2003).

The physiological function of lymphatic vascular networks in the body is to drain interstitial fluid from tissues and to return it to the blood. Furthermore, lymphatic vessels are also an essential part of the body's immune defense by directing leukocytes and antigens from tissues to lymph nodes. Structurally, lymphatic vessels are distinct from blood vessels: lymphatic capillaries are thin-walled, relatively large capillaries composed of a single layer of overlapping endothelial cells. They are not covered by smooth muscle cells and have little or no basement membrane. Lymphatic capillaries are attached to tissue stroma via elastic anchoring filaments. With increasing tissue pressure, these filaments pull on the endothelial cells, opening the gaps between the overlapping cells and thereby allowing fluid influx (Alitalo et al., 2005).

Lymphatic vessels could contribute to tumor growth in various ways. They provide a route and thereby facilitate the metastatic spread of tumor cells, and indeed, lymphogenic metastasis occurs at least as frequently as haematogenous metastasis (Cao, 2005). Because of their structural features, it might be even easier for tumor cells to invade into lymphatic vessels than into blood vessels. Since lymphatic endothelial cells (LECs), similar to blood vessel endothelial cells (BVECs) express matrix metalloproteinases (MMPs) and urokinase plasminogen (uPA), they might directly or indirectly potentiate the invasiveness of tumors. Lastly, a rather controversial hypothesis suggests that lymphatic growth into tumors is induced in order to reduce the increased interstitial fluid pressure of a given tumor. However, it is not clear yet if lymphatic vessels within tumors are fully functional (Cao, 2005).

Several molecular players important for lymphatic development and lymphangiogenesis have been identified and are described in detail elsewhere (Alitalo et al., 2005). In general, the majority of lymphangiogenic signals are mediated via the vascular endothelial growth factor receptor-3 (VEGFR-3; Kaipainen et al., 1995) a member of the (Ig) domain containing receptor tyrosine kinase (RTK) family. VEGFR-3 is primarily expressed on lymphatic endothelial cells and required for the formation of lymphatic vasculature in both, embryonic development and in the adult (Karkkainen and Petrova, 2000). High affinity ligands for VEGFR-3 are vascular endothelial growth factor-C (VEGF-C; Joukov et al., 1996) and VEGF-D (Achen et al., 1998). Both factors induce, upon binding, receptor activation. The subsequent signaling events result in

lymphangiogenesis as shown in transgenic models for both factors (Mandriota et al., 2001; Veikkola et al., 2001).

Importantly, when VEGF-C and VEGF-D transgenic mice were crossed into the Rip1Tag2 tumor mouse model, both factors induced tumor-associated lymphangiogenesis and the formation of metastases (Mandriota et al., 2001; Kopfstein et al., submitted). VEGF-C expression has been reported to be induced in tumor cells and tumor-associated macrophages, but the pathways leading to VEGF-C and VEGF-D *de novo* expression in tumors remain elusive. Interestingly, ablation of the neural cell adhesion molecule (NCAM) in Rip1Tag2 mice results in VEGF-C and VEGF-D expression, and in the induction of lymphangiogenesis and metastasis formation (Crnic et al., 2004) see also section 1.4.3.

1.4. Neural Cell Adhesion Molecule (NCAM)

1.4.1. NCAM structure, expression and function

Neural Cell Adhesion Molecule (NCAM, CD56) was the first CAM to be characterized and therefore has been studied extensively (Brackenbury et al., 1977; Cunningham et al., 1987; Crossin and Krushel, 2000). NCAM is encoded by a single gene, located on chromosome 11 in humans (Nguyen et al., 1986; Walsh et al., 1986) and on chromosome 9 in mice (D'Eustachio et al., 1985). Alternative splicing gives rise to three major isoforms, named after their relative molecular weight (*M*r) (Owens et al., 1987). NCAM140 and NCAM180 are single spanning transmembrane proteins and differ in the length of their cytoplasmic domains, whereas NCAM120 is attached to the cell membrane via a glycophosphatidyl inositol (GPI-) anchor (Figure 3). In addition to the three main isoforms, the molecule also exists in a secreted form (soluble NCAM), produced by the expression of the so called SEC-exon that contains a stop-codon, giving rise to a truncated form of the extracellular part of NCAM with a *M*r of around 115kD (Bock et al., 1987; Gower et al., 1988). Soluble NCAM also exist in a shedded form, resulting from the enzymatic removal of NCAM120 from the membrane or by proteolytic cleavage of any of the three major isoforms (He et al., 1986).

NCAM belongs to the immunoglobulin-like superfamily of adhesion molecules (Ig-CAMs): the extracellular, N-terminal part of all NCAM isoforms consists of five Ig-like domains (Ig1-5) followed by two fibronectin type III (F3) modules proximal to the membrane (Cunningham, 1995). Variability in the extracellular part is obtained by the optional insertion of additional exons, as for example the variable alternative spliced exon (VASE) in the Ig4 domain that is expressed at high levels in the adult central nervous system, serving to down-regulate axon growth (Doherty et al., 1992; Figure 3)

Further variation of NCAM expression, and therefore function, is achieved by posttranslational modifications of the protein. Attachment of the negatively charged sugar polysialic acid to the fifth Ig domain induces a shift in the adhesive properties of NCAM (Fujimoto et al., 2001), changing it form a pro-adhesive to a pro-migratory molecule, facilitating axon path-finding and plastic changes in the embryonic and adult nervous system (Hoffman et al., 1982; Angata and Fukuda, 2003; Bruses and Rutishauser, 2001). Additional post-translational modifications are found on the C-terminal, intracellular domains of NCAM140 and NCAM180. Both cytoplasmic domains can be palmitoylated, which determines NCAMs association with lipid rafts in the membrane and thereby its signaling properties (Brackenbury et al., 1987; Little et al., 1998).

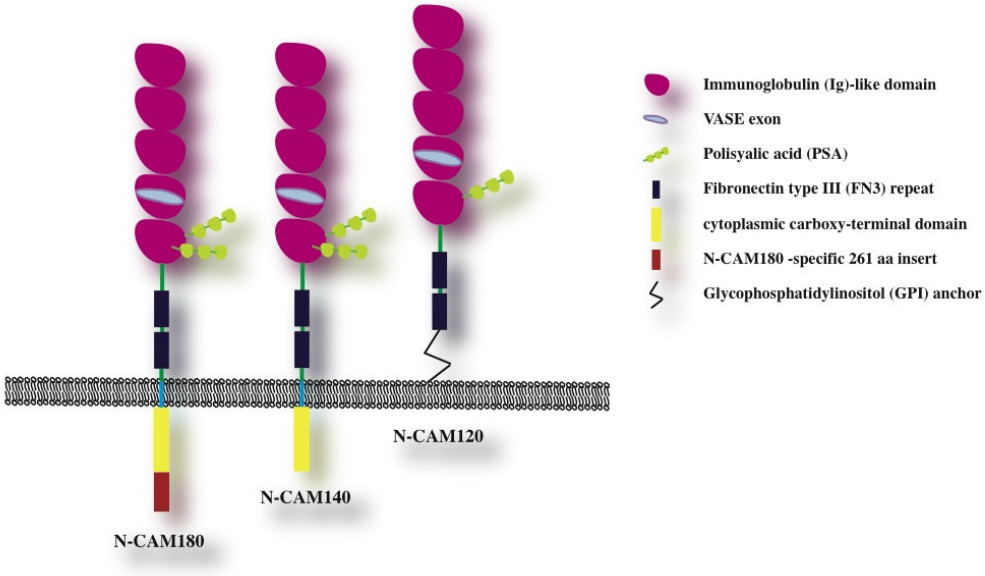


Figure 3: The three major NCAM isoforms

NCAM is expressed as three major isoforms. Two of them are transmembrane forms with either long (NCAM180) or short (NCAM140) cytoplasmic domains, while the third isoform (NCAM120), lacking a cytoplasmic domain, is anchored to the plasma membrane by a GPI-linkage. All three isoforms have five Ig-like domains and two Fibronectin type III (FN3) domains in the extracellular region. NCAM180 has an additional 261 amino acid insert at the cytoplasmic tail. The alternatively spliced VASE exon in the Ig4 domain (see text) is also indicated. All three isoforms can be post-translationally modified by the addition of polysialic acid (PSA) to the Ig5 domain.

NCAM is a Ca^{2+}-independent adhesion molecule and engaged in both, homophilic and heterophilic interactions. This, together with the above described transcriptional and post-translational modifications of the NCAM protein, results in a variety of adhesive properties and functions. During development, NCAM140 and NCAM180 are transiently expressed in the nervous system as well as in several other tissues (Crossin et al., 1985). Expression of these isoforms, also called “embryonic” isoforms, plays a pivotal role in developmental events such as neuronal cell migration, differentiation and proliferation (Walsh and Doherty, 1997; Kiss and Muller, 2001). In the adult, however, NCAM120 is the major isoform to be expressed in the nervous system (Gower et al., 1988), in skeletal muscle cells (Dickson et al., 1987) as well as some neuroendocrine tissues (Rouiller et al., 1990; Cirulli et al., 1994; Langley et al., 1989).

1.4.2. More than just an adhesion molecule-NCAM's role in signal transduction

The nature of NCAM homophilic interaction and its role in cell-cell adhesion has been studied in great detail (reviewed in Walmod et al., 2004). However, NCAM is also involved in heterophilic interaction with several proteins, both via the extracellular as well as the intracellular regions of the protein. In neuronal PC-12 cells, NCAM function results in the induction of long neuronal processes called neurites (Doherty et al., 1991). Neurite outgrowth therefore has been repeatedly used as a read-out assay to study NCAMs intracellular signaling, mediated by the induction of downstream signal transduction pathways through the direct or indirect interaction of NCAM with heterophilic ligands.

Already 10 years ago, it has been suggested that NCAMs induction of neurite outgrowth involves an interaction with the fibroblast growth factor receptor (FGFR; Doherty and Walsh, 1996; Kiselyov et al., 2003). Several studies using dominant negative versions of FGFR (Ronn et al., 2000; Saffell et al., 1997) as well as specific inhibitors of enzymes (Kolkova et al., 2000) and second messenger molecules (Williams et al., 1994a) lead to the following model (depicted in Figure 4): Upon binding to the FGFR, NCAM stimulates FGFR dimerisation and activation by auto-phosphorylation. Subsequently, several proteins dock to the receptor's cytoplasmic tail, one of them being the enzyme phospholipase Cγ (PLCγ), which, upon recruitment, becomes activated. PLCγ cleaves its substrate phosphatidylinositol 4,5-bisphosphate (PIP_2), generating the second messenger molecules inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 induces the release of Ca^{2+} by binding to intracellular Ca^{2+}-channels, whereas DAG remains at the membrane and can either activate protein kinase C (PKC) or be converted (by DAG lipase) into 2-arachidonylglycerol (2-AG) and arachidonic acid (AA), inducing various downstream signaling events (Walmod et al., 2004).

Since treatment of cells with AA has been shown to induce Ca^{2+}-influx and neurite outgrowth, AA was thought to be the signal-transmitting product downstream of DAG lipase (Williams et al., 1994b). However, it has been recently shown that FGF-induced neurite outgrowth is mediated by 2-AG (Williams et al., 2003). 2-AG can activate the cannabinoid receptors CB1 and CB2 that subsequently, among other signaling events, induce calcium influx. Addition of AA to cells might lead to increased levels of 2-AG and therefore indirectly lead to stimulation of 2-AG signaling. However, the importance of this process in NCAM-mediated neurite outgrowth remains to be determined (Williams et al., 2003).

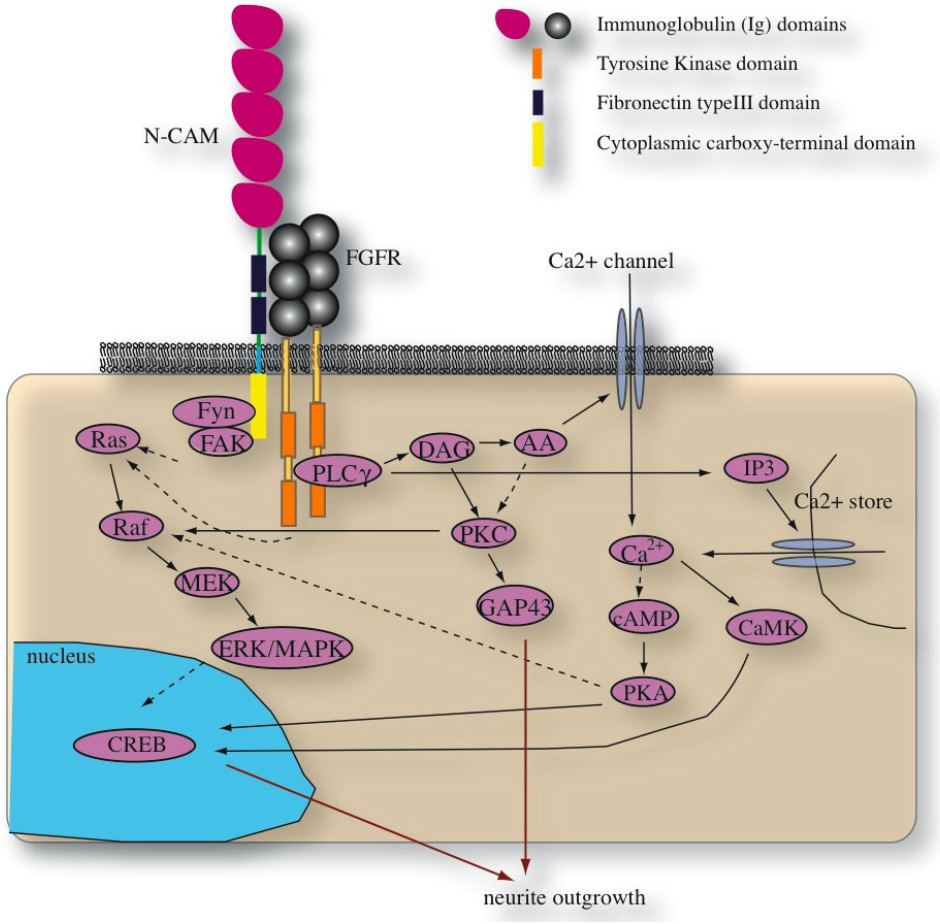


Figure 4: NCAM-mediated signal transduction pathways

NCAM induces different signal transduction pathways resulting in neurite outgrowth (see text). The structure of physical interaction between two fibronectin type III domains of NCAM (see also Figure 3) and Ig domains 2 and 3 of FGFR has been recently shown (Kiselyov et al., 2003). Dashed lines represent putative interactions. This is a simplified depiction, modified from (Povlsen et al., 2003).

In addition to signaling through FGFR, NCAM has been shown to signal and induce neurite outgrowth via non-receptor tyrosine kinases: clustering of the NCAM140 isoform in the neural plasma membrane stimulates the activating phosphorylation of mitogen-activated protein kinases (MAPKs) and the transcription factor cyclic AMP response-element binding protein (CREB). NCAM clustering transiently induces dual phosphorylation (activation) of the MAPKs ERK1 and ERK2 (extracellular signal-regulated kinases) by a pathway regulated by the focal adhesion kinase FAK and $p59^{Fyn}$, both of which have been shown to associate with NCAM140 (Beggs et al., 1997; Schmid et al., 1999; Figure 3). Furthermore, recent reports show that NCAM can interact with glial cell line derived neurotrophic factor (GDNF) and the GDNF family

receptor GFRα. The interactions induce neurite outgrowth in an FGFR-independent manner, involving signaling mediated via $p59^{Fyn}$ (Paratcha et al., 2003). However, it is not yet clear if the above mentioned signaling events involving $p59^{Fyn}$, FAK and MAPK also require GDNF and GFRα. Whether NCAM signals through FGFR/PLCγ or $p59^{Fyn}$/FAK depends on its localization on the membrane: only lipid-raft associated NCAM can induce $p59^{Fyn}$/FAK signaling, in contrast, FGFR signaling is induced by a non-raft fraction of NCAM. Still, both pathways are required for NCAM induced neurite outgrowth (Niethammer et al., 2002). Interestingly, our lab has shown that depletion of E-cadherin leads to increased expression of NCAM. Upon upregulation, NCAM molecules cluster and mediate homophilic binding, thereby modulating cell-cell contacts. Furthermore, NCAM localizes to lipid rafts and switches from a FGFR/PLCγ- to a $p59^{Fyn}$-containing complex leading to an increased number of focal contacts (Lehembre et al., submitted)

A recent study, moreover, revealed that NCAM is linked to the cytoskeleton via the linker protein spectrin. Spectrin seems to be the bridge between NCAM and PKCβII. Whereas NCAM140 and NCAM180 associate with spectrin and PKCβII independent of lipid raft integrity, NCAM120's association to these proteins is only found in a raft-dependent manner. However, FGFR-dependent formation of this complex is necessary for the induction of neurite outgrowth, indicating that the physical link of NCAM to the cytoskeleton is important for its signaling properties (Leshchyns'ka et al., 2003).

1.4.3. The role of NCAM in tumor progression: Rip1Tag2;NCAM knock-out mice

As already mentioned, NCAM is also expressed in non-neuronal tissues such as skeletal muscle cells (Dickson et al., 1987) as well as some neuroendocrine tissues (Rouiller et al., 1990; Cirulli et al., 1994; Langley et al., 1989). Interestingly, in many tumors, such as Wilm's tumor (the most common kidney cancer affecting children), colon carcinoma, Ewing sarcoma (Peripheral Primitive Neuroectodermal Tumors (PNET) of bone), neuroblastoma, small cell lung cancer and multiple myeloma, NCAM expression changes from the adult, NCAM120 isoform in normal tissue to the embryonic, NCAM140 and NCAM180 isoforms in tumors (Johnson, 1991; Kaiser et al., 1996; Lipinski et al., 1987; Moolenaar et al., 1992; Roth et al., 1988).

Furthermore, cancer progression correlates with up-regulation of NCAM in neuroblastoma and certain neuroendocrine tumors, and up-regulation of NCAM often coincides with extensive polysialylation (Komminoth et al., 1991; Angata and Fukuda, 2003; Lantuejoul et al., 1998; Lantuejoul et al., 2000; Gluer et al., 1998). In contrast, reduced or lost expression of NCAM in human astrocytoma, colorectal and pancreatic cancer has been correlated with increased tumor malignancy (Fogar et al., 1997; Sasaki et al., 1998; Huerta et al., 2001). These findings prompted our group to investigate NCAMs function during tumor progression by ablation of NCAM expression in Rip1Tag2 tumorigenesis.

Even though NCAM plays many important roles during development, NCAM knock-out mice (NCAM-/- mice) carrying a deletion in one or both alleles are born normally, are viable,

fertile and appear healthy. Adult mutants, however, show reduced brain weight and olfactory bulb size, deficits in spatial learning, altered exploratory behavior, increased intermale aggression and increased anxiety-like behavior (Stork et al., 1997; Ono et al., 1994; Tomasiewicz et al., 1993; Cremer et al., 1994; Stork et al., 1999). Moreover, in pancreatic islets of NCAM-deficient mice the normal localization of glucagon-producing α cells in the periphery of pancreatic islets is lost, resulting in a more randomized cell distribution (Esni et al., 1999). Notably, the islet-developmental phenotype is the same in NCAM+/- as well as in NCAM-/- mice, suggesting that gene dosage is important for NCAM function in this tissue (Esni et al., 1999).

During Rip1Tag2 tumorigenesis, NCAM expression changes from the adult, NCAM120 isoform to the embryonic NCAM140 and NCAM180 isoforms (Perl et al., 1999). When intercrossed with NCAM-/- mice, the resulting RipTag2;NCAM-/- mice (RT2;NC-/- mice hereafter) do not show altered tumor progression from adenoma to carcinoma. However, in 50% of NCAM-deficient tumor mice, formation of metastases to the regional lymph nodes as well as distant organs is observed (Perl et al., 1999). Importantly, formation of metastases has not been found in hundreds of Rip1Tag2 mice analyzed. The metastatic phenotype of RT2;NC-/- mice is indistinguishable from that of RT2;NC+/- mice, which is in accordance with the above mentioned haploinsufficiency of the NCAM gene in NCAM+/- mice (Esni et al., 1999).

Immunohistochemical examination revealed that NCAM-deficient Rip1Tag2 tumors exhibit up-regulated expression of the lymphangiogenic factors VEGF-C and -D and, with it, increased lymphangiogenesis (Figure 5). Repression of VEGF-C and -D function by adenoviral expression of a soluble form of their cognate receptor, VEGF receptor-3, results in reduced tumor lymphangiogenesis and lymph node metastasis (Crnic et al., 2004), indicating that loss of NCAM function causes lymph node metastasis via VEGF-C- and VEGF-D-mediated lymphangiogenesis.

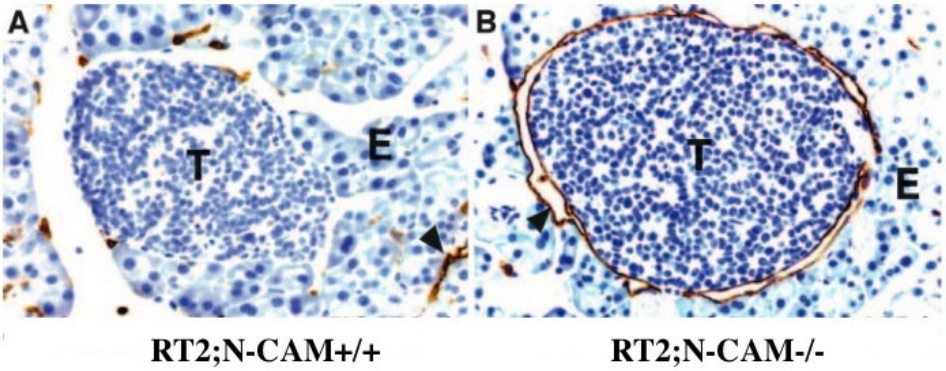


Figure 5: Lymphangiogenesis is induced in RT2;NCAM-/- and RT2;NCAM+/- mice
Immunohistochemical staining of PFA-fixed pancreatic sections. Whereas RT2;NC+/+ tumors are rarely associated with lymphatic vessels (panel A), NCAM-deficient tumors (panel B) show ongoing lymphangiogenesis, often being fully surrounded by structures positive for the lymphatic endothelial marker LYVE-1 (in brown, highlighted by arrowheads). T, tumor; E, exocrine pancreas;

Additionally to the increased lymphangiogenic phenotype, RT2;NC-/- tumors show striking alterations in tumor architecture, notably dramatic tissue disaggregation and the appearance of large hemorrhagic cavities. Clusters of tumor cells are frequently found floating in these lacunae (Cavallaro et al., 2001; Figure 6). Detailed analysis of cell lines derived from RT2 control tumors (βT2 cells) and RT2;NC-/- tumors (βTN2 cells) revealed that cell-matrix but not cell-cell adhesion is impaired in NCAM deficient tumor cells, as assayed by their capability to adhere to the ECM component Collagen IV. Moreover, βT2 cells show the formation of neurites, which are absent in βTN2 cells.

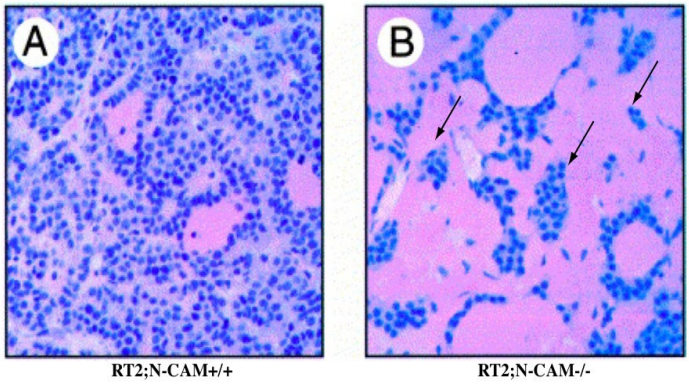


Figure 6: Tissue disaggregation in RT2;NCAM-/- tumors
H&E staining of RT2;NCAM+/+ (A) and RT2;NCAM-/- (B) tumors. In the absence of NCAM, tumors seem to fall apart, showing tissue disaggregation and the appearance of hemorrhagic lacunae filled with disseminated tumor cell clusters (indicated by arrows).

These observations suggested that NCAM is able to modulate integrin mediated matrix adhesion, and, similar to the findings in neuronal cells, might be involved in the regulation of signaling processes in β cells. Extensive biochemical analysis indeed identified the signaling

pathway(s) linking NCAM to β_1-integrin activation *in vitro*. In βT2 cells, NCAM associates with and activates a member of the fibroblast growth factor receptor (FGFR) family, FGFR-4. Interestingly, N-cadherin but not β_1-integrin is also found in the NCAM/FGFR-4 complex.

Activation of FGFR-4 results in the formation of a classical signaling complex, including phospholipase C gamma (PLC-γ), the adaptor protein FRS2, pp60 (c-src), cortactin and growth-associated protein-43 (GAP-43). Dominant-negative FGFR-4, inhibitors of FGFR signaling and anti-β_1-integrin antibodies repress matrix adhesion induced by NCAM. FGF ligands can replace NCAM in promoting matrix adhesion but not neurite outgrowth (Cavallaro et al., 2001). Taken together, the results indicate that NCAM stimulates β_1-integrin-mediated cell-matrix adhesion by activating FGFR signaling (Figure 7).

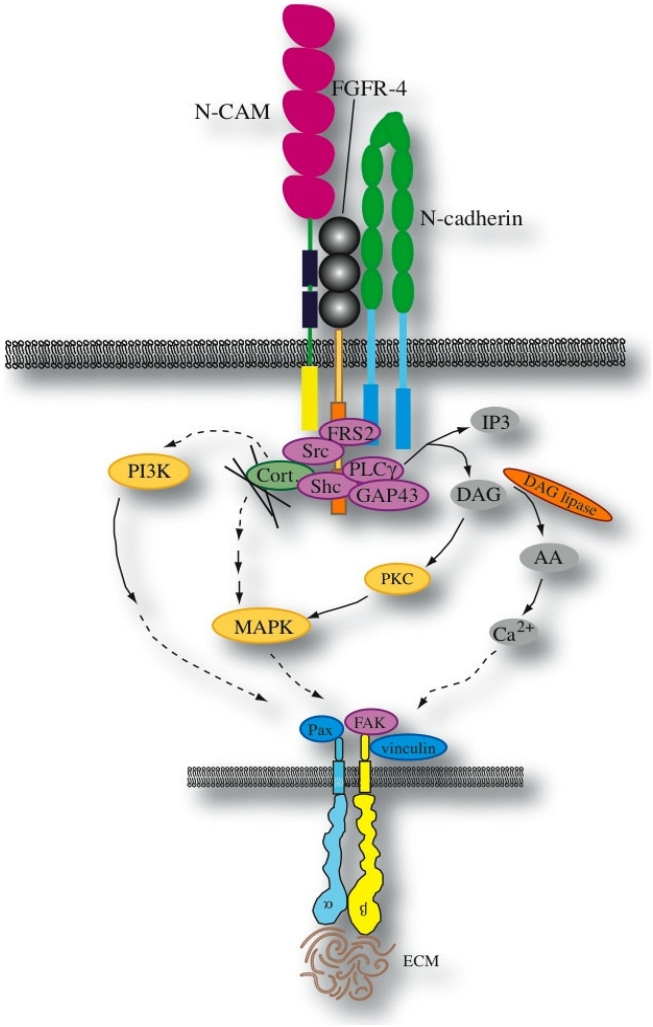


Figure 7: Model of NCAM mediated signal transduction in β cells

Upon associating with NCAM and N-cadherin, FGFR-4 is activated and recruits a classical FGFR-signaling complex (see text for description). The molecular links between NCAM and integrin activation remain to be elucidated and are indicated by dashed arrows. Adapted from (Cavallaro and Christofori, 2004).

In summary, the above described findings for the functional contribution of NCAM to Rip1Tag2 β cell tumorigenesis lead to the rather provocative suggestion of an alternative

metastatic pathway: Loss of NCAM leads to tissue disaggregation and the formation of lacunae potentially by the loss of substrate adhesion of β cells. However, the cells comprising the disseminated tumor clusters are still able to adhere to each other and exhibit a rather benign phenotype. They might be simply washed out by the blood or lymphatic circulation. Subsequently, they might be entrapped in the local lymph nodes where they grow out to form metastases.

In contrast to the current view on metastatic processes, this model of "passive metastasation" implies that, in order to metastasize, tumor cells do not necessarily have to acquire an migratory, invasive phenotype, for example by losing E-cadherin function. The finding that in the Rip1Tag2 tumor model, induction of lymphangiogenesis by transgenic expression of the lymphangiogenic factor VEGF-C alone is sufficient to induce metastasis supports this idea (Mandriota et al., 2001).

A recent report associated the occurrence of blood filled cavities in RT2;NCAM-/- mice with perturbed pericyte-endothelial cell-cell interactions. This study demonstrated that NCAM promotes pericyte recruitment during tumor angiogenesis. NCAM-deficient tumors have deficient pericyte-endothelial interactions and therefore show increased blood vessel leakage. Furthermore, pericyte deficiency per se was shown to cause haematogenous spreading of tumor cells and metastasis formation (Xian et al., 2006). Clearly, however, more experiments are needed to unravel NCAM function in inducing lymphangiogenesis, cell-matrix adhesion and the formation of metastases.

1.5. Integrins

1.5.1. Function and composition of integrins

The extracellular Matrix (ECM) is composed of an insoluble network of proteins that are secreted, assembled and remodeled throughout the lives of cells. Its function is not only to provide a shapeable, but still robust scaffold for the organization of cells in tissues. It also exerts control on the behavior of cells. Hence, it is able to dictate whether cells will proliferate or undergo growth arrest, migrate or remain stationary, or undergo apoptosis (Guo and Giancotti, 2004). Integrins are the major receptors for extracellular matrix proteins and the effects of the ECM on cells are mainly mediated by members of this large family of cell-surface receptors. By binding to ECM, integrins not only mediate adhesion but also organize the cytoskeleton and activate intracellular signaling pathways. In vertebrates, integrins also play certain roles in cell-cell adhesion (Hynes, 2002).

Each integrin consists of two type-I transmembrane subunits, one α and one β subunit. Mammals have a set of 18 α and 8 β subunits that so far are known to assemble in various combinations to form 24 distinct integrins (Figure 8). Depending on their composition, integrins bind to distinct ECM components: one set (blue in Figure 8) recognizes the tripeptide sequence RGD present in molecules such as fibronectin and vitronectin, another set (pink in Figure 8) binds to basement membrane laminins. Additionally, there is a set of integrins representing collagen receptors, and some recognize both ECM proteins, such as fibronectin, and Ig-CAM cell surface receptors, such as VCAM-1. Vertebrates also have a set of leukocyte specific integrins that also recognize Ig-superfamily counter-receptors and mediate heterotypic cell-cell adhesion. Both subunits of a given $\alpha\beta$ integrin determine the ligand specificity and it should be noted that Figure 8 is a simplified depiction.

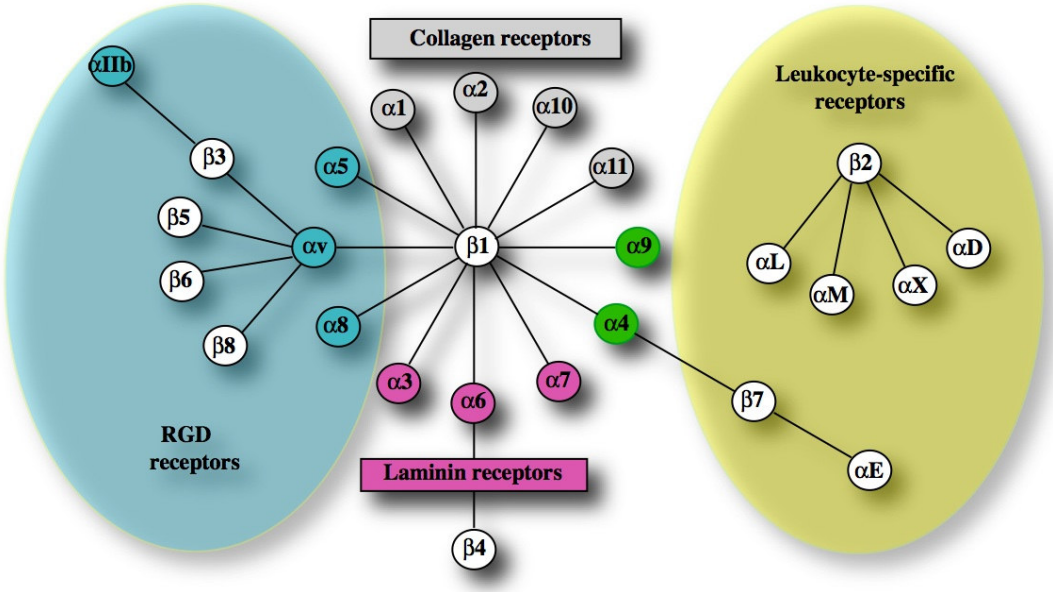


Figure 8: The integrin family in vertebrates

8 β subunits can assort with 18 α subunits to form 24 distinct integrins, which can be considered in several subfamilies, based on evolutionary relationships (coloring of subunits), ligand specificity and, in the case of β_2 and β_7 integrins, restricted expression on white blood cells. Adapted from (Hynes, 2002).

Interestingly, each integrin seems to have a specialized, unique function. All β subunits and 14 α subunits have been knocked-out in mice, and each phenotype is distinct, ranging from pre-implantation development block (β_1-integrin knock out) through major developmental defects, peri-natal lethality and defects in hemostasis, inflammation, angiogenesis as well as many others (Hynes, 2002). This indicates that, in spite of the big number of different integrins, the function of a particular integrin is non-redundant and therefore cannot be complemented by other integrins.

1.5.2. Integrin signaling

Integrins signal through the cell membrane in either direction. Extracellular binding activity to ECM proteins is dictated from signals arising from within the cells, so called inside-out signaling. For example, the main integrin on circulating platelets is $\alpha_{IIb}\beta_3$. Importantly, it is normally inactive, but upon activation by various stimuli (e.g. thrombin, ADP and others) from within the platelets, $\alpha_{IIb}\beta_3$ binds to its ligands fibrinogen, van Willebrand factor and fibronectin.

If it were not inactive in the place, platelets would bind fibrinogen from the plasma and aggregate, leading to thrombosis (Giancotti and Ruoslahti, 1999; Ginsberg et al., 2005).

As already indicated, all integrin subunits are type I transmembrane proteins with large extracellular domains, a single-pass transmembrane (TM) domain and a small cytoplasmic tail. The cytoplasmic tails are devoid of enzymatic features, therefore, integrin-signals are transduced by adaptor proteins that connect integrins to the cytoskeleton, to the cytpolasmic kinases and finally to transmembrane growth factor receptors. Once an integrin has bound to the ECM, it elicits signals that are transmitted into the cell (outside-in signaling). Upon binding, integrins cluster in the plane of the cell membrane and associate with a cytoskeletal signaling complex that promotes assembly of actin filaments. The re-organization of actin filaments into larger stress fibers in turn causes more integrin clustering, enhancing matrix binding. The resulting aggregates of ECM proteins, integrins and cytoskeletal proteins are known as focal adhesions (Burridge and Chrzanowska-Wodnicka, 1996) and this is what integrins were named after: they serve as integrators of the ECM and the cytoskeleton (Giancotti and Ruoslahti, 1999)

The molecular players involved in integrin signaling are too many, and the networks are too complex to be discussed here in detail. Generally, as described in Figure 9, most integrins signal through Src-family kinases (SFK) that get recruited and activated via the activation of focal adhesion kinase (FAK), which in turn is dependent on the assembly of focal adhesions. FAK also activates signaling through phosphatidylinositol 3-kinase (PI3K) and therefore AKT/protein kinase B (PKB). Src can phosphorylate $p130^{CAS}$ and paxillin, which engages the Crk-DOCK180 complex and results in the activation of Rac. FAK also activates extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) by employing the RAP1 guanine nucleotide exchange factor (GEF) C3G through Crk, resulting in the activation of ERK/MAPK by B-Raf.

In an alternative pathway, FAK activates ERK/MAPK via the recruitment of the growth factor receptor bound-2 (GRB2) and son-of-sevenless (SOS) complex (Schlaepfer and Hunter, 1998; Cary et al., 1999). Some integrins are able to directly interact with SFKs with their β subunits (Arias-Salgado et al., 2003), whereas others are coupled to palmitoylated SFKs (such as Fyn and Yes) through their α subunits. The palmitoylated SFKs activate the adaptor SHC, which combines with GRB2-SOS to activate ERK/MAPK signaling from Ras (Wary et al., 1996). In this pathway, caveolin is needed to facilitate the recruitment of Fyn and Yes (Wary et al., 1998). The pathways that integrins activate through SFKs are sufficient to induce cell migration and survival signals (see below).

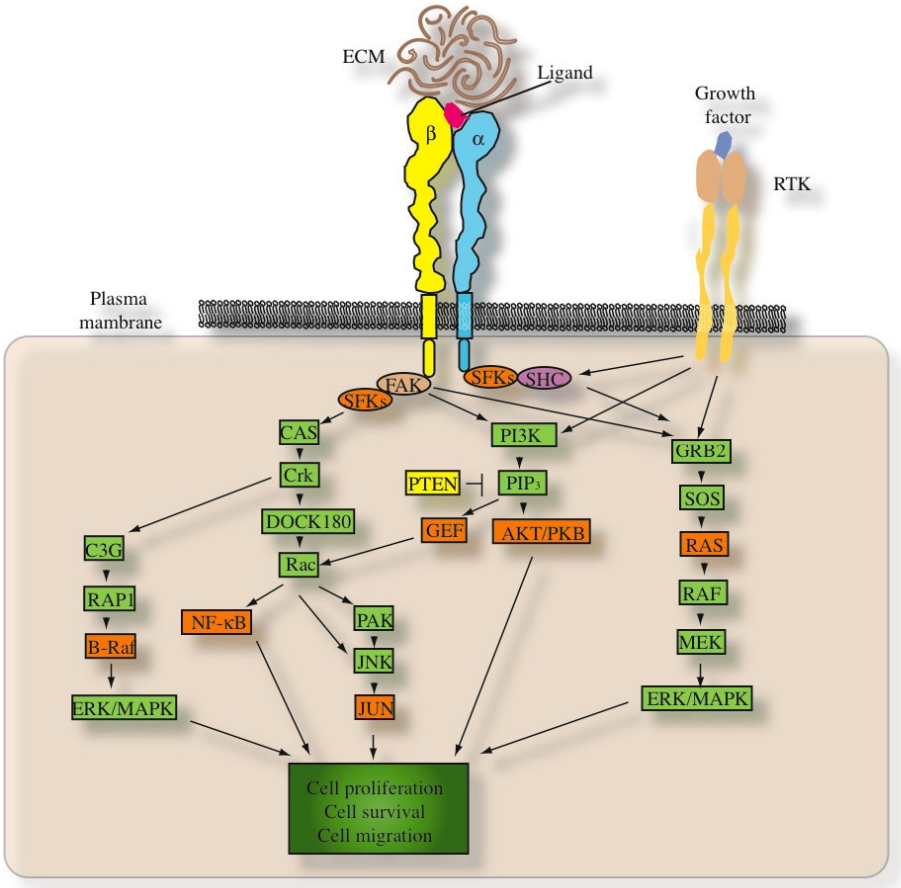


Figure 9: Pathways employed by integrin signaling

Integrins employ several pathways to transduce signals resulting in different cellular behavior: Focal adhesion kinase (FAK) can activate (either directly or indirectly) ERK/MAPK via the Grb2/Ras/Raf or the Crk/Rap1/B-Raf pathway. Alternative pathways via Rac result in JUN and NFκB activation or AKT/PKB activation via PI3K (see text). Adapted from (Guo and Giancotti, 2004).

Integrins not only signal on their own; they are also necessary for optimal growth factor activation. Integrin clustering and association with the cytoskeleton gives rise to integrin-growth factor receptor complexes. Only under appropriate cell attachment conditions, growth factor receptors such as platelet derived growth factor receptor (PDGFR), VEGFR, and epidermal growth factor receptor (EGFR) are optimally activated by their respective ligands, which describes the basic mechanisms of anchorage dependence of cell survival and proliferation.

1.5.3. The functions of integrin signaling

What are the results of integrin signaling? Integrin signals are essential for cells to traverse the cell division cycle (progression through G_1). This is, among other factors, mediated through cyclin D1 expression, since cells that are not properly attached show suppressed cyclin D1 levels. The composition of the ECM is important in this respect: myoblasts, for example, proliferate on fibronectin but stop proliferation and form myotubes on laminin, implying that integrin signaling also regulates, or at least is necessary, for differentiation.

Loss of attachment causes apoptosis in many cell types, a phenomenon called anoikis. Just like cell growth, anoikis can be regulated in an integrin-specific manner, meaning that integrin signaling induces apoptosis in non-attached cells, but gives rise to survival signals in properly located cells (Meredith et al., 1993; Frisch et al., 1996). Importantly, most cells in adult organisms are not actively dividing, therefore it is likely that other cell adhesion proteins override the growth-promoting but not the survival-promoting effects of integrins. This contact inhibition of growth, together with integrin-induced anoikis or cell survival respectively, ensures the development and maintenance of proper tissue architecture.

Integrins also regulate cell spreading and migration: a cell that comes in contact with ECM usually extends filopodia. Integrins at the tips of these filopodias bind to the ECM and induce the formation of focal adhesions. Subsequently, actin-rich lamellipodia are generated and the cells spread on the ECM, followed by the full development of focal adhesions and actin stress fibers. During cell migration, these same events occur as cells extend lamellipodia and form focal adhesions to derive the traction necessary for movement (Giancotti and Ruoslahti, 1999). Integrins regulate these events by activating the Rho-family of small guanine nucleotide-binding proteins, in which Cdc42 induces filopodia, Rac lamellipodia, and Rho induces focal adhesion and associated stress fibers, each of them further controlling the cytoskeleton by associating with downstream effectors (Hall, 1998).

Taken together, integrins can be seen as one class of “master regulators”, being important for the establishment and maintenance of tissue architecture.

1.5.4. Integrins in tumor progression

As already mentioned in section 1.1 and 1.3, cells that have undergone neoplastic transformation are much less dependent on the ECM for their proliferation and survival. To eventually form metastases, cancer cells have to undergo critical changes affecting their invasive properties. Given the important role of integrins, it is not surprising that in many tumors mutation in genes playing a role in integrin signaling have been identified (Guo and Giancotti, 2004).

Importantly, despite their relative anchorage independence, tumor cells can still take advantage of integrin signals by preferentially expressing integrins that favor proliferation, survival and migration. These changes in integrin expression are complex and depend on the

tissue of origin of the tumor, its histological type and the stage of progression of the disease (Zutter et al., 1995; Albelda et al., 1990; Gladson and Cheresh, 1991). Cell type-dependent changes in integrin signaling make it impossible to assign each of the integrins to the "anti-neoplastic" or "pro-neoplastic" category. It seems that $\alpha_2\beta_1$ and $\alpha_3\beta_1$, at least in some cases, suppress tumor progression, whereas $\alpha_v\beta_3$, $\alpha_v\beta_6$, and $\alpha_6\beta_4$ often promote it (Guo and Giancotti, 2004). A recent study, however, demonstrated that β_1-integrin is essential for the formation of mammary tumors in a mouse model of human breast cancer, but not for the initial stages of mammary ductal outgrowth, suggesting that the primary function of β_1-integrin is to promote cell proliferation (White et al., 2004).

As discussed in section 1.3.1, loss of E-cadherin-mediated adhesion is required for malignant conversion. Increasing evidence indicates that joint integrin-receptor tyrosine kinase (RTK) signaling contributes to disrupting cell-cell adhesion in cancer cells. Blocking β_1-integrin in a 3D culture of breast carcinoma cells induced these cells to re-assemble adherens junctions and deposit a basement membrane, giving rise to acini characterized by a distinct polarity (Weaver et al., 1997), whereas over-expression of β_1-integrin caused the disruption of adherens junction in normal epithelial cells (Gimond et al., 1999).

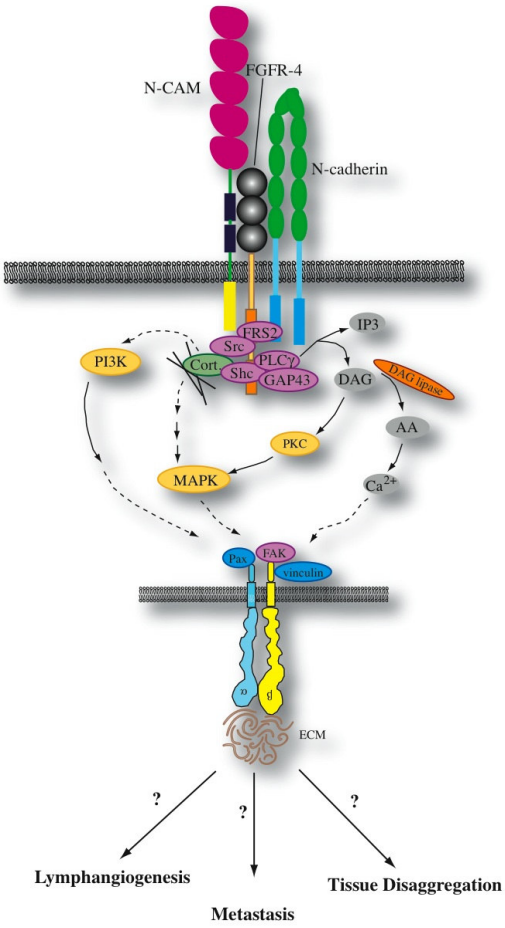
Two mechanisms seem to be involved in disrupting cell-cell adhesions: activated RTKs and SFKs induce tyrosine phosphorylation of components of the E-cadherin-β-catenin complex (see also section 1.3.1). The tyrosine-phosphorylated complex is recognized by the E3 ubiquitin protein ligase Hakai and therefore down-regulated by endocytosis (Fujita et al., 2002). This process requires integrin function and FAK phosphorylation in v-Src transformed cells, suggesting that v-Src promotes endocytosis of E-cadherin by enhancing integrin signaling (Avizienyte et al., 2002). Secondly, integrin signaling operates through SNAIL/SLUG to suppress E-cadherin expression and consequently disturb adherens junctions. Both, integrin linked kinase (ILK) and in another study SFKs have been implicated in this mechanism (Novak et al., 1998; Tan et al., 2001; Zhang et al., 2003)

In normal cells, loss of cell-matrix adhesion induces anoikis (see section 1.5.3). Resistance to anoikis is essential for metastatic dissemination of tumor cells. FAK promotes the survival of cells by signaling through PI3K to AKT/PKB (Frisch et al., 1996; Khwaja et al., 1997) and many invasive human cancers have elevated levels of FAK (Gabarra-Niecko et al., 2003), which also has been implicated in a more migratory phenotype of cells. FAK also promotes the expression of anti-apoptotic and suppression of pro-apoptotic stimuli (Guo and Giancotti, 2004). Integrins contribution to tumor progression have been implicated in many more processes, such as angiogenesis or matrix remodeling, which is described in great detail elsewhere (Guo and Giancotti, 2004; Hood and Cheresh, 2002; Hynes, 2002).

Summarizing the effects of integrins in tumor development, it seems that each tumor type undergoes characteristic and dynamic changes in integrin expression and function during tumor progression. Future studies might uncover the full complexity of these changes. Testing the relevance of these changes in integrin expression and signaling in mouse models of cancer could open the way to the development of (potentially anti-integrin) compounds for tumor therapy.

1.6. Aims of the study

Deletion of NCAM in Rip1Tag2 tumors results in the induction of lymphangiogenesis, tissue disaggregation and the formation of metastases. In β tumor cells, NCAM associates with FGFR-4 and N-cadherin, thereby the receptor is activated and signaling cascades, leading to the activation of β_1-integrin and hence cell-matrix adhesion are induced. We therefore wanted to investigate whether ablation of β_1-integrin function in Rip1Tag2 tumors pheno-copied the NCAM deletion.



Specific research goals:

- To determine whether the *in vitro* observed lack of β_1-integrin activation in NCAM deleted tumor cells leads to tumor tissue disaggregation *in vivo*, we planned to interfere with β_1-integrin expression specifically in the Rip1Tag2 tumor model.

- Using this approach, we furthermore analyzed whether increased lymphangiogenesis in NCAM knock-out tumors lies downstream of β_1-integrin inactivation and/or tissue-disaggregation or whether it is the result of an alternative, β_1-integrin-independent pathway.

- By interfering with β_1-integrin expression we furthermore wanted to determine whether loss of NCAM-dependent activation of β_1-integrin alone is sufficient to induce the formation of metastasis.

- Using specific inhibitors or stimulating reagents on cells and assaying for cell-matrix adhesion we wanted to dissect and compare the pathways underlying NCAM and FGF induced β_1-integrin activation *in vitro*.

- In co-expression and immunoprecipitation studies, we aimed to investigate whether NCAM can associate with and modulate additional receptor tyrosine kinases.

NCAM binds to the PDGFRβ and potentially reduces its activity

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Abstract

Neural cell adhesion molecule has been studied mainly in neurons where it influences processes, such as neurite outgrowth, axon guidance and pathfinding by homophilic and heterophilic interaction with the fibroblast growth factor receptor FGFR-1. We have previously shown that NCAM modulates FGFR-4 signaling also in non-neuronal tissues by exerting stimulating effects as well as inhibiting ligand-induced signaling. Here, we show that NCAM associates with a number of receptor tyrosine kinases (RTKs). In particular, NCAM binding to the platelet derived growth factor receptor β (PDGFRβ) represses its activity. Our results suggest that NCAM acts as a general modulator of Ig-domain containing RTKs.

2. NCAM binds to the PDGFRβ and potentially reduces its activity

2.1. Introduction

The neural cell adhesion molecule (NCAM) is a member of the immunoglobulin-like superfamily of Ca^{2+} -independent adhesion molecules (Ig-CAMs; Rutishauser, 1993). Three main isoforms of NCAM are produced through alternative splicing of a single gene, namely NCAM120, NCAM140 and NCAM180, where the numbers refer the isoforms relative molecular weight (Owens et al., 1987). NCAM140 and NCAM180 are single spanning transmembrane proteins, in contrast, NCAM120 is linked to the cell membrane via a glycophosphatidyl inositol (GPI-) anchor. The N-terminal, extracellular parts of all isoforms are composed of five Ig-like domains followed by two fibronectin type III (F3) modules proximal to the membrane. The intracellular domain of NCAM180 differs to the one of NCAM140 in having an additional, 261 amino acids long insert (Cunningham, 1995). Variation of NCAM expression and therefore function of all three isoforms is attained through post-translational modifications. For example, the negatively charged sugar polysialic acid can be attached to the fifth Ig domain (Fujimoto et al., 2001). Moreover the cytoplasmic domains of NCAM140 and NCAM180 can be palmitoylated, which determines the proteins association with lipid rafts (Little et al., 1998).

NCAM is mainly expressed in neuronal tissues, both during development and in the adult organism. It is involved in processes such as the migration of neuronal progenitor cells, axon growth and pathfinding, synaptic plasticity and long-term potentiation (Walsh and Doherty, 1997). The effects of NCAM are mediated through its homophilic binding as well as its heterophilic interactions with other Ig-CAMs, extracellular matrix proteins and cell surface receptors. NCAM binding has been shown to affect several intracellular signaling pathways, such as the mitogen activated protein kinase (MAPK), phospholipase C-γ (PLC-γ), protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3K), diacylglycerol and arachidonic acid (reviewed in Povlsen et al., 2003). It has been shown that NCAM association with the fibroblast growth factor receptor 1 (FGFR-1) and the resulting induction of signaling cascades is crucial for neurite outgrowth in neuronal cells. A recent study identified the interaction domains of FGFR-1 and NCAM to be located in the third Ig domain of FGFR-1 and the second F3 repeat of NCAM (Kiselyov et al., 2003).

However, NCAM expression is not only reduced to the nervous system but also found in skeletal muscle cells as well as some neuroendocrine tissues (Dickson et al., 1987; Rouiller et al., 1990). We have previously shown that NCAM associates with the FGFR-4 in fibroblasts and pancreatic β tumor cells (Cavallaro et al., 2001). In these cellular systems, NCAM acts as a ligand for FGFR-4, inducing its activation and downstream signaling cascades upon ligation with the receptor. In this way, NCAM is able to modulate β_1-integrin activity and hence cell-matrix adhesion. These findings extended NCAMs function in modulating receptor tyrosine kinase (RTK) signaling to another member of the FGFR family in non-neuronal tissues.

RTK are single transmembrane spanning proteins, all comprising an intracellular tyrosine kinase activity and varying extracellular domains, which groups them into several subfamilies. The FGFR subfamily is composed of four members, FGFR1-4 (Klint and Claesson-Welsh, 1999). Structurally, the extracellular domains are composed of two or three Ig-like domains. Similar to the FGFR family, platelet derived growth factor receptors (PDGFRα and PDGFRβ) are composed of five Ig-like repeats (Heldin and Westermark, 1999), whereas other receptors, such a the epidermal growth factor receptor (EGFR) (Ullrich and Schlessinger, 1990) or the hepatocyte growth factor receptor (c-Met), do not display these structures in their extracellular domain (Giordano et al., 1989). RTKs usually get activated by binding to specific ligands, i.e. growth factors. Dimerisation of receptor monomers upon ligand binding is a prerequisite for activation of receptor trans-phosphorylation. Upon phosphorylation, a number of signal transduction molecules are binding to the receptors cytoplasmic tails, triggering a variety of downstream signaling events (Ullrich and Schlessinger, 1990). However, as mentioned above, regulation of RTK signaling is also achieved by extracellular binding of other factors to RTKs.

We hypothesized that NCAM could act as a general modulator of RTK signaling by interacting with and influencing various members of the RTK family. We show here that NCAM can bind to several RTKs. Specifically, NCAM likely acts as a negative regulator of PDGFRβ signaling. Our findings extend NCAM's functions to other RTKs and demonstrate that NCAM can have different (stimulating and inactivating) effects on RTK signaling.

2.2. Materials and Methods

2.2.1. Antibodies and reagents

Anti-FGFR-2 (C-17), FGFR-3 (C-15), FGFR-4 (C-16), PDGFRα (C-20), PDGFRβ (958 and P-20,) all from Santa Cruz; anti-V5 (Invitrogen), anti-P-MAPK and MAPK (Sigma), anti human NCAM (OB11, Sigma), anti mouse NCAM (5B8, generous gift from U. Cavallaro), anti-P-Tyr (PY-20, Transduction Laboratories). PDGF-BB was obtained from Sigma, Protein G Sepharose from Amersham.

2.2.2. Tissue culture

HEK 293T cells were grown in DMEM, 10% FCS, 1% Glutamine, 1% Penicillin/Streptomycin (Sigma). NIH 3T3 cells were grown in DMEM, 10% CS, 1% Glutamine, 1% Penicillin/Streptomycin (Sigma).

2.2.3. Stable transfection of NIH 3T3 cells

For constructing a shRNA vector against mouse N-CAM: Forward primer: 5'-GATCCCCGTACAAGGCTGAGTGGAAGTTCAAGAGACTTCCACTCAGCCTTGTACT TTTTGGAAA-3; reverse primer: 5'-AGCTTTTCCAAAAAGTACAAGGCTGAGTGG-AAGTCTCTTGAACTTCCACTCAGCCTTGTACGGG-3'; after annealing, the sequence was cloned into pSuperRetro-neo. As control, a sequence directed against human E-cadherin was used (forward primer: 5'-GATCCCCATCTGAAAGCGGCTGATACT-TCAAGAGAGTATCAGCCGCTTTCAGATTTTTTGGAAA-3' , reverse primer: 5'-AGCTTTTCCAAAAAATCTGAAAGCGGCTGATACTCTCTTGAAGTATCAGCCGCTT TCAGATGGG-3'), after transfection, cells were selected using 200 ng/ml G418 (Sigma), resistant clones were expanded for further analysis.

2.2.4. Cell stimulation, protein extraction and Western Blot

NIH3T3 cells were cultured in 6-well plates in medium containing serum, then serum-starved overnight in serum-free medium. The day of the experiment, cells were stimulated with 30 ng/ml PDGF-BB for the time indicated. After the stimulation, cells were lysed in lysis buffer (20 mM Tris/HCl pH 8.0, 160 mM NaCl, 1 mM $CaCl_2$, 10 µg/ml aprotinin, 1% Triton X-100, 1 µg/ml leupeptin, 1 mM PMSF, 10 mM NaF, and 1 mM sodium orthovanadate). Following centrifugation, the protein concentration of cell lysates was determined using the Bio-Rad DC Protein Assay (BioRad, Hercules, CA). Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Milipore). Proteins of interest were visualized using specific antibodies, followed by peroxidase-conjugated

secondary antibodies and by an enhanced chemiluminescence kit (Amersham, Little Chalfort, UK).

2.2.5. Immunoprecipitation

For immunoprecipitation analyses, cell lysates (1 mg) were pre-cleared with non-immune IgG (Sigma) plus Protein G-Sepharose (Amersham). Lysates were then incubated with specific antibodies overnight at 4 °C, followed by the addition of Protein G-Sepharose and further incubation for 2 h at 4 °C. After four washing steps in lysis buffer, proteins were eluted with Laemmli buffer and resolved by SDS-PAGE, followed by immunoblotting.

2.3. Results

2.3.1. NCAM associates with all members of the FGFR family

We previously showed that all three NCAM isoforms are found in a complex with FGFR-4 in fibroblastic L cells (Cavallaro et al., 2001). To investigate whether NCAM is also found in association with FGFR-4 in other cell types, FGFR-4 and either of the three NCAM isoforms (NCAM120, NCAM140 and NCAM 180) were transiently expressed in human embryonic kidney (HEK 293T) cells. Complex formation was assayed by investigating proteins associated with the FGFR-4 in co-immunoprecipitation experiments or, conversely, by identifying proteins that bind to NCAM. When immunoprecipitated with antibodies directed against FGFR-4, all three NCAM isoforms were found to bind to FGFR-4 (Figure 1A). The same observation was made in the reverse experiment: All three NCAM isoforms were able to co-immmunoprecipitate FGFR-4 (Figure 1B). Albeit expression levels of NCAM180 were high in both experiments, this isoform was least efficient in binding FGFR-4. These results confirm our previous observations that all NCAM isoforms can associate with FGFR-4 in various cellular system

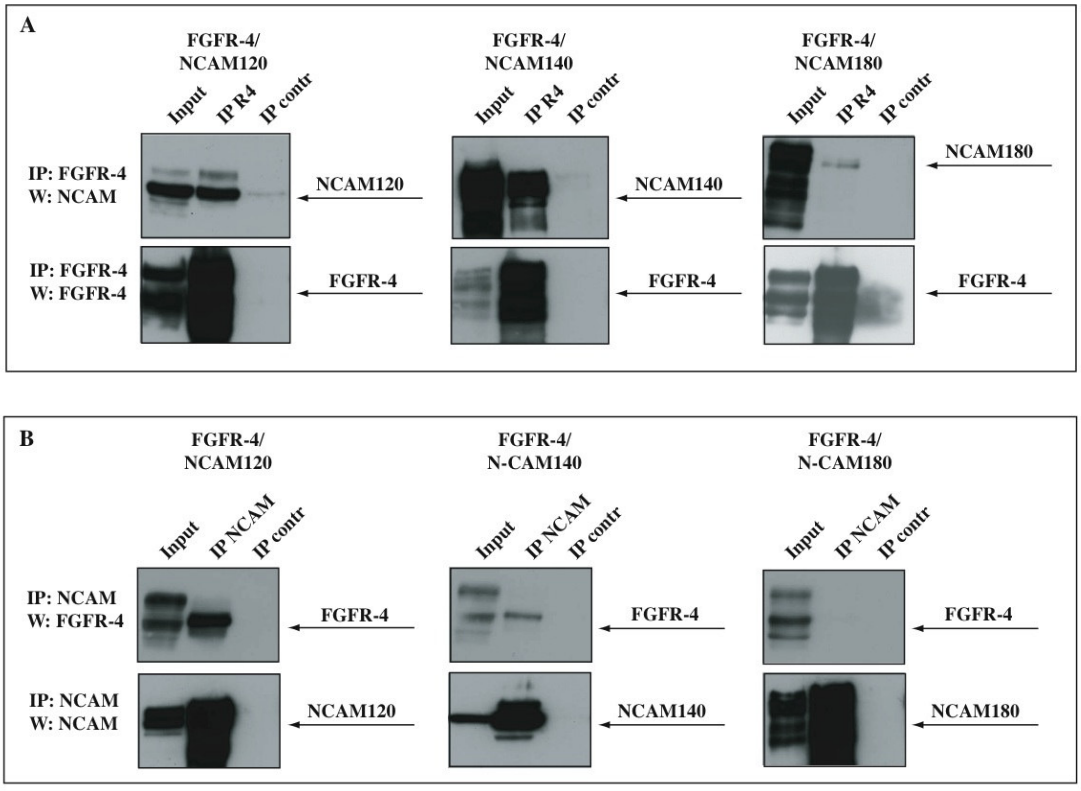

Figure 1: All three NCAM isoforms associate with the FGFR-4

Immunoblotting analysis following immunoprecipitations of lysates transfected with FGFR-4 and NCAM 120 (left panel), FGFR-4 and NCAM 140 (middle panel) or FGFR-4 and NCAM 180 (right panel). A) Lysates were incubated with an antibody against FGFR-4 (IP R4) or an unrelated antibody (IP contr). B) Lysates were incubated with an antibody directed agains NCAM (IP NCAM) or an unrelated antibody (IP contr). Input: total lysate for expression control.

In addition to FGFR-4, a direct interaction between FGFR-1 and NCAM was recently demonstrated (Kiselyov et al., 2003). We thus hypothesized that NCAM can bind to all members of the FGFR family. To test this, FGFR-3 (Figure 2, upper panel) and FGFR-2 (Figure 2, lower panel) were co-expressed with the three NCAM isoforms in HEK 293T cells. Using anti-FGFR-2 or FGFR-3 antibodies for immunoprecipitation, all three NCAM isoforms were found in a complex with the respective FGFR (Figure 2). Hence, we could observe that NCAM can bind to all four FGFR family members.

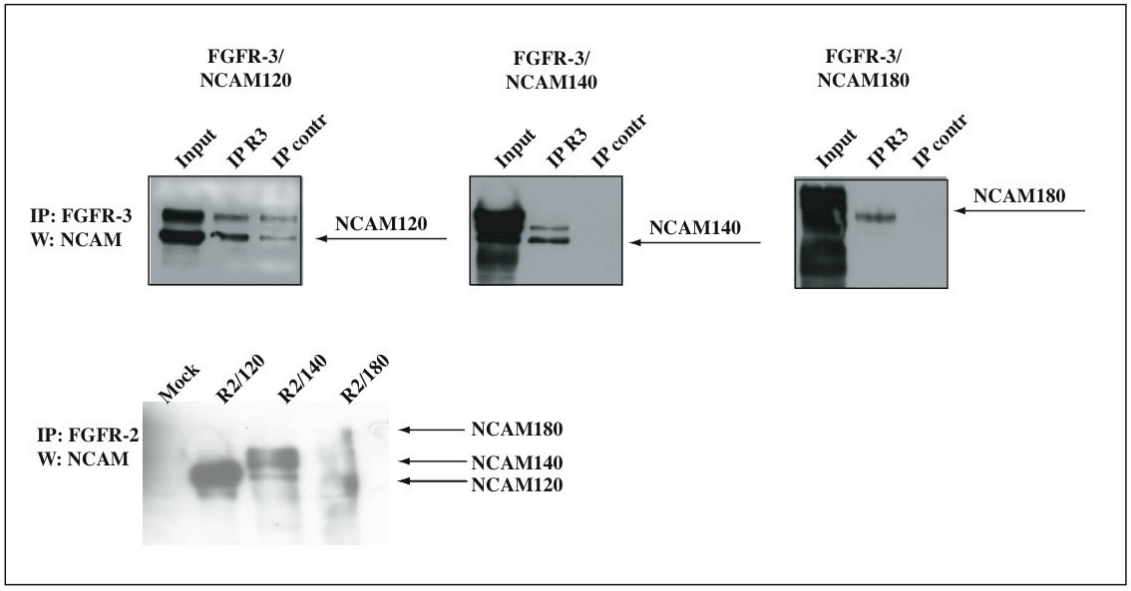

Figure 2: NCAM binds to FGFR-2 and FGFR-3

HEK 293T cells were transiently transfected with cDNA for FGFR-3 (upper panel), FGFR-2 (lower panel) and the three major NCAM isoforms. Co-immunoprecipitations using anti-FGFR-3 antibodies (upper panel) or anti-FGFR-2 antibodies (lower panel) and subsequent Immunoblotting analysis for NCAM was performed. IP R3, IP for FGFR-3; R2/120, R2/140, R2/180, lysates expressing FGFR-2 and NCAM120, NCAM140 or NCAM180; Mock, lysates transfected with a control plasmid; Input, total lysate;

2.3.2. NCAM associates with PDGFRβ

All FGFR family members consist of an extracellular domain containing Ig-like repeats. Interaction between NCAM and FGFR-1 is dependent of the third Ig-domain of the receptor. We therefore investigated if other Ig-domain containing RTKs can bind to NCAM. HEK 293T cells were transfected with cDNAs coding for either of the two platelet derived growth factor receptors PDGFRα and PDGFRβ, and NCAM140. Furthermore we tested complex formation between NCAM and two receptors that do not contain Ig repeats, the hepatocyte growth factor receptor (c-Met) and the epidermal growth factor receptor (EGFR).

In repeated experiments we never found c-Met or EGFR to associated with NCAM140 (data not shown). However, both PDGFRα and PDGFRβ were found to bind NCAM140 (Figure 3A and Figure 3B). PDGFRβ binding to NCAM was not restricted to the NCAM140 isoform, since NCAM120 and NCAM180 could also be immunoprecipitated together with PDGFRβ (Figure 3C). Taken together, PDGFRα and PDGFRβ can form a complex with NCAM.

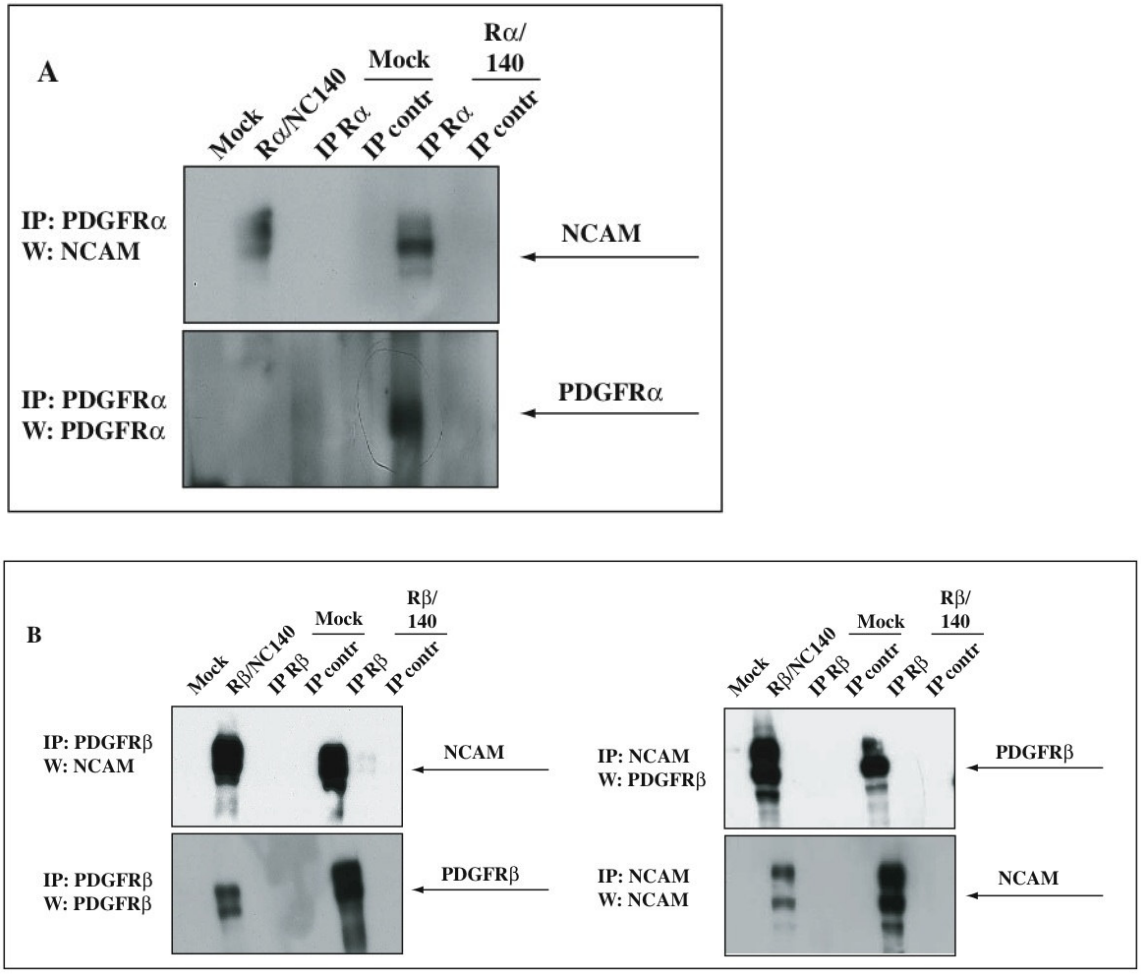

Figure 3 A and B: NCAM associates with PDGFRα and PDGFRβ

Western blot analysis of co-immunoprecipitations (co-IPs) of HEK 293T cells expressing either a control plasmid (Mock), PDGFRα and NCAM140 (Rα/140, Figure 3A) or PDGFRβ and NCAM140 (Rβ/140, Figure 3B). IP Rα, IP Rβ, IP using anti-PDGFRα or anti-PDGFRβ antibodies; IP contr, IP using unrelated antibodies.

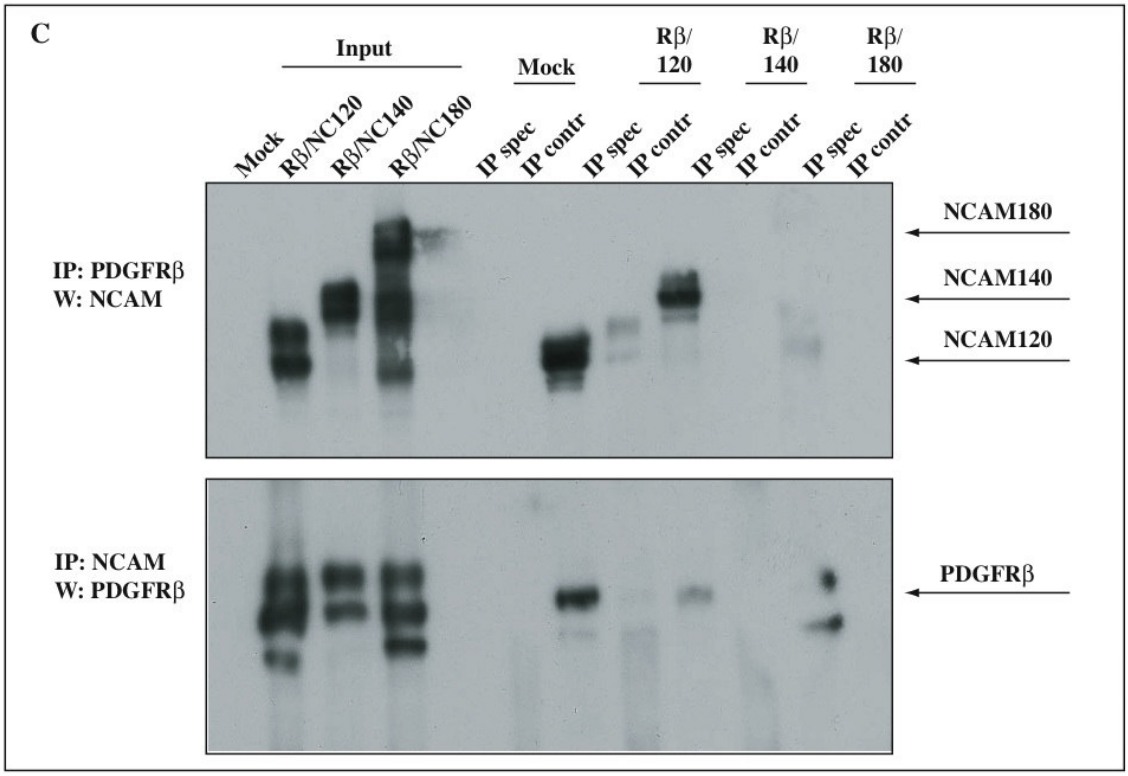

Figure 3C: NCAM associates with PDGFRα and PDGFRβ

HEK 293T were transfected with a control plasmid (Mock), PDGFRβ and NCAM120 (Rβ/120), PDGFRβ and NCAM140 (Rβ/140) or PDGFRβ and NCAM180 (Rβ/180). IPs were performed using either a specific antibody (IP spec) against PDGFRβ (upper panel), NCAM (lower panel) or an unrelated antibody (IP contr) and analyzed by Western blot with the indicated antibodies.

2.3.3. NCAM reduces tyrosine phosphorylation on the PDGFRβ

The complex formation of PDGFRβ and NCAM, but not of PDGFRα and NCAM, was confirmed in a number of alternative cellular systems, such as L-cells and NIH 3T3 fibroblasts (data not shown) and therefore the effects of NCAM on PDGFRβ signaling was further investigated. We first examined whether the cellular tyrosine phosphorylation pattern was altered in cells expressing PDGFRβ in the presence or absence of NCAM. The experiments were performed under serum starvation (no PDGF-BB) and receptor stimulation (addition of PDGF-BB) conditions. Phospho-tyrosine signals could only be detected in cells expressing PDGFRβ. Interestingly, the signal was reduced in cells co-expressing PDGFRβ and NCAM. Upon PDGF-BB treatment, phosphos-tyrosine levels were increased. However, NCAM reduced phospho-tyrosine levels equally in serum free or PDGF-BB-treated cells. Thus, it seems that tyrosine phosphorylation reduction by N-CAM is not specific for ligand stimulated receptors (Figure 4A, 1st panel). The total amounts of PDGFRβ (Figure 4A, 2nd

panel) and NCAM140 (Figure 4A, 3rd panel) were unchanged, suggesting that the observed effects were not a result of reduced receptor levels.

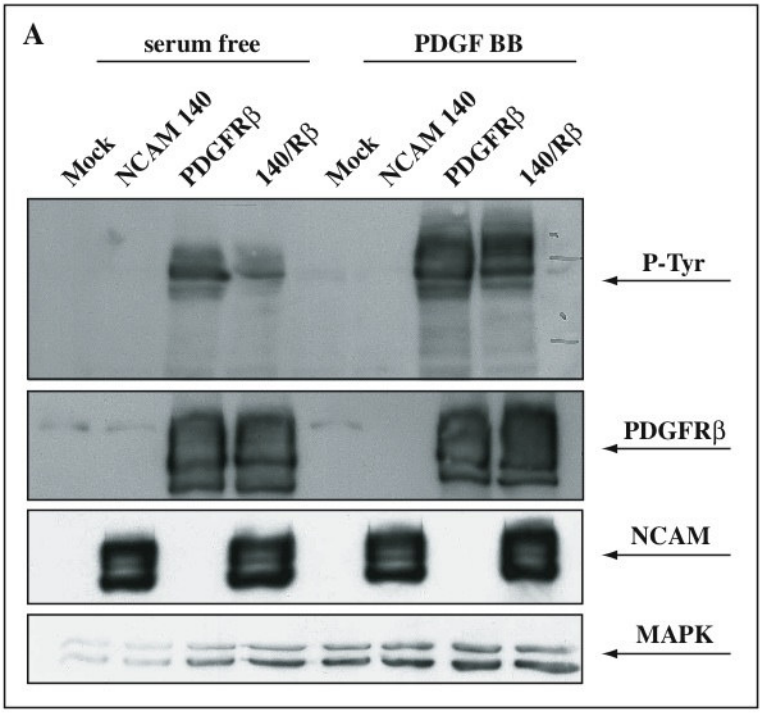

Figure 4A: Phosphotyrosine levels are reduced upon NCAM140 expression

Lysates from HEK 293T cells transfected with the indicated cDNAs and grown in the absence (serum free) or presence of PDGF-BB were analyzed for the expression levels of the indicated proteins. P-Tyr, phospho-tyrosine; 140/Rβ, NCAM140 and PDGFRβ). Equal loading was confirmed by immunoblotting for MAPK.

To investigate whether the alleviated phospho-tyrosine levels correlated with a decrease in PDGFRβ activation, the PDGFRβ-specific phosphorylation pattern was analyzed in the presence or absence of NCAM. As shown in Figure 4B, tyrosine phosphorylation levels of PDGFRβ were slightly lessend in cells that co-express NCAM as compared to PDGFRβ solely expressing cells. Similar to our previous observation, this could be observed in both, serum free and PDGF-BB stimulated conditions, confirming that the presence of the ligand does not alter the effects exhibited by NCAM. Taken together, NCAM associates with PDGFRβ in a ligand independent manner and negatively influences its tyrosine phosphorylation.

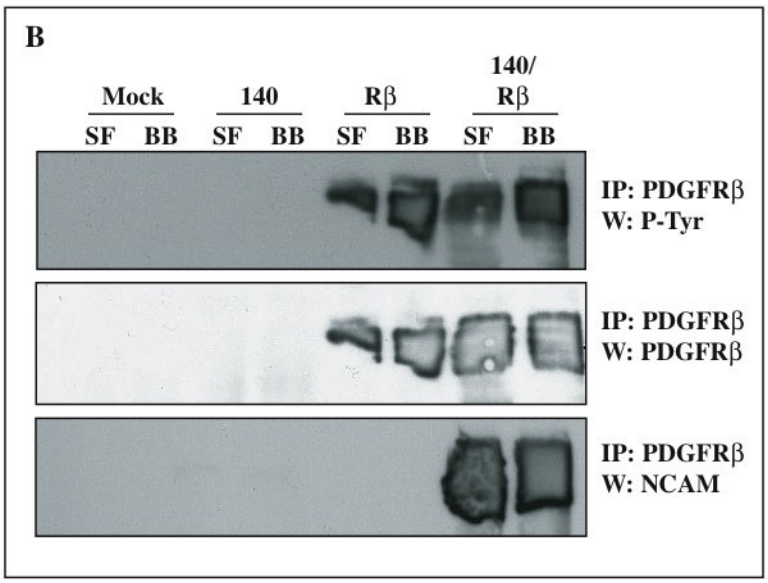

Figure 4B: Reduced tyrosine phosphorylation levels of PDGFRβ in the presence of NCAM
Lysates were prepared from HEK 293T grown in the absence (SF) or presence (BB) of PDGF-B, transfected with a control plasmid (Mock), NCAM140 (140), PDGFRβ (Rβ) or PDGFRβ and NCAM140 (140/ Rβ). IPs were performed using a specific antibody against PDGFRβ and analyzed by immunoblotting with the indicated antibodies.

2.3.4. Loss of NCAM enhances PDGF-BB induced activation of MAPK

Forced expression of proteins usually yields high amounts of the respective protein. In the case of RTKs, this often leads to receptor phosphorylation by dimerisation in absence of an appropriate stimulus, which was also observed in the case of PDGFRβ expression under serum starved conditions (Figure 4A and Figure 4B). Furthermore, not every receptor molecule associates with NCAM and vice versa, not every NCAM molecule is engaged in receptor binding, since both, NCAM or PDGFRβ were also found in cleared lysates after immunoprecipitation (data not shown). Therefore, detecting the effect of NCAM on a sub-population of all PDGFRβ proteins might be undermined by the NCAM-unbound fraction of PDGFRβ. These drawbacks together with the fact that HEK 293T cells are not PDGF-BB responsive, forced us to choose an alternative approach to investigate the effect of N-CAM on PDGFRβ signaling.

Interfering with NCAM expression using an shRNA approach in NIH 3T3 mouse fibroblast cells allowed for analyzing NCAMs influence on PDGFRβ signaling in a system expressing physiological levels of both proteins. We generated stable clones expressing three different shRNAs specific for mouse NCAM. One of the tested shRNAs was effective in degrading NCAM mRNA, leading to almost quantitative loss of NCAM protein expression (clone 3-1, Figure 5, upper panel). As control, an unrelated shRNA was stably introduced into NIH 3T3 cells (clone 6-3, Figure 5), having no effect on NCAM expression levels. Stimulating NIH 3T3 cells with PDGF-BB results in extracellular signal-regulated kinase1

and 2 (Erk1/2, referred to MAPK hereafter) activation and can therefore be used to analyze PDGFRβ signaling. We used clone 6-3 and clone 3-1 to compare PDGFRβ signaling upon stimulation in the presence and absence of NCAM. When grown under serum free conditions, loss of NCAM resulted in slightly increased levels of phosphorylated Erk1/2 (P-MAPK hereafter) (lane 0 in Figure 5, middle panel). Upon addition of PDGF-BB, P-MAPK levels were induced in control cells, showing highest MAPK activation after ten minutes of ligand addition and dropping to almost serum starved levels after 30 minutes. In contrast, in NCAM-deficient cells, P-MAPK was induced to maximum level already after five minutes and activation was sustained throughout the entire duration of the time course. Furthermore, over all P-MAPK levels were increased in cells having lost NCAM expression. These results indicate that N-CAM negatively affects PDGFRβ-induced MAPK-signaling in the absence and presence of PDGF-BB.

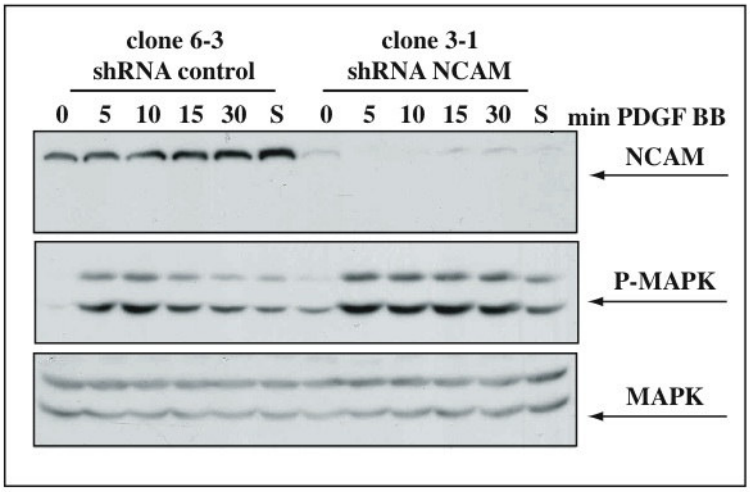

Figure 5: NCAM reduces PMAPK levels in NIH 3T3 cells

Immunoblotting analysis of cells expressing control shRNA (clone 6-3) and shRNA against mouse NCAM (clone 3-1). 0, 5, 10, 15, 30: minutes of incubation with PDGF-BB. S, serum control.

To determine whether the induction of MAPK was a specific consequence of NCAM down-regulation and did not reflect an unspecific effect resulting from the shRNA, human NCAM140 tagged with the V5 epitope was transiently re-introduced into clone 3-1. Transfection efficacy was rather low since only about 5-10% of all cells expressed the V5-tag and thus human NCAM140 (Figure 6, lower left panel). When the cells were stimulated with PDGF-BB, P-MAPK levels were not significantly reduced upon re-expression of NCAM (Figure 6, 3rd right panel). Furthermore, the kinetics of the stimulation was similar to the original, NCAM deficient clone 3-1. However, since only a small percentage of all analyzed cells were successfully transfected, the result of NCAM re-introduction may not be detectable. Therefore, this experiment is not conclusive.

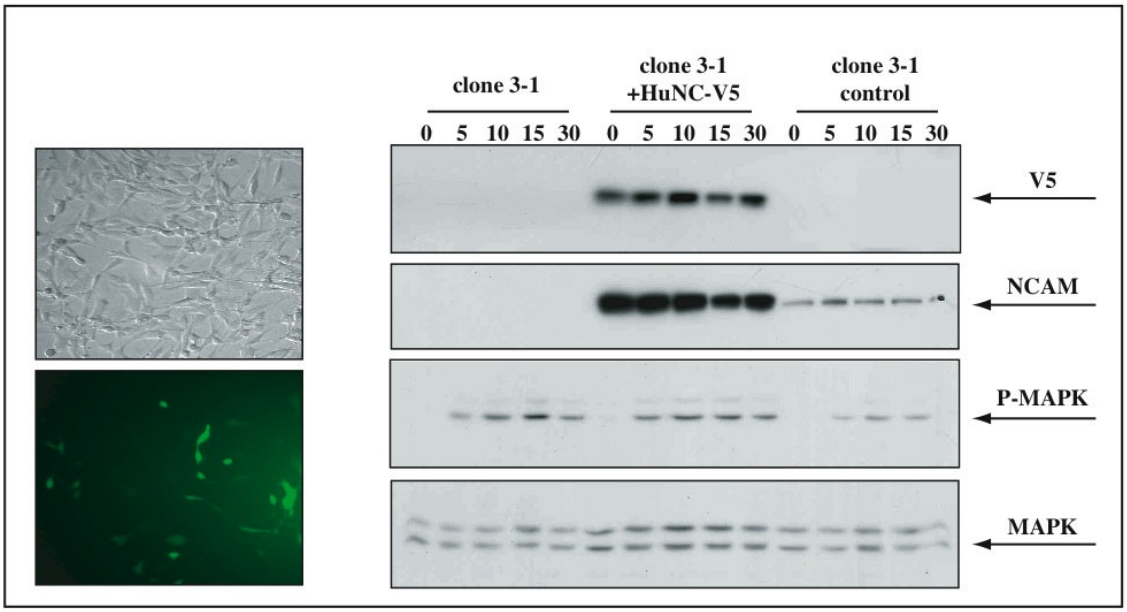

Figure 6: Partial re-introduction of NCAM does not result in P-MAPK reduction

Right panel: Immunoblotting analysis with the indicated antibodies of clone 3-1 transiently transfected with human NCAM140 cDNA in comparison to original clone 3-1 and control clone 6-3. 0, 5, 10, 15, 30: minutes of incubation with PDGF-BB. Left panel: control of transfection efficacy. Up: Cell density at time point of harvesting cells, determined by light microscopy. Down: Immunofluorescence staining of clone 3-1 transfected with human NCAM140 carrying a V5 tag with an antibody recognizing the V5 epitope.

2.4. Discussion

A direct interaction between NCAM and FGFR-1 and 4 was recently demonstrated by others and our group (Kiselyov et al., 2003; Francavilla et al., in preparation). In the case of FGFR-1, the interaction is dependent on the third Ig domain of the receptor. Among the huge family of RTKs, several receptors carry varying numbers of Ig-domains in their extracellular parts. Members of the FGFR family for instance are composed of two (Ig domain II and III) or three Ig domains (Ig domain I, II and III), depending on the particular splice variant (Klint and Claesson-Welsh, 1999). Other receptors such as PDGFRα and PDGFRβ are characterized by five Ig domains (Heldin and Westermark, 1999) or, in the case of the vascular endothelial growth factor receptor (VEGFR) family, seven Ig domains. We show here that all NCAM isoforms are able to associate with three members of the FGFR family (FGFR-2, -3 and -4), as well as PDGFRα and PDGFRβ. The interaction of NCAM and RTKs seems to be dependent on the presence of an Ig domain, since RTKs lacking this structural feature (EGFR and c-MET) cannot associate with NCAM. In next steps, it will be important to investigate if VEGFRs are also interacting with NCAM to support the idea that NCAM can act as a ligand for Ig domain containing RTKs. Furthermore, the identification of the exact domain or structure of Ig domain III on FGFR-1 necessary for NCAM binding might allow to identify additional binding proteins of NCAM.

In L-cells, stimulation of the FGFR-4 by NCAM or FGF leads to increased cell-matrix adhesion in a MAPK dependent manner. Using this assay it was recently shown that in the absence of FGF, N-CAM acts on FGFR-4 in a stimulating way, inducing MAPK activation. However, NCAM acts as a negative regulator of FGF-induced MAPK activation and subsequent cell-matrix adhesion (Francavilla et al., in preparation, Chapter 3 in this work). We do not know if NCAM binding to FGFR-2 and 3 influences the receptor's activity in a similar way as observed for FGFR-4. Using a system that is based on the forced expression of RTKs is sub-optimal to study signaling events, since the un-physiological receptor-levels lead to auto-phosphorylation and hence activation of RTK signaling in the absence of appropriate stimuli. It will therefore be crucial to identify or generate an appropriate system to unravel the influence of NCAM on FGFR-2 and FGFR-3 signaling.

Several experiments indicate that NCAM acts as a negative modulator of PDGFRβ signaling. Co-expression of NCAM and PDGFRβ leads to reduced tyrosine phosphorylation of the receptor, both, in the absence and presence of PDGF-BB. Furthermore, interfering with NCAM function in NIH 3T3 cells influenced the kinetics and levels of PDGF-BB stimulation. Whereas in the absence of NCAM the ligand elicits a strong, sustained P-MAPK response, the presence of NCAM allows only a short, transient and weaker activation of MAPK by PDGF-BB. This is very similar to what can be observed in FGF induced FGFR-4 signaling in L-cells (Francavilla et al., in preparation, Chapter 3) and suggests that NCAM influences PDGFRβ similarly to FGFR-4. However, in contrast to FGFR-4 signaling, NCAM does not induce PDGFRβ signaling in the absence of PDGF-BB.

The specificity of the results obtained with shRNA transfected NIH 3T3 cells remains to be confirmed. To exclude that the observed effects on PDGFRβ signaling are due to unspecific effects of the transfected shRNA, we currently try to establish stable expression of

human NCAM140 in the NCAM-deficient clone 3-1. The finding that NCAM associates with all FGFR proteins as well as PDGFRα and PDGFRβ indicates that NCAM might act as a general RTK signaling modulator in a variety of cell types. Furthermore, the results described here and in Chapter 3 extend NCAMs function from stimulating RTKs to also inhibiting ligand induced RTK signaling. Future work will focus on the identification of suitable cellular systems to dissect the distinct effects of NCAM on the respective RTK.

NCAM acts as a molecular switch for FGFR signaling

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Abstract

Neural cell adhesion molecule (NCAM) mediates cell-cell adhesion in the central nervous system. However, NCAM is also expressed in non-neural tissues where its function has in most parts remained elusive. Using a transgenic mouse model of pancreatic β cell carcinogenesis (Rip1Tag2), we have previously reported that NCAM stimulates cell-matrix adhesion by activating fibroblast growth factor receptor (FGFR) signaling. Here, we demonstrate that the direct binding of NCAM to FGFR is necessary and sufficient for triggering FGFR activity, which results in mitogen-activated protein kinase (MAPK)-mediated cell-matrix adhesion. Moreover, our studies reveal that NCAM and FGFs elicit distinct FGFR-mediated signaling cascades which account for the differential cell responses observed with the two molecules. In addition, NCAM negatively regulates FGF-induced cell-matrix adhesion, cell proliferation and MAPK activation. Our results indicate an unexpected role of NCAM in modulating FGFR functions, thus introducing a novel type of control mechanism for receptor tyrosine kinase activity.

3. NCAM acts as a molecular switch for FGFR signaling

3.1. Introduction

Neural cell adhesion molecule (NCAM) belongs to the immunoglobulin-like superfamily of adhesion molecules (Ig-CAMs), and is a cell surface glycoprotein involved in calcium-independent intercellular adhesion. The extracellular portion of NCAM contains five Ig-like domains (Ig1-5) and two fibronectin type-III (F3) repeats. Alternative splicing yields three main NCAM isoforms, two of which are endowed with a transmembrane and a cytoplasmic region (NCAM140 and 180), while NCAM120 is linked to the membrane through a glycosylphosphatidyl inositol (GPI) anchor (reviewed in Walmod et al., 2004). Posttranslational modifications of the protein add further complexity to NCAMs expression pattern and function. In particular, the NCAM ectodomain can be polysialylated in a time and space-dependent manner, and this process induces a shift in NCAM function from pro-adhesive to pro-migratory (Angata and Fukuda, 2003).

NCAM is widely expressed in the central nervous systems, where it plays an important role in various processes, both during embryonic development and in adulthood (reviewed in Hinsby et al., 2004). Indeed, NCAM is involved in the migration of neural progenitor cells, axonal growth and pathfinding, synaptic plasticity and long-term potentiation. At the cellular level, NCAM exerts these functions by controlling intercellular recognition and adhesion, neurite outgrowth, cell migration, proliferation and survival. These events are triggered by the homophilic interaction of NCAM molecules on adjacent cells as well as by the heterophilic binding of NCAM to other Ig-CAMs, extracellular matrix components, and cell surface receptors. NCAM's homophilic and heterophilic interactions affect a complex network of signaling cascades, which involve many crucial pathways, such as mitogen-activated protein kinases (MAPK), phospholipase C-γ (PLCγ), diacylglycerol and arachidonic acid, protein kinase C (PKC), and phosphatidyl inositol 3-kinase (PI3K) (reviewed in Walmod et al., 2004).

Pioneering studies performed by the group of P. Doherty and F. Walsh have highlighted a functional interplay between NCAM and the fibroblast growth factor receptor (FGFR) in neuronal cells, which underlies NCAM-dependent neurite outgrowth (Walsh and Doherty, 1997). Further support for these results came from surface plasmon and nuclear magnetic resonance studies that have demonstrated a direct binding of NCAM to FGFR and identified the interaction domains in the second F3 repeat of NCAM and the third Ig domain of FGFR (Kiselyov et al., 2003). We have confirmed and extended the NCAM/FGFR interplay in non-neuronal and tumor cells, such as pancreatic beta tumor cells and fibroblasts, implying that it is not restricted to the nervous system (Cavallaro et al., 2001). Our data have revealed that the formation of the NCAM/FGFR complex induces FGFR autophosphorylation and stimulates an FGFR-dependent signaling cascade that leads to the modulation of β_1-integrin-mediated cell-matrix adhesion. Interfering with this process by abrogating the expression of NCAM in a transgenic mouse model of β cell tumorigenesis resulted in the

disruption of the tumor tissue architecture, tumor-associated lymphangiogenesis, and lymph node metastasis (Perl et al., 1999; Cavallaro et al., 2001; Crnic et al., 2004). These and other studies imply that the cross-talk between adhesion molecules and receptor tyrosine kinases has important implications and that its dysregulation can play a pathogenetic role in diseases such as cancer and neurological disorders (Cavallaro and Christofori, 2004).

The intracellular signaling pathways elicited by the NCAM/FGFR complex and their impact on cellular physiology have been elucidated only to a certain extent, and all the information available so far derives from studies on neuronal cell types. In particular, it has been shown that, upon NCAM binding to FGFR, a number of intracellular effectors are recruited to the phosphorylated residues of the FGFR's cytoplasmic tail. Among these, PLCγ becomes activated when bound to FGFR and then cleaves phosphatidylinositol 4,5-biphosphate to generate inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). While IP_3 induces an increase in intracellular Ca^{2+} concentration, DAG can either activate PKC or be converted into arachidonic acid (Walmod et al., 2004). In neurons, it has been shown that the cytoplasmic tail of NCAM180 and 140 binds to and activate the $PKC\beta_{II}$ isoenzyme via spectrin, a scaffolding protein that contributes to the organization of membrane microdomains and to the anchorage of membrane proteins to the cytoskeleton (Leshchyns'ka et al., 2003). The formation of this NCAM/spectrin/$PKC\beta_{II}$ complex was reported to be FGFR-dependent. PKC acts as an important nodal point in the signaling cascade elicited by the NCAM/FGFR interplay: it activates the neuritogenic protein GAP-43 (involved in signaling and in cytoskeletal rearrangements) and it can also stimulate Raf kinase activity. The latter activity enables PKC to link the NCAM/FGFR complex to the Raf-MEK-MAPK pathway. Notably, homophilic NCAM interactions, which underlie NCAM-mediated cell-cell adhesion, also elicit MAPK activation via non-receptor tyrosine kinases, such as $pp60^{c\text{-}src}$ or focal adhesion kinase (FAK). However, the mutual influence between the signaling cascades elicited by NCAM-mediated intercellular adhesion and homophilic interaction and those downstream of the NCAM-induced FGFR activation remains elusive. Here, we report novel implications of the NCAM/FGFR cross-talk, by showing that *i*) the direct binding of NCAM to FGFR is necessary and sufficient to stimulate MAPK-dependent cell-matrix adhesion; *ii*) NCAM and FGF activate FGFR signaling in a distinct manner, i.e. they induce different FGFR-mediated pathways; and *iii*) NCAM can also act as a negative regulator of the cellular response to FGF stimulation. Our results highlight a novel, NCAM-dependent mechanism for the modulation of FGFR activity.

3.2. Experimental Procedures

3.2.1. Cell lines and reagents

NCAM-null and NCAM-positive β tumor cells were isolated from the pancreatic tumors of Rip1Tag2;NCAM-/- or NCAM+/+ mice and cultured as previously described (Cavallaro et al., 2001). Mouse fibroblastic L cells were maintained in DMEM, 10% fetal calf serum, L-glutamine and antibiotics. FGF-1 and FGF-2 were purchased from Peprotech (London, UK). Heparin, PMA and arachidonic acid were purchased from Sigma (St. Louis, MO). The FGFR inhibitor PD173074 (Skaper et al., 2000) was kindly provided by Pfizer (Groton, CT). The PKCβ_{II} inhibitor CGP53353 was kindly provided by D. Fabbro (Novartis, Basel, Switzerland). The MEK inhibitor PD98059 and the broad-spectrum PKC inhibitor Calphostin C were from Sigma. The PKC α/γ inhibitor HBDEE and the Src inhibitor PP1 were from Calbiochem. Antibodies: mouse anti-phospho-Erk1/2, rabbit anti-phospho-FRS2 and rabbit anti-phospho-Src (recognizing the activated form of most Src kinases) were from Cell Signaling Technology (Danvers, MA); rabbit anti-Erk1/2 was from Sigma; rabbit anti-PKCα and anti-PKCβ_{II}, rabbit anti-FRS2 and mouse anti-Src kinases were from Santa Cruz Biotechnologies; rabbit anti-phospho-PKCβ_{II} was from Abcam (Cambridge, UK); mouse anti-NCAM was from BD Biosciences (San Jose, CA). The pIg3 vectors containing the cDNA for the ectodomain of human NCAM, either full-length or with the deletion of the second F3 module (ΔFN2), fused to the Fc fragment of human IgG, were kindly provided by L. Needham (Duke University). The vectors were used to transiently transfect HEK 293 cells, and the recombinant proteins were purified from the conditioned medium of transfected cells using Protein G-Sepharose chromatography. The FGL peptide from the second F3 module of NCAM and its mutated version carrying two alanine substitutions (FGL_{mut}; Kiselyov et al., 2003) were a generous gift from ENKAM Pharmaceuticals (Copenhagen, Denmark).

3.2.2. Stable transfection of L cells

The cDNA for mouse NCAM140 was subcloned into the pcDNA3.1 expression vector (Invitrogen). L cells were transfected with the pcDNA-NCAM plasmid (L-NCAM) or with the empty vector (L-mock) using Lipofectamine 2000, followed by selection with 0.8 mg/ml G418 (Invitrogen) and cloning by limiting dilution.

3.2.3. Cell stimulation, protein extraction and Western Blot

Cells were cultured in 6-well plates in DMEM with 10% FCS, then serum-starved overnight in serum-free medium. The day of the experiment, cells were stimulated with the following molecules at the indicated concentrations: 20 μg/ml NCAM-Fc, 20 μg/ml ΔFN2-Fc, 20 μg/ml FGL or FGL_{mut}, 20 ng/ml FGF-1 plus 10 μg/ml heparin, 20 ng/ml FGF-2. If not

indicated otherwise, cells were stimulated for 10 minutes. When needed, cells were pre-incubated for 1-2 hours with specific inhibitors at the following concentrations: 100 nM PD173074, 25 μM PD98059, 10 μM CGP53353, 100 μM HBDEE, 400 nM Calphostin C, 20 μM PP1. Control cells were pre-incubated with vehicle alone. After the stimulation, cells were lysed in lysis buffer (20 mM Tris/HCl pH 8.0, 160 mM NaCl, 1 mM $CaCl_2$, 10 μg/ml aprotinin, 1% Triton X-100, 1 μg/ml leupeptin, 1 mM PMSF, 10 mM NaF, and 1 mM sodium orthovanadate). Following sonication and centrifugation to remove cell debris, the protein concentration of cell lysates was determined using the Bio-Rad DC Protein Assay (BioRad, Hercules, CA). Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Protran, Biosciences). Proteins of interest were visualized using specific antibodies, followed by peroxidase-conjugated secondary antibodies and by an enhanced chemiluminescence kit (Amersham, Little Chalfort, UK).

3.2.4. Immunoprecipitation

For immunoprecipitation analyses, cell lysates (2 mg) were pre-cleared with non-immune IgG (Sigma) plus Protein G-Sepharose (Pharmacia, Uppsala, Sweden). Lysates were then incubated with specific antibodies overnight at 4°C, followed by the addition of Protein G-Sepharose and further incubation for 2 h at 4°C. After four washing steps in lysis buffer, proteins were eluted with Laemmli buffer and resolved by SDS-PAGE, followed by immunoblotting.

3.2.5. Cell adhesion assays

Matrix adhesion assays were performed on collagen type-IV, a substrate that mediates NCAM-dependent cell adhesion (Cavallaro et al., 2001). Twenty-four well plates were coated with 5 μg/cm^2 of mouse collagen IV **(**BD Biosciences). Cells cultured in 6-well plates were stimulated with appropriate compounds, and 10^5 cells were seeded in 24-well plates coated with 4 mg/cm2 of collagen IV. After 2 hours, non-adherent cells were removed by washing with PBS. Adherent cells were fixed for 30 minutes with 2% formalin, then stained with crystal violet, washed and air-dried. Bound dye was solubilized with 10 % acetic acid and absorbance measured at 595 nm. Cell-free wells served as blanks. The assays were performed in quadruplicate and repeated at least three times.

3.2.6. Cell proliferation

L cells were seeded in triplicate on 24-well plates at $5x10^3$ cells/well, serum-starved overnight and treated for 4 days with 10% fetal calf serum or 20 ng/ml FGF-2, which were added every 24 hours. At each time point, cells were washed with PBS, fixed and stained with crystal violet as described for the adhesion assay. The absorbance at 595 nm was measured,

and the ratio between stimulated and non-stimulated L cells was determined for each time point. The assays were performed in quadruplicate and repeated at least three times.

3.3. Results

3.3.1. NCAM-induced cell-matrix adhesion requires the binding of NCAM to FGFR.

We have previously reported that membrane-bound NCAM promotes neurite outgrowth and cell-matrix adhesion in pancreatic β tumor cells isolated from the Rip1Tag2 transgenic mouse model of β cell tumorigenesis (Cavallaro et al., 2001). Interestingly, similar effects were obtained by treating NCAM-/- β tumor cells with the soluble extracellular portion of NCAM fused to the Fc fragment of human IgG (Figure 1A). The Fc fragment alone had no effect on cell adhesion (not shown). To verify the cell type-specificity of NCAM functions, we stably transfected L cells, a fibroblastic cell line which expresses no NCAM (Cavallaro et al., 2001), with NCAM140, and subjected the cells to adhesion assays. As shown in Figure 1B, NCAM expression stimulated the adhesion of L cells to collagen IV.

Moreover, by analogy to β tumor cells, soluble NCAM-Fc promoted matrix adhesion of mock-transfected L cells (Figure 1C), while very little effect was obtained in NCAM-expressing cells (not shown). These data imply that both the anchorage of NCAM to the cell surface and its cytoplasmic tail are dispensable for NCAM-induced cell-matrix adhesion, and that this functional property of NCAM is not restricted to β tumor cells.

Both NCAM-/- β tumor cells and L cells lack endogenous NCAM. Hence, the pro-adhesive effect of NCAM-Fc is not due to homophilic NCAM-NCAM interactions but rather implicates a cross-talk of soluble NCAM-Fc with other effectors. We have previously reported that NCAM-induced neurite outgrowth and matrix adhesion in β tumor cells are mediated by FGFR signaling (Cavallaro et al., 2001). These observations were now extended to L cells, where the FGFR inhibitor PD173074 repressed NCAM-dependent matrix adhesion, confirming the NCAM/FGFR crosstalk (Figure 1B). NCAM and FGFR have been previously found to co-immunoprecipitate from whole cell lysates (Cavallaro et al., 2001), yet the implications of their direct physical interaction have remained elusive. To address this issue, we took advantage of the recent mapping of the FGFR-binding motifs in the second F3 repeats of the NCAM's extracellular portion (Kiselyov et al., 2003), and treated NCAM-/- β tumor cells with a version of NCAM-Fc lacking this domain (ΔFN2-Fc). As shown in Figure 1A, ΔFN2-Fc failed to induce cell adhesion to collagen IV. In agreement with our previous observations (Cavallaro et al., 2001), FGF-1 was able to induce cell-matrix adhesion almost to the same extent as full-length NCAM-Fc. FGFR activation promoted matrix adhesion also in fibroblastic L cells, as demonstrated by stimulation with FGF-2 (Figure 1C). The latter was used in all the experiments with L cells because this cell type, unlike β tumor cells, is more responsive to FGF-2 than FGF-1 (CF and UC, unpublished observations). As expected, the FGFR inhibitor PD173074 repressed the adhesion of L cells stimulated with FGF-2. The inhibitory effect of PD173074 was specific for FGFR signaling, since the drug did not affect the basal adhesion activity of L cells (Figure 1C). By analogy to β tumor cells, ΔFN2-Fc failed to promote L cell adhesion to collagen IV. In addition, NCAM-Fc-induced adhesion of L cells was repressed by PD173074 (Figure 1C), thus confirming the essential role of FGFR in NCAM-mediated signaling. Furthermore, matrix adhesion of L cells was also stimulated by

the FGL peptide, which mimics NCAM binding to and activation of FGFR, while a mutated version of the peptide unable to bind to FGFR (Kiselyov et al., 2003) showed no effect (Figure 1C). Together, the results indicate that the direct association of NCAM to FGFR is not only necessary but also sufficient for NCAM-dependent cell-matrix adhesion.

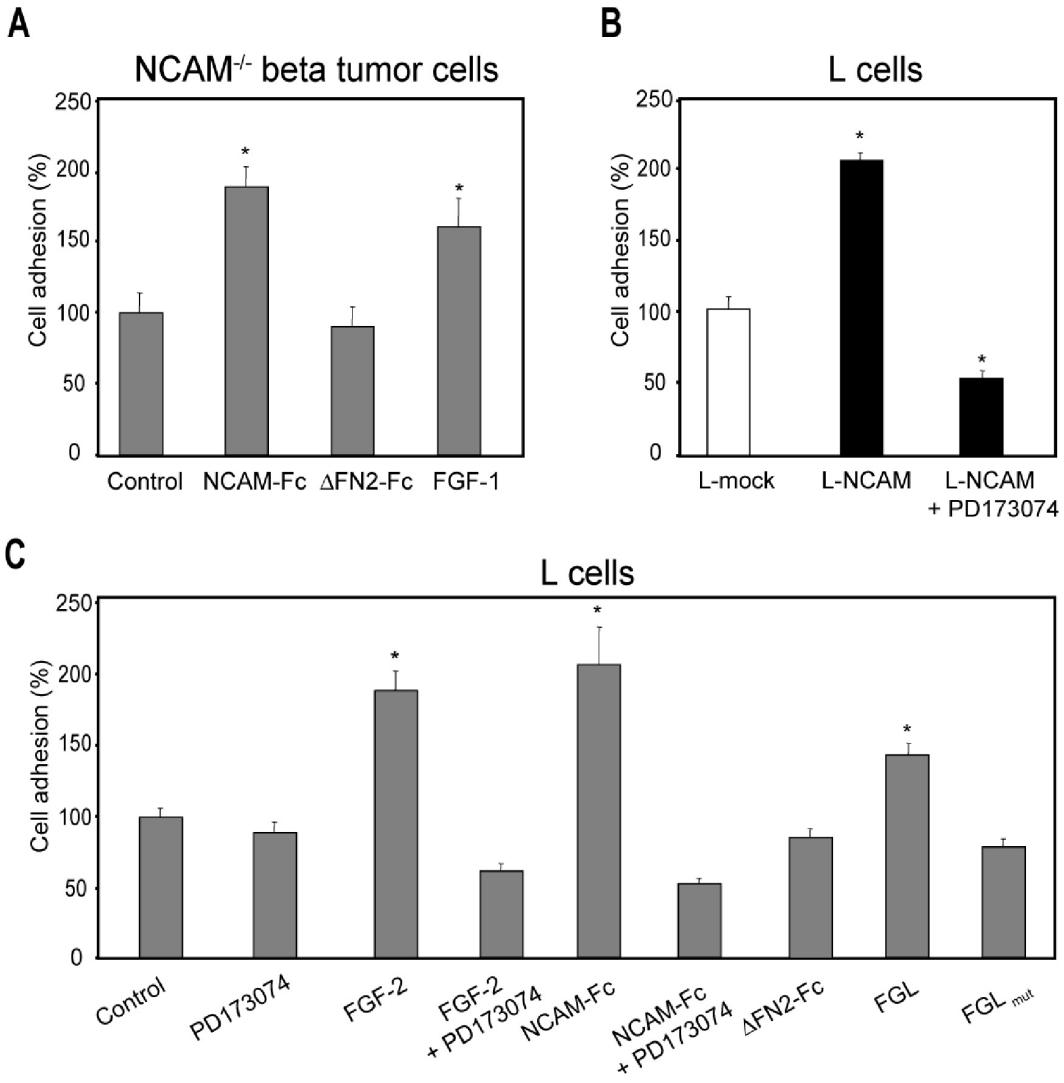

Figure 1: NCAM induces cell-matrix adhesion by binding to FGFR

*NCAM-/- β tumor cells (A) or L cells (B and C) were stimulated for 10 minutes with NCAM-Fc, ΔFN2-Fc, FGL or FGF_{mut}, FGF-1, or FGF-2. When needed, cells where pre-incubated with PD173074 or DMSO alone for 2 hours prior to the stimulus. After the treatment, cells were subjected to adhesion assays on collagen IV-coated wells. Adherent cells were counted, and the results are presented percentage of control, untreated cells ± standard deviation. Experiments were performed in quadruplicate. *p<0.005.*

3.3.2. NCAM-induced cell-matrix adhesion is mediated by Erk1/2 activation

We and others have shown that various signaling pathways are implicated in neurite outgrowth stimulated by NCAM (Cavallaro et al., 2001; Walmod et al., 2004). However, the intracellular cascade(s) that underlies NCAM-induced cell-matrix adhesion has been only partially unraveled. Since the activation of extracellular signal-regulated kinase 1 and 2 (Erk1/2) is one of the most prominent effects elicited by membrane-bound NCAM (Cavallaro et al., 2001), we initially focused on this signal transduction pathway. To elucidate the role of Erk1/2 in NCAM-dependent adhesion, NCAM-/- β tumor cells were treated with NCAM-Fc in the presence of the Erk1/2 inhibitor PD98059 prior to the adhesion assay on collagen IV. As shown in Figure 2A, PD98059 blocked the matrix adhesion of NCAM-Fc-treated cells, implicating Erk1/2 as key mediators of NCAM-induced cell adhesion. Interestingly, also the adhesion stimulated by FGF-1 required Erk1/2 activity (Figure 2A), which supports the hypothesis that NCAM and FGF elicit common signaling pathways. Very similar results were obtained in L cells stimulated with NCAM-Fc or FGF-2, where matrix adhesion was repressed in the presence of PD98059 (Figure 2B). Based on these data, we verified whether soluble NCAM-Fc stimulates Erk1/2 activation. In agreement with our previous result (Cavallaro et al., 2001), NCAM-expressing β tumor cells exhibited constitutive activation of Erk1/2, which was not enhanced by the treatment with NCAM-Fc (Figure 2C).

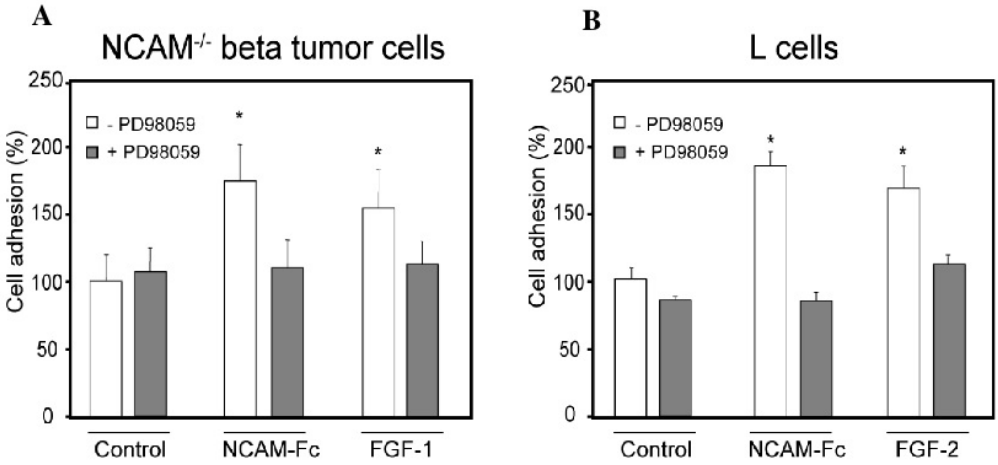

Figure 2, A and B: NCAM signaling is mediated by Erk1/2

*NCAM-/- β tumor cells (A) or L cells (B) were stimulated for 10 minutes with NCAM-Fc, FGF-1 or FGF-2. Prior to stimulation, cells where pre-incubated with PD98059 (grey bars) or DMSO alone (white bars) for 30 minutes. After treatment, cells were subjected to adhesion assays on collagen IV-coated wells. Adherent cells were counted and are indicated as percentage of control, untreated cells ± standard deviation. Experiments were performed in quadruplicate. *p<0.005.*

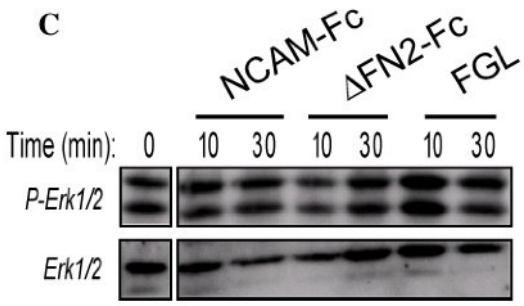

Figure 2C: Constitutive activation of β tumor cells

NCAM+/+ β tumor cells were stimulated for 10 or 30 minutes with NCAM-Fc, DFN2-Fc, or FGL. Cells were then lysed and immunoblotted for phospho-Erk1/2, followed by stripping and immnoblotting for total Erk1/2.

In contrast, NCAM-Fc strongly induced Erk1/2 activation in NCAM-/- β tumor cells, an effect that was also recapitulated by the FGL peptide alone, while ΔFN2-Fc was markedly less effective (Figure 2D). Once again, the role of NCAM in Erk1/2 activation was not restricted to β tumor cells, since we obtained comparable results with fibroblastic L cells. Indeed, cells treated with either NCAM-Fc or FGL, but not with ΔFN2-Fc, showed a high level of Erk1/2 activation (Figure 2E). This effect was readily inhibited by PD173074 (Figure 2D), supporting the crucial role of FGFR downstream of NCAM. Besides confirming that the membrane insertion is not necessary for certain functions of NCAM, these data imply that the direct binding to FGFR is essential for NCAM to stimulate Erk1/2 activation.

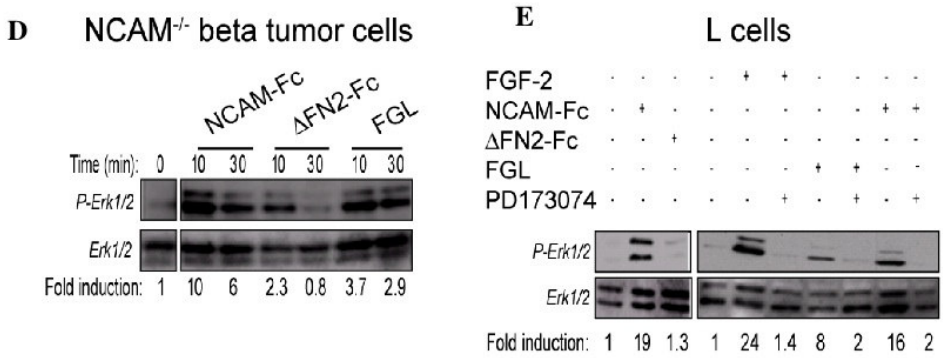

Figure 2, D and E:

(D) NCAM-/- β tumor cells were stimulated for 10 or 30 minutes with NCAM-Fc, ΔFN2-Fc, or FGL. Cell lysates were then immunoblotted for phospho-Erk1/2 (top panel), followed by stripping and immunoblotting for total Erk1/2 (bottom panel). The densitometric ratio between phosphorylated and total Erk1/2 was measured for each time point, and the induction of Erk activation relative to untreated cells (time 0) is indicated as arbitrary units. (E) L cells were stimulated for 10 minutes with NCAM-Fc, ΔFN2-Fc, FGL, or FGF-2. Cells where pre-incubated for 2 hours with PD173074 prior to the stimulus as indicated. Cells were then lysed and immunoblotted for phospho-Erk1/2 (top panel), followed by stripping and immunoblotting for total Erk1/2 (bottom panel). The densitometric ratio between phosphorylated and total Erk1/2 was measured for each treatment, and the induction of Erk activation relative to untreated cells is presented as arbitrary units.

3.3.3. NCAM and FGF activate distinct, FGFR-mediated signaling pathways

We have previously reported that NCAM induces both cell-matrix adhesion and neurite outgrowth in β tumor cells in an FGFR-dependent manner (Cavallaro et al., 2001). However, only cell-matrix adhesion and not neurite outgrowth could be rescued in NCAM-deficient cells treated with FGF, implying that the interaction of NCAM with FGFR induces a downstream signaling that is distinct, although partially overlapping, from that of FGF. Based on the observation that NCAM-induced Erk1/2 activation was inhibited by the broad-spectrum PKC inhibitor calphostin-C in both β tumor cells and L cells (Figure 3A and B), we investigated NCAM- and FGF-mediated differences in protein kinase C (PKC) signaling. The biological significance of NCAM-induced PKC activation was underscored by the observation that the PKC activator phorbol 12-myristate 13-acetate (PMA) induced both Erk1/2 activation (not shown) and adhesion of NCAM-/- β tumor cells to collagen IV (Figure 3C). However, calphostin-C also repressed the Erk1/2 activation induced by FGF-1 in β tumor cells and FGF-2 in L cells (Figure 3A and B), indicating that both NCAM and FGF signal via PKC. Nevertheless, given that several PKC family members with distinct functions and partners are normally co-expressed in most cell types (Jaken and Parker, 2000), the activation of different PKC isoenzymes may account for the differential FGFR-mediated signaling stimulated by NCAM and FGF. To test this hypothesis, we first set out to identify the PKC acting downstream of NCAM. Since $PKC\beta_{II}$ has been previously implicated in NCAM signaling in neurons (Leshchyns'ka et al., 2003), we verified whether this applied also to our cellular systems. Indeed, immunoblotting analyses on L cell lysates with an antibody against activated $PKC\beta_{II}$ revealed that NCAM-Fc induces the activation of $PKC\beta_{II}$. Notably, this effect was inhibited by PD173074 (Figure 4A), indicating that NCAM-Fc-induced activation of $PKC\beta_{II}$ requires FGFR signaling. Based on the implication of $PKC\beta_{II}$ in NCAM signaling, we assessed whether the two molecules are physically associated. Co-immunoprecipitation analysis on NCAM-positive β tumor cells showed that NCAM associated with $PKC\beta_{II}$, but not with PKCα, which is also expressed by these cells (Figure 4B, left). The formation of a NCAM/$PKC\beta_{II}$ complex was also confirmed in L cells upon transfection with NCAM (Figure 4B, right), indicating that it is not a phenomenon restricted to β tumor cells. In contrast to NCAM-Fc, FGF failed to promote $PKC\beta_{II}$ activation (Figure 4A), supporting the hypothesis of a dichotomy in the FGFR-mediated signaling pathways downstream of NCAM and FGF.

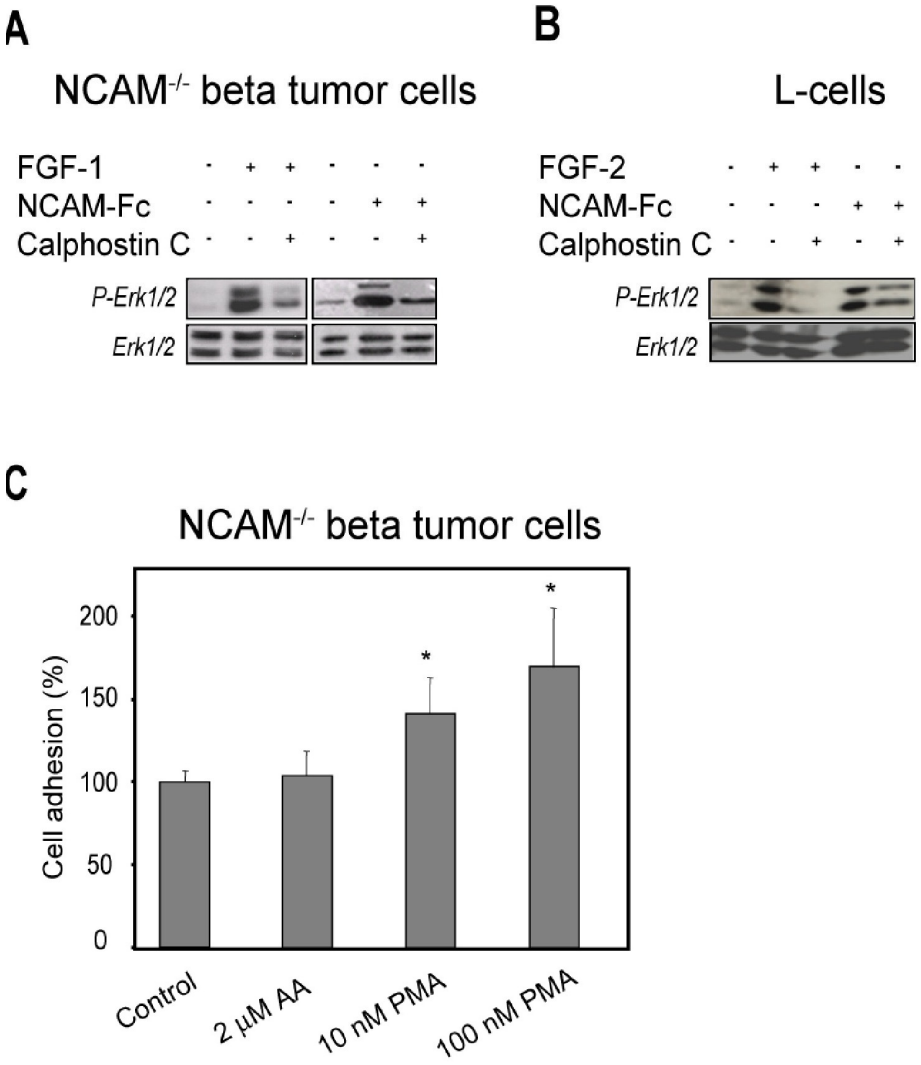

Figure 3:PKC stimulates Erk1/2 and adhesion

*(A and B) NCAM-/- β tumor cells (A) or L cells (B) were stimulated for 10 minutes with NCAM-Fc, FGF-1 or FGF-2. When needed, cells were pre-incubated with 400 nM Calphostin C for 30 minutes. Cells were then lysed and immunoblotted for phospho-Erk1/2, followed by stripping and immunoblotting for total Erk1/2. (C) NCAM-/- β tumor cells were treated with the indicated concentrations of PMA or arachidonic acid for 1 hour, followed by adhesion assays on collagen IV-coated wells. Adherent cells were counted, and the results presented as percentage of control, untreated cells ± standard deviation. Experiments were performed in quadruplicate. *p<0.005.*

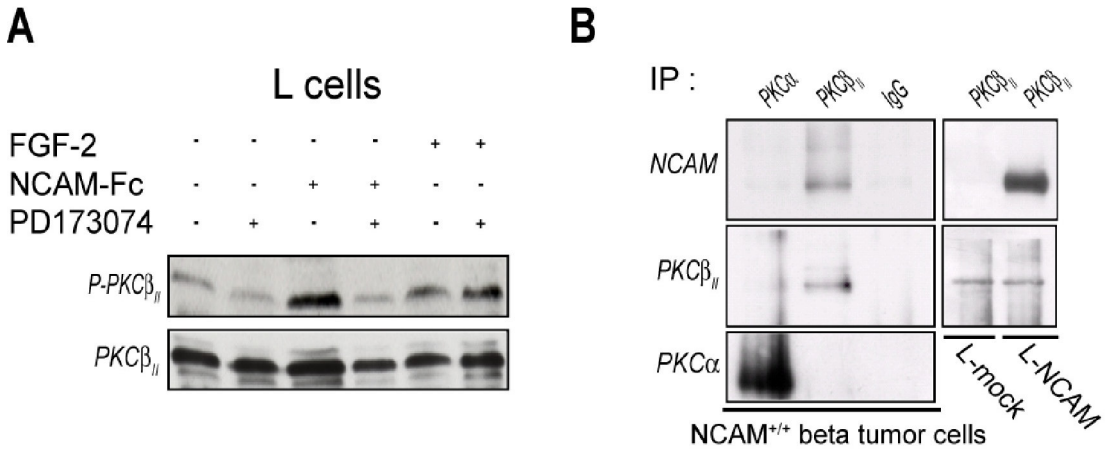

Figure 4. NCAM-activates PKCβ_{II} in an FGFR-dependent manner

(A) L cells were stimulated for 10 minutes with NCAM-Fc or FGF-2, with or without a pre-treatment with PD173074 for 2 hours. Cells were then lysed and immunoblotted for phospho-PKC$_{\beta II}$ (top panel), followed by stripping and immunoblotting for total PKC$_{\beta II}$ (bottom panel). (B) PKC isoforms were immunoprecipitated from protein extracts of NCAM+/+ β tumor cells (left panels) or of mock- or NCAM-transfected L cells (right panels). Immunoprecipitates were then probed with antibodies against NCAM or against the specific PKC isoforms as indicated.

To gain further insight into the biological relevance of NCAM-induced PKCβ_{II} activation, we took advantage of CGP53353, a chemical compound that selectively inhibits PKCβ_{II} function (Kouroedov et al., 2004). CGP53353 was used to investigate the role of PKCβ_{II} in NCAM-induced cell adhesion. As shown in Figure 5A and B, CGP53353 repressed cell-matrix adhesion of both NCAM-/- β tumor cells and L cells stimulated with NCAM-Fc, supporting the role of PKCβ_{II} as an effector of NCAM signaling. In contrast, CGP53353 showed no effect on FGF-induced cell-matrix adhesion (Figure 5A and B), confirming that PKCβ_{II} is not involved in the signaling cascade elicited by FGF. Rather, FGF appeared to induce the activation of PKCα and/or γ, since the compound HBDEE, which selectively inhibits PKCα and γ (Kashiwada et al., 1994), blocked the matrix adhesion of L cells stimulated with FGF-2, but not with NCAM-Fc (Figure 5C). A similar picture emerged when we analyzed the Erk1/2 activation pathways in both NCAM-/- β tumor cells and L cells: while CGP53353 inhibited the phosphorylation of Erk1/2 in cells treated with NCAM-Fc, but not with FGF, HBDEE showed the opposite effect (Figure 5D). Finally, in agreement with previous reports on the brain-restricted expression of PKCγ (Musashi et al., 2000), we did not detect this PKC isoenzyme in our cellular systems (not shown), indicating that in the experiments described above HBDEE acted as a selective PKCα inhibitor. Taken together, these results indicate that NCAM activates PKCβ_{II} in an FGFR-dependent manner whereas FGF activates PKCα, strongly supporting the notion that the two molecules induce distinct signaling cascades.

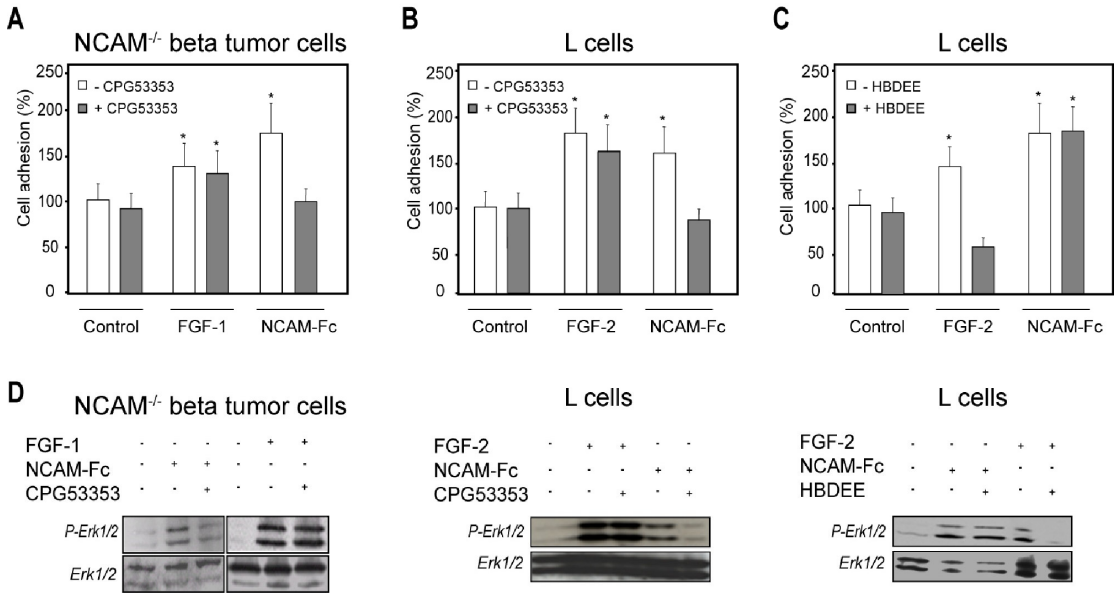

Figure 5: NCAM and FGF activate different PKC isoenzymes.

*(A) NCAM-/- β tumor cells were stimulated for 10 minutes with NCAM-Fc or FGF-1, in the absence (white bars) or presence of CGP53353 (grey bars). Cells were then subjected to adhesion assays on collagen IV. Adherent cells were counted and the results are presented as percentage of control, untreated cells ± SD. Experiments were performed in quadruplicate. *p<0.005. (B and C) L cells were stimulated for 10 minutes with NCAM-Fc or FGF-2, in the absence (white bars) or presence (grey bars) of 10 μM CGP53353 (B) or 100 μM HBDEE (C). Cells were then subjected to adhesion assays on collagen IV. Adherent cells were counted and are indicated as percentage of control, untreated cells ± SD. Experiments were performed in quadruplicate. *p<0.005. (D) NCAM-/- β tumor cells (left panel) were stimulated for 10 minutes with NCAM-Fc or FGF-1, with or without a pre-treatment with CGP53353. L cells (middle and right panels) were stimulated for 10 minutes with either 20 μg/ml NCAM-Fc or 20 ng/ml FGF-2, with or without a pre-treatment with 10 μM CGP53353 (middle panel) or 100 μM HBDEE (right panel), as indicated. Treated cells were lysed and immunoblotted for phospho-Erk1/2, followed by stripping and immunoblotting for total Erk1/2.*

To gain further insight into the dichotomy in the FGFR signaling activated by NCAM and FGF, we focused on additional candidate effectors. Our previous results indicated that non-receptor tyrosine kinases of the Src family associate with the NCAM/FGFR complex in β tumor cells. Moreover the inhibition of Src kinases with the compound PP1 resulted in the neutralization of NCAM function (Cavallaro et al., 2001), thus implicating Src in the signaling elicited by the NCAM/FGFR complex. This hypothesis has been confirmed biochemically, based on the observation that treating NCAM-negative L cells with NCAM-Fc induced Src activation, as revealed by the use of an antibody that specifically recognizes the active form of the kinase (Figure 6A). The antibodies that were used in these experiments cross-reacted with various members of the Src family, excluding a clear distinction between the specific Src family kinase(s) activated by NCAM. NCAM-Fc-induced activation of Src

kinases was mediated by FGFR, since it was abolished by PD174074 (Figure 6A). Moreover, as shown in Figure 6B, PP1 inhibited the activation of Erk1/2 in cells treated with NCAM-Fc, implicating Src kinases as signaling effectors of the NCAM/FGFR complex upstream of Erk1/2. Notably, when cells were treated with FGF-2, no Src activation was detected (Figure 6A). In agreement with this, the Src inhibitor PP1 showed no effect on FGF-induced activation of Erk1/2 (Figure 6B). Therefore, NCAM induced Src activation in an FGFR-dependent, whereas FGF was unable to regulate Src activity. We have also focused on another classical target of FGFR signaling, namely FGF-receptor substrate-2 (FRS2) that is known to undergo tyrosine phosphorylation upon activation of FGFR (Eswarakumar et al., 2005). Unlike PKCβ_{II} and Src, both NCAM-Fc and FGF-2 were able to induce FRS2 phosphorylation in L cells, as demonstrated by immunoblotting the lysates of stimulated cells with an antibody against phospho-FRS2 (Figure 6C). Finally, PD173074 abolished FRS2 phosphorylation in cells stimulated either with NCAM-Fc or with FGF-2, confirming the involvement of FGFR in the signaling elicited by both proteins.

Taken together, these results support the notion that both NCAM and FGF induce FGFR activation, yet the receptor responses and the downstream signaling pathways evoked by the two stimuli are remarkably different.

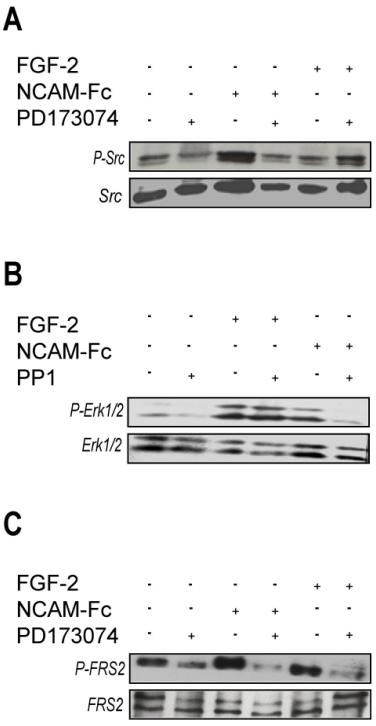

Figure 6: NCAM and FGF activate distinct, FGFR-mediated signaling pathways

L cells were stimulated for 10 minutes with NCAM-Fc or FGF-2, with or without a pre-treatment with PD173074 for 2 hours (A and C) or with PP1 for 30 minutes (B). Cells were then lysed and immunoblotted for phospho-Src (A, top panel), phospho-Erk1/2 (B, top panel) or phospho-FRS2 (C, top panel), followed by stripping and immunoblotting for total Src, Erk1/2 or FRS2, respectively (bottom panels).

3.3.4. NCAM regulates the cellular response to FGF

Since both NCAM and FGF stimulate FGFR signaling, we next assessed whether NCAM modulates the cellular response to FGF. Although NCAM *per se* stimulated cell-matrix adhesion (Cavallaro et al., 2001; Figures 1 and 7A), it strongly inhibited the adhesion induced by FGF. Moreover, serum-induced adhesion was also repressed upon expression of NCAM (Figure 7A). Finally, NCAM-Fc also slightly represses the adhesion of NCAM-transfected cells (Figure 7A), suggesting that NCAM homophilic interactions do not account for NCAM-dependent cell-matrix adhesion.

We have previously reported that FGF induces MAPK activation in NCAM-/- β tumor cells, whereas wild-type cells, which exhibited a constitutive activation of the MAPK pathway, did not further respond to FGF treatment (Cavallaro et al., 2001), suggesting that NCAM may interfere with FGF function. However, those results were obtained on cell lines derived from tumors of different animals, raising the possibility that the different responses to FGF were due to intrinsic differences between the two cell lines, rather than to the expression of NCAM. Hence, we performed these experiments on mock- vs. NCAM-transfected L cells. Serum-starved L-mock cells showed a very low level of basal activation of Erk1/2, which was strongly enhanced by FGF-2. In contrast, the forced expression of NCAM resulted in constitutive MAPK activation that was only slightly increased by FGF-2 (Figure 7B, upper panel). This differential response to FGF-2 by L-mock and L-NCAM cells recapitulated that of NCAM-/- vs. wild-type β tumor cells (Cavallaro et al., 2001). Furthermore, in time-course experiments L-mock cells exhibited a strong and sustained response to FGF-2 for at least 4 hours, while the small peak of Erk1/2 activation in L-NCAM cells declined rapidly to the basal level (Figure 7B, bottom panel). Since MAPK activation often represents a proliferative signal, we verified whether NCAM affected the proliferation of L cells. Indeed, while L cells showed a strong proliferative response to recombinant FGF-2, this effect was markedly inhibited by the expression of NCAM (Figure 7C). A similar inhibitory effect was observed when cells were stimulated with fetal bovine serum. Thus, NCAM exerts a negative regulation on FGF-induced cell adhesion, MAPK activation and cell proliferation, supporting a novel role for NCAM in the modulation of FGFR response to its classical ligand FGF.

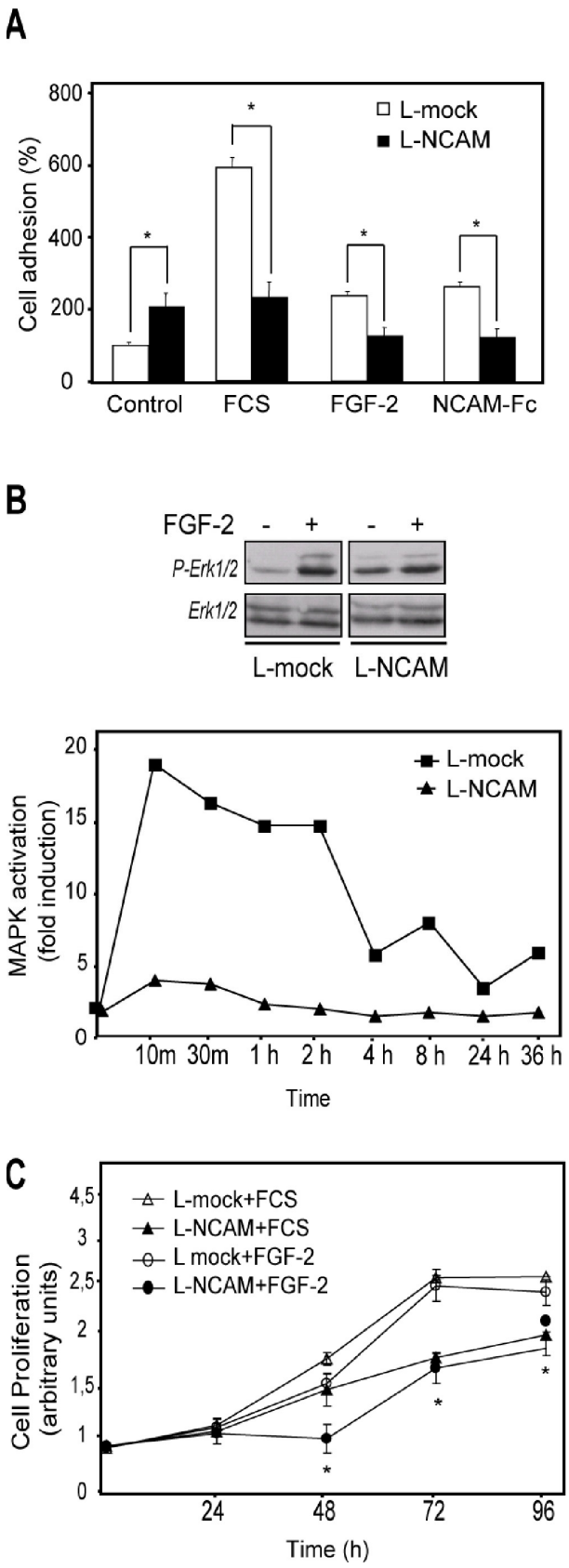

Figure 7: NCAM regulates the cellular response to FGF

*(A) Mock (open bars) or NCAM-transfected L cells (solid bars) were stimulated for 10 minutes with either 10% fetal calf serum, 20 ng/ml FGF-2, or 20 μg/ml NCAM-Fc, followed by adhesion assays on collagen IV-coated wells. Adherent cells were counted and the results are presented as percentage of control, untreated L-mock cells ± standard deviation. Experiments were performed in triplicate. *p<0.005. (B) Upper panel: mock- or NCAM-transfected L cells were stimulated for 10 minutes with FGF-2, followed by cell lysis and immunoblotting for phospho-Erk1/2 and then for total Erk1/2. Bottom panel: mock- (solid squares) or NCAM-transfected L cells (solid triangles) were treated with 20 ng/ml FGF-2 for the indicated time lengths. After the treatment cells were lysed and subjected to SDS-PAGE and immunoblotting for phospho-Erk1/2, followed by stripping and immunoblotting for total Erk1/2. The densitometric ratio between phosphorylated and total Erk1/2 was measured for each time point. The values relative to the basal activation of Erk1/2 (time 0) in a representative experiment are shown. The experiment was repeated three times with similar results. (C) Mock- (open symbols) or NCAM-transfected L cells (solid symbols) were treated daily with 10% fetal calf serum (triangles) or 20 ng/ml FGF-2 (circles) for 4 days. Cells were counted every 24 hours and the ratio with non-stimulated L-mock cells was determined for each time point. Experiment were performed in quadruplicate ± SD. *p<0.005 (L-NCAM vs. L-mock).*

3.4. Discussion

While NCAM has long been known to control various functions in the nervous systems, which include progenitor cell migration, axon guidance, mossy fiber fasciculation and spatial learning (Hinsby et al., 2004), its role in non-neuronal tissue has remained elusive. We have previously shown that the loss of NCAM in pancreatic β cell tumors results in tissue disaggregation and lymph node metastasis (Cavallaro et al., 2001). The latter event is likely due to the tumor-associated lymphangiogenesis caused by NCAM deficiency (Crnic et al., 2004). Tissue disaggregation and tumor cell detachment reflect a deficit in β_1-integrin-mediated cell-matrix adhesion. Indeed, we have previously reported that NCAM stimulates the activation of β_1-integrin and, hence, matrix adhesion by triggering a signaling cascade mediated by FGFR. These observations led us to the identification of a novel signaling complex in which NCAM associates with FGFR and with N-cadherin (Cavallaro et al., 2001). Strong support for this model came from protein-protein interaction studies that revealed the direct binding of NCAM to FGFR, with the interaction domains mapping in the two membrane-proximal F3 repeats of NCAM and in the second and third Ig loops of FGFR (Kiselyov et al., 2003). Thus, given that NCAM homophilic interactions are mediated by the membrane-distal Ig1-2-3 domains (Soroka et al., 2003), one should be able to investigate the NCAM/FGFR cross-talk independently from the cell-cell adhesive properties of NCAM. In this study, we show that the binding of NCAM to FGFR and an intact FGFR signaling are essential for the stimulation of specific events such as Erk1/2 activation and cell-matrix adhesion. An interplay between adhesion molecules, including NCAM, and FGFR has long been proposed in the nervous system (Walsh and Doherty, 1997). We provide further experimental support for this model by showing that NCAM acts as a direct inducer of FGFR function in non-neuronal cells. Based on our results with soluble NCAM-Fc, the membrane localization of NCAM is dispensable for its interaction with FGFR. NCAM shedding has been described in cultured cells (Deak et al., 2005; Diestel et al., in press), and soluble forms of NCAM have been detected in human cerebrospinal fluid and serum, with high levels in severe neurological disorders and in cancer patients (Gower et al., 1988; Ledermann et al., 1994; Lynch et al., 1997; Torado et al., 2004). Moreover, the production of soluble NCAM in mice lacking membrane-associated NCAM results in embryonic lethality (Rabinowitz et al., 1996), thus highlighting the biological relevance of NCAM's heterophilic interactions. In this context, our data point to FGFR as a major effector of soluble NCAM, raising the intriguing hypothesis that NCAM acts as a *bone fide* ligand for FGFR *in vivo*. Such a novel ligand-receptor interaction needs further investigation, in particular to verify whether it plays a pathogenetic role in diseases characterized by excessive release of soluble NCAM, potentially resulting in aberrant activation of FGFR and/or the inhibition of FGF-induced FGFR signaling.

An interesting implication of our data is that, although both NCAM and FGF stimulate FGFR activity, only a subset of FGFR-dependent events is elicited by both molecules. Indeed, while NCAM induces neurite outgrowth in β tumor cells by binding to and activating FGFR, FGF is unable to rescue this process in NCAM-deficient cells (Cavallaro et al., 2001). In contrast, both NCAM and FGF stimulate FGFR-mediated matrix

adhesion and Erk1/2 activation in NCAM-/- tumor β cells and in L cells. Finally, FGF exerts a proliferative effect that is not recapitulated by NCAM, either membrane-associated (Cavallaro et al., 2001) or as a soluble molecule (our unpublished data). To gain insights into the molecular mechanisms that account for this dichotomy between NCAM and FGF-induced FGFR signaling, we have dissected the biochemical cascades elicited by the two molecules. Both NCAM and FGF induce the activation of two classical FGFR substrates, namely PLCγ and FRS2. However, the signaling pathways elicited by NCAM and FGF were clearly divergent at the level of PKC, in that NCAM induced $PKC\beta_{II}$ whereas FGF stimulated PKCα. The activation of $PKC\beta_{II}$ was not due to NCAM homophilic interactions, since it was induced by stimulating NCAM-negative cells (either β tumor cells or fibroblastic cells) with soluble NCAM-Fc. Moreover, the inhibition of FGFR signaling repressed NCAM-induced $PKC\beta_{II}$ activation, clearly indicating that it is mediated by FGFR, as previously shown in neurons (Leshchyns'ka et al., 2003). The induction of PKC occurs also upon NCAM homophilic binding, i.e. during NCAM-mediated cell-cell adhesion and neurite outgrowth. However, in that case NCAM activates multiple PKC isoenzymes, including PKCα (Kolkova et al., 2005). Hence, NCAM-NCAM and NCAM-FGFR interactions elicit different signaling pathways, as it was recently proposed by Kiryushko and co-workers (Kiryushko et al., 2006). In that study, however, NCAM-induced activation of Src kinases appeared to be independent of FGFR, while we have clearly shown that FGFR signaling is required downstream of NCAM in order to stimulate Src activation. Such a discrepancy might be due to the cell type-specificity of NCAM functions, given that Kiryushko and co-workers analysed NCAM signaling in neurons, while our studies were performed in non-neuronal cells. Our data on the divergent FGFR signaling pathways downstream of NCAM and FGF are also supported by the observation that the adaptor protein ShcA is phosphorylated in an FGFR-dependent manner upon NCAM-induced neurite outgrowth, but not following FGF stimulation (Hinsby et al., 2004).

Hence, NCAM and FGF induce FGFR signaling in a different manner, and future work should address the molecular basis of this divergence. For example, it would be insightful to elucidate whether NCAM, especially when associated to the membrane, induces the clustering of FGFR, a property that would not be shared with FGF. Indeed, lateral clustering of NCAM has been described and involves the first three Ig domains (Soroka et al., 2003). Thus, the integration of NCAM *cis*-oligomerization with FGFR binding would appear as a novel mechanism of FGFR activation. Alternatively, NCAM, unlike FGF, might recruit FGFR to specific cell surface compartments, implying that the divergence between NCAM and FGF signaling is dictated by spatial parameters that are also fulfilled by soluble NCAM-Fc.

Our studies demonstrate that NCAM not only directly stimulates FGFR activity, but also exerts a regulatory function on the cellular response to FGF. Indeed, we have provided experimental evidence that various FGF-induced effects, including matrix adhesion, MAPK activation and cell proliferation, are repressed upon concomitant expression of NCAM. An inhibitory effect of NCAM on FGF-stimulated cell proliferation has been described in astrocytes and in neural progenitor cells. In the first case, it was attributed to NCAM's homophilic interactions (Krushel et al., 1998), whereas heterophilic partners were implicated

in the anti-proliferative function of NCAM in neural progenitors (Amoureux et al., 2000). Based on the data presented here, we propose that NCAM exerts a tight control on the cellular response to FGF, which not only extends beyond the central nervous system (we observed it in pancreatic β cells and in fibroblasts), but is also not restricted to the control of cell proliferation, in that it also modulates cell-matrix adhesion (see Figure 7A). FGFs induce a wide variety of cellular processes that depend on a complex network of signaling and transcriptional events that is cell type-specific. Notably, the control mechanisms that have been invoked so far to explain the differential responses to FGFs are mostly intracellular (Dailey et al., 2005). Our data implicate membrane-associated NCAM as a novel and important regulator of FGF signaling, adding a further level of modulation of FGFR activity.

The aberrant expression and/or function of NCAM have been described in several pathological conditions, ranging form neurological to neoplastic diseases (Vawter, 2000; Mikkonen et al., 2001; Cavallaro and Christofori, 2004). In addition, NCAM-/- mice exhibit significant developmental and behavioral defects (Cremer et al., 1994; Cremer et al., 1997; Stork et al., 1997; Stork et al., 1999). The pathogenetic role of NCAM in these disorders has been attributed to the dysregulation of its adhesive properties. However, based on the data presented here, aberrant FGFR function needs to be considered as an additional consequence of NCAM alterations, and investigated as a possible pathogenetic factor. For example, excessive FGFR signaling in tumors induces cancer cell proliferation, survival and invasion, together with angiogenesis and metastasis (Grose and Dickson, 2005). Together with the notion that NCAM expression is reduced during the progression of certain tumor types (Cavallaro and Christofori, 2004) and that its loss induces metastasis (Perl et al., 1999), this implies that NCAM might act as a tumor suppressor by negatively regulating FGFR signaling. Indeed, NCAM-dependent inhibition of FGFR activity has been observed not only in β tumor cells (Cavallaro et al., 2001), but also in other cancer cell types (S. Zecchini, A. Godwin, M. Bianchi, P, Nuciforo, and U. Cavallaro, manuscript in preparation).

In summary, we have shown that NCAM can act as an activating ligand for FGFR and as a modulator of the cellular response to FGF stimulation. Future studies should address the molecular basis of these additional functions of NCAM, thus unraveling a novel mechanism for the regulation of FGFR activity and hopefully leading to innovative therapeutic approaches for those diseases caused by dysfunction of NCAM and/or FGFR.

β_1-integrin deletion induces tumor cell dissemination and reduction of tumor burden in the Rip1Tag2 model

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Abstract

Neural cell adhesion molecule (NCAM) has been mainly studied in the central nervous system where it mediates processes, such as neurite outgrowth and axon guidance. However, NCAMs function in non-neuronal tissues has remained elusive. Using the Rip1Tag 2 model of multistage tumorigenesis, we have previously reported that loss of NCAM induces tissue disaggregation, lymphangiogenesis and metastasis. These processes might result from the loss of FGFR signaling–dependent activation of β_1-integrin in NCAM-deficient cells. Here we show that interference with β_1-integrin function in the Rip1Tag2 model leads do tumor cell cluster dissemination into lymphatics but not to increased lymphangiongenesis. Moreover, β_1-integrin-deficient cells are not able to metastasize, and tumors with reduced β_1-integrin expression are smaller, probably due to the induction of senescence. Our results indicate a so far unknown role of β_1-integrin in senescence.

4. β_1-integrin deletion induces tumor cell dissemination and reduction of tumor burden in the Rip1Tag2 model

4.1. Introduction

The Neural Cell Adhesion Molecule NCAM is a member of the family of Ca^{2+}-independent cell adhesion molecules, mediating homotypic cell-cell as well as heterotypic cell-matrix adhesion (Cunningham, 1995; Rutishauser, 1993). NCAM is expressed during development and its involvement in developmental processes has been studied in great detail (Walsh and Doherty, 1997). In the adult, NCAM expression is mainly found in neuronal tissues but also in skeletal muscle cells (Dickson et al., 1987) as well as some neuroendocrine tissues such as pancreas (Rouiller et al., 1990; Langley et al., 1989; Cirulli et al., 1994). In many human cancers, NCAM expression changes from the adult, 120kD GPI-anchored isoform to the embryonic, 140kD and 180kD transmembrane isoforms (Johnson, 1991; Kaiser et al., 1996; Lipinski et al., 1987; Moolenaar et al., 1992; Roth et al., 1988). Furthermore, reduced overall expression of NCAM has been correlated with poor prognosis in astrocytomas, colon and pancreatic cancer (Fogar et al., 1997; Huerta et al., 2001; Sasaki et al., 1998). Besides its function as a cell-cell adhesion molecule, recent research has focused on NCAMs role in signal transduction (reviewed in Walmod et al., 2004).

We have previously employed a transgenic mouse model of β cell carcinogenesis (Rip1Tag2; Hanahan, 1985) to study NCAM function during tumor progression. In Rip1Tag2 mice (RT2 mice), the Simian Virus large T oncogene is expressed under the control of the Rat insulin promoter, resulting in the reproducible development of β cell tumors following a multistage tumorigenesis pathway. These mice usually do not form metastases. However, when crossed to NCAM knock-out mice (NCAM-/- mice), formation of metastasis could be observed in 50% of the resulting RT2;NCAM-/- mice (Perl et al., 1999). Further investigations revealed that in NCAM-deficient tumors, tumor-associated lymphangiogenesis is induced via *de novo*-expression of the lymphangiogenic factors VEGF-C and VEGF-D. Repression of VEGF-C and VEGF-D function by adenoviral expression of a soluble form of their cognate receptor (VEGFR-3) resulted in reduced metastasis formation in RT2;NCAM-/- mice, suggesting that loss of NCAM promotes metastasis by induction of lymphangiogenesis (Crnic et al., 2004).

Another feature of NCAM-/- tumors is the occurrence of alterations in tissue architecture, namely tumor tissue disaggregation and the appearance of hemorrhagic cavities. Clusters of tumor cells are found floating in these lacunae. Cell lines derived from NCAM expressing RT2 tumors (βT2 cells) and NCAM-deficient RT2;NCAM-/- tumors (βTN2 cells) revealed that NCAM deficiency leads to impaired cell-matrix adhesion but does not alter cell-cell adhesive properties *in vitro*. Extensive biochemical analysis identified a potential mechanism by which NCAM could affect cell-matrix adhesion. In βT2 cells, NCAM associates and activates FGFR-4, leading to the assembly of a classical signaling complex.

The activation results, via as yet unidentified pathway(s), in the stimulation of β_1-integrin dependent adhesion to ECM proteins such as collagen IV (Cavallaro et al., 2001).

β_1-integrin belongs to the family of integrin transmembrane receptors consisting of 8 β and 18 α subunits that assemble as heterodimers to form 24 distinct integrins. The main ligands for integrins are extracellular matrix proteins and cellular counter-receptors. In their role as the major adhesion receptors, integrins signal across the plasma membrane in both directions: high affinity ligand binding requires integrins to become activated by undergoing conformational changes regulated by inside-out signals. In turn, integrin ligation triggers outside-in signals that regulate different aspects of cell behavior such as cell survival, control of transcription, cell proliferation, cell motility and cytoskeletal organization (Hynes, 2002). Due to their broad spectrum of features, integrins and integrin signaling have been shown to contribute to tumor progression in various ways (Guo and Giancotti, 2004). Recent reports have shown that β_1-integrin expression is critical for the initiation of mammary tumorigenesis *in vivo*, and for maintaining the proliferative capacity of late stage tumor cells .

Our aim was to investigate the role of β_1-integrin in inducing the phenotypes observed in RT2;NCAM-/- mice, namely tissue disaggregation and increased lymphangiogenesis. To address this question, we employed the RT2 tumor mouse model carrying a conditional, β cell specific knock-out of the β_1-integrin locus. We show that a partial deletion of β_1-integrin in β cell tumorigenesis leads to tumor cluster dissemination into lymphatics but does not induce lymphangiogenesis and metastasis. Mice having lost β_1-integrin expression in β cells display reduced tumor burden, most likely through the induction of senescence. Furthermore, tumor cells lacking β_1-integrin are not able to form tumors and metastases in transplantation experiments.

4.2. Materials and Methods

4.2.1. Histopathological analysis

The following antibodies were used for immunohistochemistry or immunofluorescence on paraffin sections: guinea pig anti-insulin (DakoCytomation, Glostrup, Denmark), rabbit anti-mouse LYVE-1 (Reliatech, Braunschweig, Germany), biotinylated mouse anti-BrdU (Zymed, South San Francisco, CA) for detection of proliferating cells, In Situ Cell Death Detection Kit, (TUNEL, Roche, Basel, Switzerland) for visualization of apoptotic cells. All biotinylated secondary antibodies for immunohistochemistry (Vector, Burlingame, CA) were used at a 1:200 dilution, and positive staining was visualized with the ABC horseradish peroxidase kit (Vector) and DAB Peroxidase Substrate (Sigma Chemical Co., St. Louis, MO) according to the manufacturer's recommendations. For analysis of tissue morphology, slides were slightly counterstained with hematoxylin or eosin. Alexa Fluor 568- and 488-labeled secondary antibodies (Molecular Probes, Eugene, OR) diluted 1:400 were used for immunofluorescence analysis. 6-Diamidino-2-phenylindole (DAPI) was used for nuclear staining in immunofluorescence stainings. All paraffin-embedded sections were subject to antigen retrieval with 10 mM citrate buffer (microwave) except for insulin and glucagon (10 min in 0.2% Triton X-100 in PBS), BrdU (1 h in 2N HCl and subsequently 1 h 1x trypsin at room temperature), and TUNEL (10 min 2 µg/ml Proteinase K (Fluka) at room temperature). Stained sections were viewed on a Axioskop 2 plus light microsope (Zeiss, Feldbach, Switzerland) using the axiovision 3.1. software (Zeiss, Feldbach, Switzerland) or a Nikon Diaphot 300 immunofluorescence microscope (Nikon, Egg, Switzerland) using the Openlab 3.1.7. software (Improvision, Coventry, England).

For BrdU labeling, 100 µg BrdU (Sigma Chemical Co., St. Louis, MO) per gram body weight were injected 90 min before sacrificing the mice. To determine tumor cell proliferation/apoptotic indices, BrdU-/TUNEL-positive nuclei were counted per randomly chosen 40x magnification field of tumor tissue, respectively. Approximately 10 fields/mouse were counted.

Lymphangiogenesis was quantified by assessing the extent by which LYVE-1-positive lymphatic vessels surrounded the tumor perimeter. Tumors from all mice of a genotype were grouped into five classes: tumors that were not in contact with any lymphatic vessel (0%), tumors that were surrounded less than 10% (< 10%), less that 25% (< 25%), less than 50% (< 50%), and tumors that were surrounded more than 50% of the tumor perimeter by lymphatics (> 50%).

Tumor grading: Tumors were categorized into following sub-classes: normal/hyperplastic islet (including normal as well as enlarged islets), adenoma (larger than 1 mm in diameter, well differentiated tumor cells, encapsulated tumor, no invasive tumor edges), carcinoma grade 1 (well differentiated, one invasive tumor edge), carcinoma grade

2 (partially dedifferentiated, tumor capsule largely absent, more than one invasive tumor edge), carcinoma grade 3 or anaplastic tumor (complete loss of tumor cell differentiation).

The islet area was measured on pictures of insulin and glucagon stained slides using the ImageJ software of the National Institutes of Health (http://rsb.info.nih.gov/ij/). All statistical analyses were performed using the GraphPad software.

4.2.2. Mouse tissue processing

Animal care was in accordance with Swiss Animal Protection Ordinance issued by the Swiss Federal Veterinary Office. All mice were sacrificed between 12 and 13 weeks of age. Tumor incidence per mouse was determined by counting all macroscopically apparent tumors with a minimal diameter of 1 mm. Tumor volume was defined as total tumor volume per mouse in mm^3, calculated by measuring the tumor diameters assuming a spherical or elliptical shape of the tumors. Tumors and pancreata were fixed overnight in 4% paraformaldehyde in PBS, dehydrated in a Microm Spin Tissue Processor STP-120 (Microm, Volketswil, Switzerland) and paraffin-embedded. 5 µm paraffin-embedded tissue sections were deparaffinized and re-hydrated prior to usage according to standard procedures.

4.2.3. Tissue culture

All cell lines were grown in DMEM supplemented with 10% fetal bovine serum, 2 mmol/L glutamine and 100 units/mL penicillin. Tumor cell lines were established from insulinomas of twelve week-old RT2;$\beta_1^{fl/fl}$ and RCre;RT2;$\beta_1^{fl/fl}$ mice as described in (Cavallaro et al., 2001). In brief, tumors were excised from pancreata and single-cell suspended in cold PBS. The suspension was mixed 1:1 with DMEM supplemented with 10% fetal bovine serum, 10 % horse serum, 2 mmol/L glutamine and 100 units/mL penicillin. After 1 minute of sedimentation at room temperature, the supernatant was transferred to a new tube, re-mixed 1:1 with medium and let sediment another 10 min at room temperature. The pellet containing the tumor cells was resuspended and cells were plated on 24-well plates for further expansion. For transplantation of tumor cells, 10^6 cells in PBS were injected subcutaneously into the two flanks of C57 mice, or intravenously into athymic nude mice anesthetized with isoflurane (Minrad Inc., Buffalo, NY).

4.2.4. Cell adhesion

Matrix adhesion assays were performed on collagen IV, a substrate that mediates NCAM-dependent cell adhesion (Cavallaro et al., 2001). 96-well plates were coated with 5 µg/cm^2 of mouse collagen IV (BD Biosciences). $6x10^4$ cells were seeded per well, after

90 minutes, non adherent cells were removed by washing with PBS. Adherent cells were fixed for 20 minutes with 25% glutaraldehyde (Sigma), stained with crystal violet, washed and air-dried. Bound dye was solubilized with 10 % acetic acid and absorbance measured at 595 nm. Cell-free wells served as blanks. The assays were performed in triplicates.

4.2.5. Cell proliferation

10^5 cells were seeded onto 24 well plates at t = 0 hours. About every 24 hours, 100 µl MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 5mg/ml in PBS) was added to the investigated well. After incubation for 90 minutes at 37 °C, medium was removed and 500 µl solubilization buffer (95% isopropanol, 5% formic acid) was added and incubated for 5 minutes at RT. Absorption of the solution was determined at 570 nm.

For growth in 3D culture, $5x10^4$ cells were mixed in matrigel and seeded on a layer of solidified matrigel. After solidification of the upper, cell containing matrigel layer, normal growth medium was added to the culture.

4.2.6. FACS analysis of tumors

Tumors were dissected out of pancreata, put into ice cold PBS and minced into small pieces. After washing with PBS, tumor pieces were incubated with a collagenase mix (DMEM, 5% NU-serum (Becton Dickinson), 0.16 mg/mL DNase I, 1 mg/mL collagenase D, H and collagenase/dispase (Roche), 0.5 mg/mL Collagenase I (Sigma)) to obtain single cell suspensions for 30 minutes at 37 °C, followed by filtration and washing with FACS-PBS (1x PBS, 2% FCS). Tumor cell suspensions were incubated with anti- β_1-integrin-FITC (Serotec) and anti-CD31-PE (Pharmingen) antibodies and analyzed with a FacsScan (Becton Dickinson) using the CellQuest software.

4.3. Results

4.3.1. β_1-integrin is required for islet cell sorting

A complete knock out of the β_1-integrin gene results in embryonic lethality. We therefore employed mice carrying conditional β_1-integrin alleles, $\beta_1^{fl/fl}$ mice, (Fassler and Meyer, 1995), and crossed them to mice expressing the Cre recombinase under the control of the β cell specific Rat insulin promoter, RipCre, (Ahlgren et al., 1998). This mating gave rise to RipCre;$\beta_1^{fl/fl}$ mice that lack β_1-integrin specifically in the β cells of the pancreatic islets of Langerhans, enabling investigation of its function specifically in this tissue. The effect of β_1-integrin deletion was first looked at with respect to normal islet development. Islets of RipCre;$\beta_1^{fl/fl}$ mice were compared to either wild type C57 or $\beta_1^{fl/fl}$ control mice. In mice, most islets adopt their final shape, namely insulin expressing β cells located in the center, and non-β cells (glucagon expressing α cells, somatostatin producing γ cells and pancreatic polypeptide (PP) cells) in the islet periphery, from the age of four to five weeks.

Histopathological analysis by H&E staining of islets from 8-10 weeks old RipCre;$\beta_1^{fl/fl}$ mice showed no apparent changes in islet number, size and architecture (Figure 1, upper panel and data not shown). A closer examination by staining pancreata for insulin and glucagon, however, revealed differences in the organization of islet cells (Figure 1, lower panel). In control animals, most islets (82.4%) displayed a normal phenotype, namely α cells located within the three most peripheral cell layers (Figure 1, lower left panel). Only in a small percentage of control islets α cells were also found within the center of islets (Table1, 17,6%), hereafter referred to as mixed phenotype. In contrast, most islets of RipCre;$\beta_1^{fl/fl}$ mice were of the mixed phenotype (81% mixed vs. 19% normal phenotype, Figure 1, lower right panel and Table 1). When calculating the average number of α cells per islet area, we found that in RipCre;$\beta_1^{fl/fl}$ islets, total number of α cells/1000 μm^2 is increased (1.307 α cells/1000 μm^2 vs 2.205 α cells/1000 μm^2 , Table 1, $P<0.005$). Thus, deletion of β1-integrin leads to disturbances in cell type segregation during islet development.

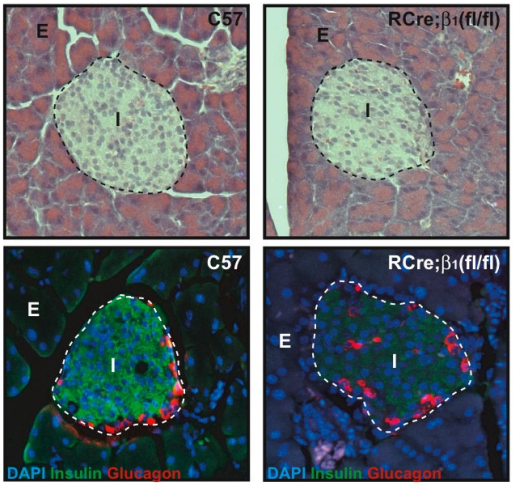

Figure 1: Sorting phenotype in RipCre;$\beta_1^{fl/fl}$ islets
Immunohistochemical analysis of wild type (upper left) and β_1-integrin deleted PFA-fixed sections of pancreatic islets by H&E staining show no alterations in islet architecture. Immunofluorescence co-stainings for insulin (green), glucagon (red) and nuclei (DAPI, blue) reveal that α cells are not properly located to the islet periphery in β_1-integrin deficient islets.

Table I: Quantification of sorting phenotype

	C57/β_1(fl/fl)		RCre;β_1(fl/fl)
N	28		28
% normal	82,4%		19,0%
% mixed	17,6%		81%
α-cells per 1000µm^2	1,307 +/-0,646	**P<0.005***	2,205 +/- 1.122

**: unpaired t-test*

4.3.2. Deletion of β1-integrin reduces tumor mass

To investigate the role of β_1-integrin *in vivo* during Rip1Tag2 tumorigenesis, we crossed Rip1Tag2 (RT2) mice into the $\beta_1^{fl/fl}$ background, resulting in RT2;$\beta_1^{fl/fl}$ control mice. To excise of the $\beta_1^{fl/fl}$ allele specifically in the β cells of the islets of Langerhans, RT2;$\beta_1^{fl/fl}$ mice were further crossed to RipCre-mice. Efficient recombination of the β_1-integrin gene in the resulting RCre;RT2;$\beta_1^{fl/fl}$ experimental and RT2;$\beta_1^{fl/fl}$ control mice was monitored by determining β_1-integrin protein levels in tumors of 12 weeks old mice. Tumor cells were subjected to FACS analysis by performing a CD31 (endothelial cell marker) and β_1-integrin co-staining, allowing exclusion of endothelial cells from the analysis. More than 95% of the cells derived from RT2;$\beta_1^{fl/fl}$ tumors show β_1-integrin expression (Region R2 in Figure 2 and Table 2), whereas in RCre;RT2;$\beta_1^{fl/fl}$ tumors a second, β1-integrin negative population appears (R1). This population represents around 40% of all analyzed cells (Table 2). From these experiments, we conclude that the $\beta_1^{fl/fl}$ allele is efficiently recombined in about 40% of all tumor cells.

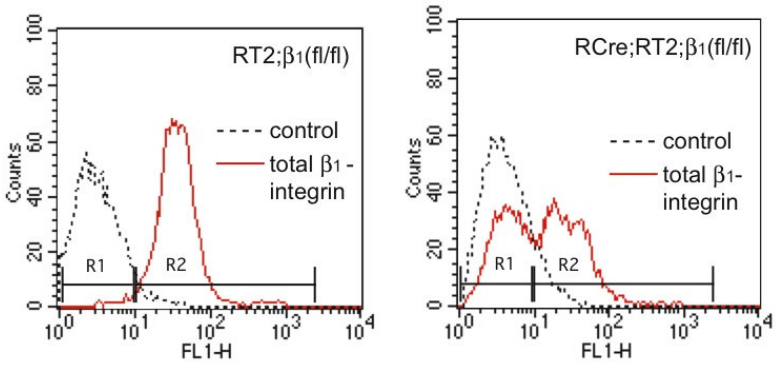

Figure 2: Histogram of β_1-integrin surface levels

FACS analysis of RT2;$\beta_1^{fl/fl}$ (left panel) and RCre;RT2;$\beta_1^{fl/fl}$ (right panel) tumor cell suspensions stained for β_1-integrin. Dashed and red lines represent control (unstained) cells and β_1-integrin stained cells respectively.

Table II: Quantification of β_1-integrin surface levels in RT2;$\beta_1^{fl/fl}$ and RCre;RT2;$\beta_1^{fl/fl}$ tumor cell

suspensions

	% of cells in R1	**% of cell in R2**
N	3	3
RT2;$\beta_1^{fl/fl}$	2,64 +/- 2,08%	96,42 +/- 2.34
RCre;RT2;$\beta_1^{fl/fl}$	39,24 +/- 3,07%	56,07 +/- 2,62%

The effects of β_1-integrin depletion on RipTag2 tumorigenesis were examined by immunohistochemical and immunopathological analysis. Control and experimental mice were sacrificed at the age of 12 weeks. Tumor-bearing pancreata were excised and tumor incidence was scored by counting and measuring macroscopically visible (>1 mm) tumors. Tumor incidence (i.e. number of tumors per mouse, Figure 3A) remained unchanged in RCre;RT2;$\beta_1^{fl/fl}$ mice as compared to control mice. However, when the total tumor volumes were calculated and compared, tumor burden was found to be significantly reduced in RCre;RT2;$\beta_1^{fl/fl}$ mice (Figure 3B).

We next investigated whether the decreased tumor sizes or masses respectively were due to a proliferation defect or increased apoptosis in β_1-integrin deleted tumors. For this purpose, PFA-fixed, paraffin embedded tissue sections of pancreata from 12 weeks old mice were prepared followed by immunohistochemical stainings for BrdU (as proliferative marker) and TUNEL (marker for cells undergoing apoptosis). RCre;RT2;$\beta_1^{fl/fl}$ tumors were not only significantly less proliferative (Figure 3C), but also showed a decreased apoptotic rate (Figure 3D). Interestingly, tumor progression was not affected since tumors of both genotypes showed a similar incidence of adenomas and grade 1, 2 and 3 carcinomas (Figure 3E).

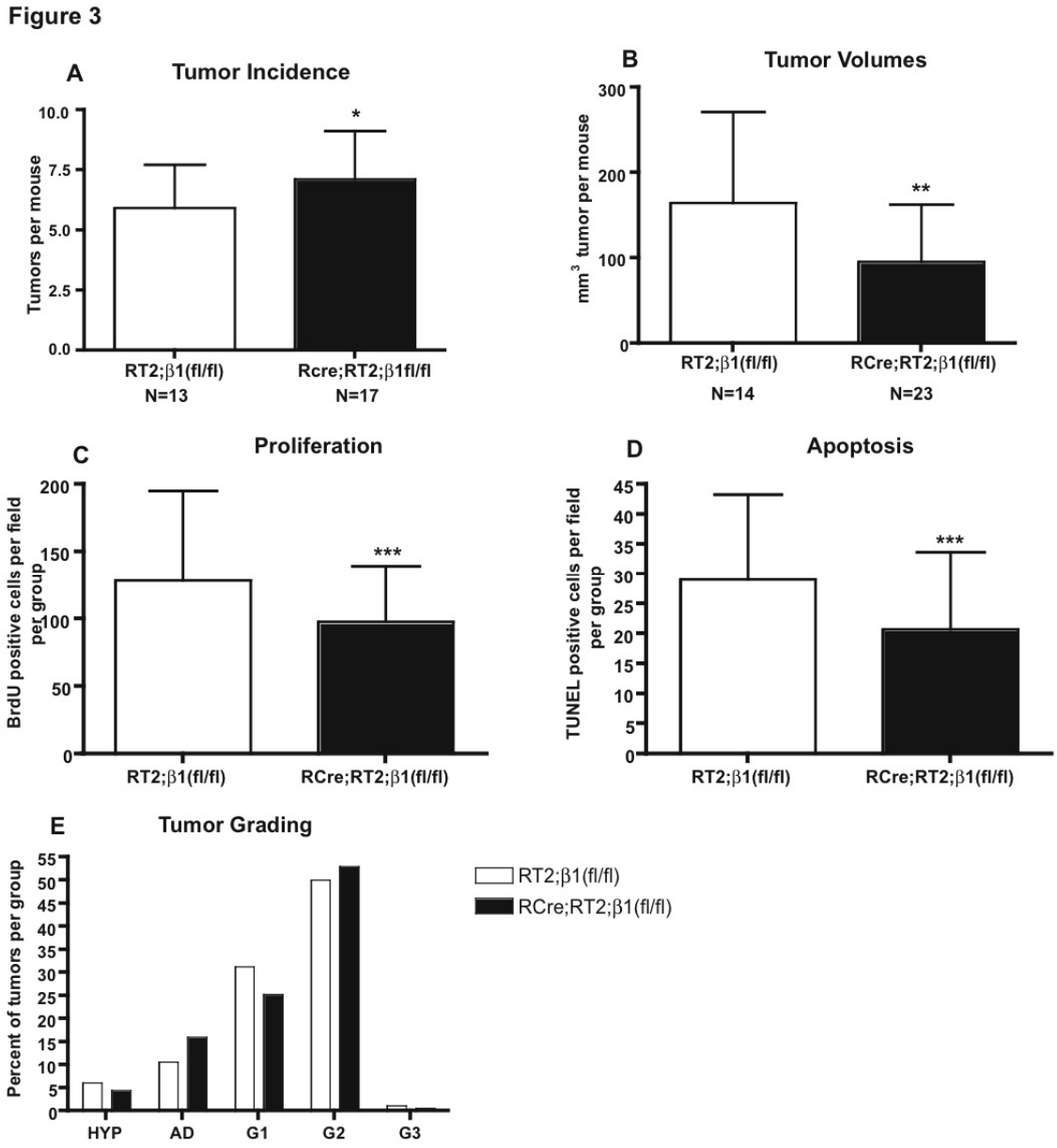

Figure 3: Statistics of histopathological and histochemical analyses of RT2;$\beta_1^{fl/fl}$ and RCre;RT2;$\beta_1^{fl/fl}$ tumors

*Tumor incidences (panel A) and tumor volumes (panel B) of RT2;$\beta_1^{fl/fl}$ control tumors and RCre;RT2;$\beta_1^{fl/fl}$ experimental tumors. Proliferating cells were visualized by BrdU staining (panel C) and apoptotic cells by the TUNEL reaction (panel D). BrdU or TUNEL positive cells per a defined area were counted. For tumor grading (panel E), tumors of H&E stained sections were classified according to their histological grading. White bars, RT2;$\beta_1^{fl/fl}$ control tumors; black bars; RCre;RT2;$\beta_1^{fl/fl}$ experimental tumors; HYP, hyperplastic islets; AD, adenoma; G1, carcinoma grade1; G2, carcinoma grade 2; G3, carcinoma grade 3; *, P>0.1; **, P<0.05; ***, P<0.01; N, number of analyzed mice;*

4.3.3. Loss of β1-integrin induces tumor cell dissemination into lymphatics

Previously, we have reported that NCAM- deficient insulinomas show increased tumor associated lymphangiogenesis (Crnic et al., 2004), as well as tumor tissue disaggregation and increased metastasis (Cavallaro et al., 2001; Perl et al., 1999). To investigate whether loss of β_1-integrin leads to increased lymphangiogenesis, we analyzed sections of 11-13 weeks old mice for the presence of lymphatic vessels using the α-LYVE-1 antibody (Banerji et al., 1999). Tumors were divided into five groups according to the degree of lymphatic vessel lining (no lymphatic vessels = 0 %, 1-10 %, 10-25 %, 25-50 % or > 50 % of tumor perimeter covered by lymphatic vessels, Figure 4). Statistical analysis revealed that no significant changes in lymphangiogenesis were observed for any group. Moreover, as asseyed by immunohistochemical staining, VEGF-C levels are not altered in β_1-integrin deleted tumors (data not shown). We conclude that loss of β_1-integrin function does not induce lymphangiogenesis and the expression of lymphangiogenic factor VEGF-C.

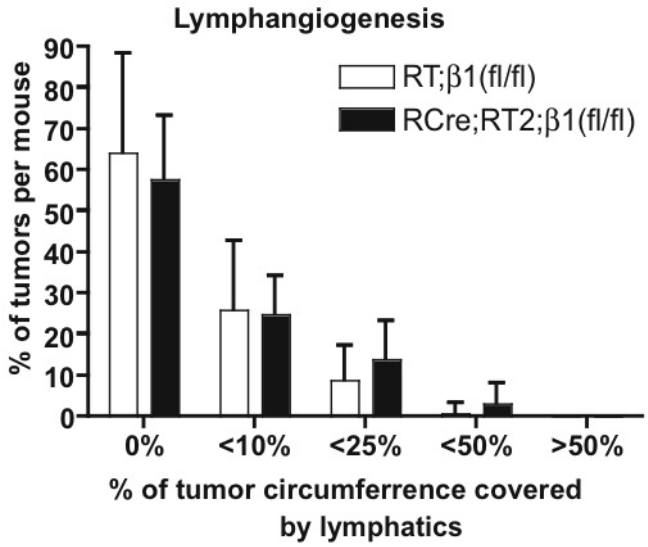

Figure 4: Lymphatic lining of RT2;$\beta_1^{fl/fl}$ and RCre;RT2;$\beta_1^{fl/fl}$ tumors

RT2;$\beta_1^{fl/fl}$ (white bars) and RCre;RT2;$\beta_1^{fl/fl}$ (black bars) tumors were categorized according to the degree of lymphatic coverage of the tumor circumference (see text). Statistical analysis (unpaired t-test) indicated that differences within groups are not statistically significant.

Ablation of NCAM in the Rip1Tag2 model leads to a severe change in tumor architecture, namely marked tissue disaggregation and the appearance of hemorrhagic lacunae (Cavallaro et al., 2001; Xian et al., 2006) Extensive biochemical analysis of NCAM-/- tumor cell lines revealed that NCAM-deficient cells have an impaired capability to adhere to extra-cellular matrix *in vitro*, arising from the lack of NCAM dependent, FGFR-4 mediated activation of β_1-integrin. We thus investigated if deletion of β_1-integrin in this tumor model induces tissue disaggregation by analyzing H&E stained sections of RT2;$\beta_1^{fl/fl}$ and

RCre;RT2;$\beta_1^{fl/fl}$ tumors. We did not observe significantly increased appearance of hemorrhagic lacunae and therefore tissue disaggregation within RCre;RT2;$\beta_1^{fl/fl}$ tumors. Interestingly though, during the analysis of sections stained for LYVE-1 (see above), we found disseminated tumor cell clusters enclosed by lymphatic vessels in 60% of 15 analyzed RCre;RT2;$\beta_1^{fl/fl}$ mice and only in 7.7% of 12 control mice (Figure 5A and 5B). The tumor cell clusters were predominantly found in close vicinity to the tumors (Figure 5C, left panel) or tumor edges (Figure 5C, right panel). Importantly, immunohistopathological analysis did not reveal the occurrence of metastases.

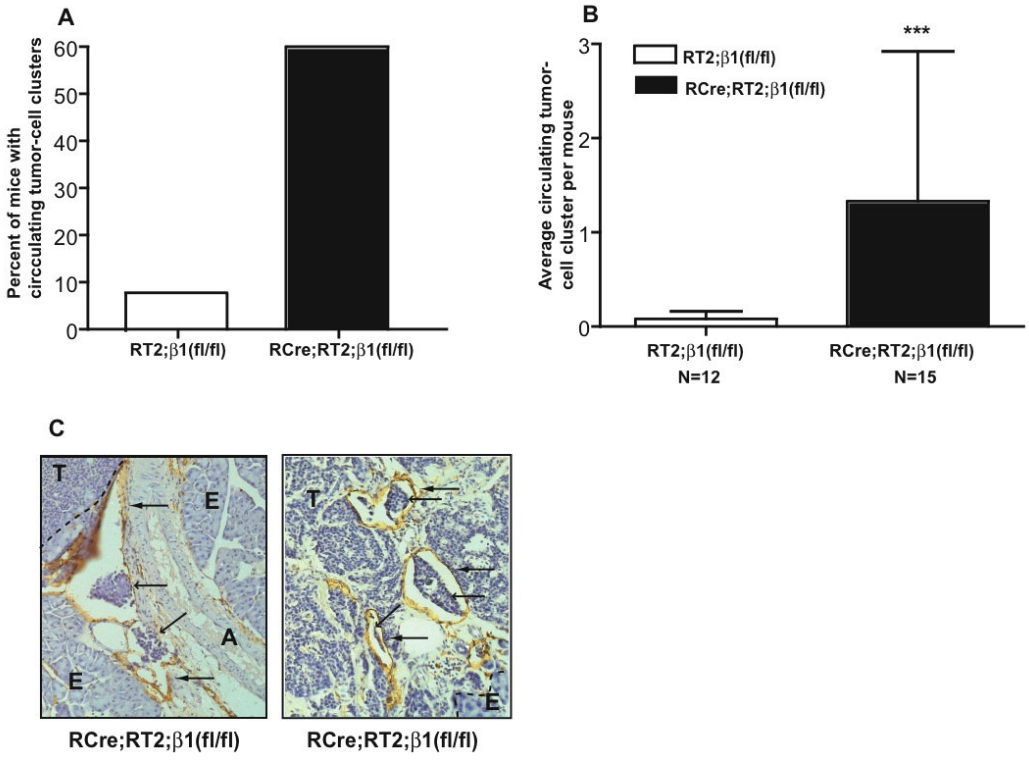

Figure 5: Disseminated tumor cell clusters in RCre;RT2;$\beta_1^{fl/fl}$ mice

*Percent of mice (panel A) showing disseminated tumor cell clusters and average number of disseminated tumor cell clusters per mouse (panel B) in RT2;$\beta_1^{fl/fl}$ (white bars) and RCre;RT2;$\beta_1^{fl/fl}$ (black bars) mice. N, number of analyzed mice; ***, P<0.02; C: LYVE-1 staining (brown, big arrowheads) of RCre;RT2;$\beta_1^{fl/fl}$ tumors. Circulating tumor cell clusters are highlighted with small arrowheads. A, artery; E, exocrine pancreas; T, tumor;*

4.3.4. β1-integrin-deficient β tumor cells exhibit proliferation deficiencies

To investigate how the deletion of β_1-integrin contributes to the reduction of tumor burden and might limit the metastatic potential of disseminated tumor cells, we established cell lines from RCre;RT2;$\beta_1^{fl/fl}$ experimental as well as RT2;$\beta_1^{fl/fl}$ control tumors. PCR analysis confirmed the presence of two floxed β_1-integrin alleles in control cells (βTifl/fl) and two deleted alleles in cells derived from experimental tumors (βTiΔ, Figure 6A, upper panel). FACS analysis demonstrated that βTiΔ cells lost β_1-integrin expression. Cell lines derived from RT2;NCAM+/+ (βT2) and RT2;NCAM-/- (βTN2) tumors (Cavallaro et al., 2001), both carrying the wild type β_1-integrin alleles, showed β_1-integrin expression levels comparable to those of the βTifl/fl cell line (data not shown). Importantly, NCAM levels in these cell lines vary in that βT2 cells express very high levels of NCAM, whereas in the βTifl/fl and βTiΔ cell lines NCAM levels are rather low (Figure 6).

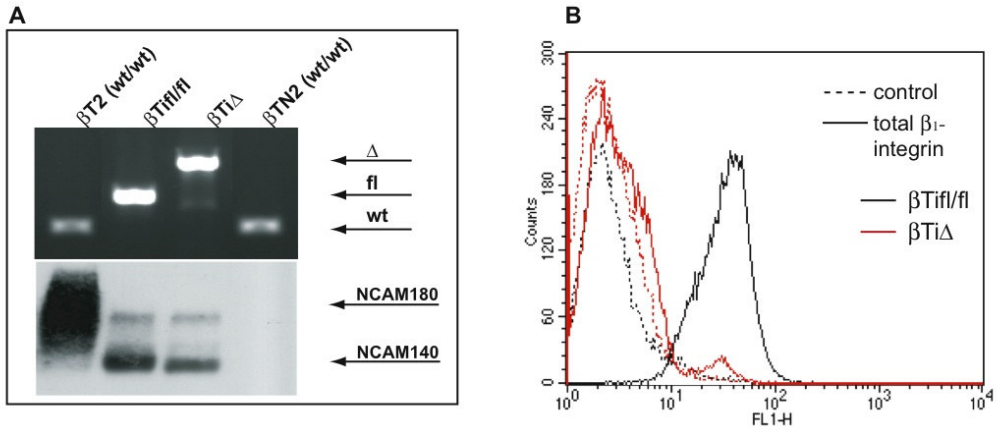

Figure 6: Cell lines derived from RT2;$\beta_1^{fl/fl}$ and RCre;RT2;$\beta_1^{fl/fl}$ tumors

A, top: Genotyping of cell lines derived from RT2 (βT2), RT2;$\beta_1^{fl/fl}$ (βTifl/fl), RCre;RT2;$\beta_1^{fl/fl}$ (βTiΔ) and RT2;NCAM-/- (βTN2) tumors by PCR analysis. βT2 and βTN2 both carry the wild type β_1-integrin alleles (wt), βTifl/fl carry the conditional alleles (fl) and almost all βTiΔ cells have undergone recombination and therefore mainly carry the deleted (Δ) alleles. A, bottom: Western Blot analysis of NCAM levels in βT2, βTifl/fl, βTiΔ and βTN2 cells. NCAM levels in βT2 cells are very high, whereas βTifl/fl and βTiΔ cells have low NCAM expression. βTN2 served as negative control. B: β_1-integrin surface expression levels of βTifl/fl (black line) and βTiΔ (red line) cells, assayed by FACS analysis. βTiΔ cells have no more β_1-integrin surface expression. Dashed line, control (unstained cells);

Using adhesion of cells to collagen IV (a specific substrate for β_1-integrin) as an assay for β_1-integrin activation, we have previously shown that activation of β_1-integrin is abolished in NCAM-deficient βTN2 cells and can be re-established by re-introducing NCAM (Cavallaro et al., 2001). We thus compared the adhesion capabilities of βTifl/fl and βTiΔ cell lines to those of βT2 cells in the presence or absence of NCAM. Equal amounts of cells were seeded onto either uncoated or collagen IV coated 96-well plates and cells were allowed to

adhere for 90 min. Intrinsic adhesion to collagen IV of the βTiΔ cell line was very low (6.76 % of βT2, Figure 7) and adhesion could not be increased significantly by re-introducing NCAM140 (9.86%). Similarly, adhesion of Mock-transfected βTifl/fl cells was only 16% of βT2 cells, however, re-introducing NCAM140 significantly stimulated adhesion to the β_1-integrin specific ligand collagenIV (16% Mock transfected vs. 32% NCAM 140 transfected cells) but not to uncoated plastic. Interestingly, even in βT2 cells that express very high amounts of NCAM, adhesion could be further stimulated by NCAM transfection. Thus, NCAM signaling lies upstream of β_1-integrin activation in β tumor cells.

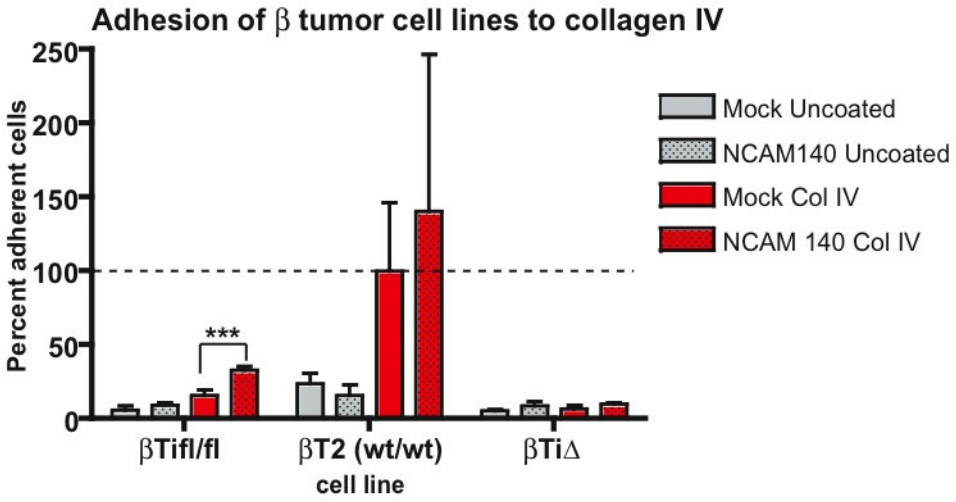

Figure 7: Adhesion assay of β tumor cells

*Mock-transfected (clear bars) or NCAM140 transfected (dotted bars) β tumor cells were seeded on either uncoated (grey bars) or collagen IV coated (red bars) dishes. Adherent cells were counted as described in Materials and Methods. Adhesion of Mock-transfected βT2 cells to collagen was taken as 100% for reference. ***: increase in adhesion is statistically significant (P<0.003, unpaired t-test).*

Since deletion of β_1-integrin in RT2 tumors leads to a reduction of tumor volumes, decreased proliferation and apoptosis, we analyzed growth rates of β_1-integrin deleted β-tumor cells in comparison to control cells. As shown in Figure 8, βT2, βTN2 and βTifl/fl display very similar growth curves and linear regression curves have comparable slopes (0.0024 for βT2, 0.0025 for βTifl/fl and 0.0027 for βTN2). Growth of the β_1-integrin deleted cell line (βTiΔ) however, was significantly slower (0.0014, $P < 0.0001$, unpaired t-test of linear regression curves).

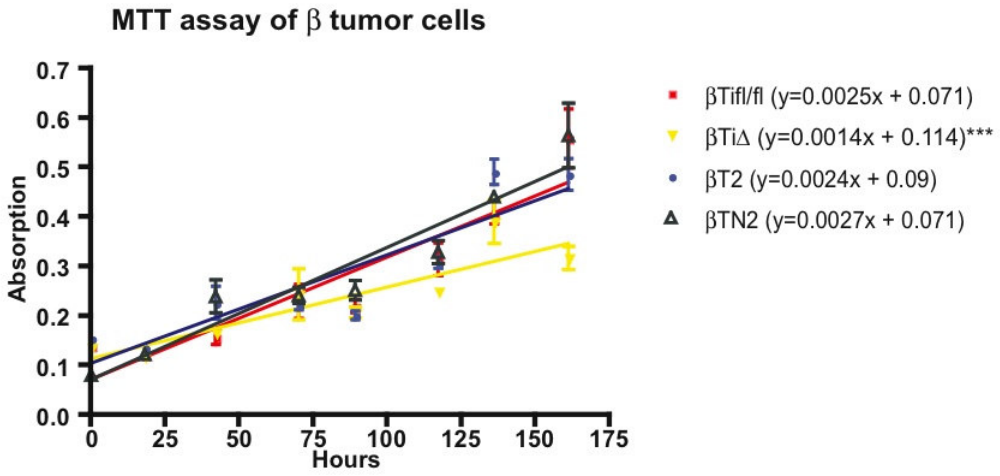

Figure 8: Growth curves of β tumor cells in 2D-culture

*MTT assay of β-tumor cell lines. Absorption as an indirect measure for cell counts is plotted against the time after seeding cells (0h). Linear regression curves were calculated and are displayed for each cell line. *** indicates that decrease in growth rate of βTiΔ cells is statistically significant compared to all other cell lines (P<0.001, unpaired t-test of linear regression curves)*

Since integrins are the major receptors for extracellular matrix (ECM), we hypothesized that culturing βTifl/fl and βTiΔ tumor cells in a 3D matrigel culture could reflect the potential effects of β_1-integrin deletion on cell growth closer to an *in vivo* system. In fact, already two days after seeding the cells in matrigel, differences in cell and cell-cluster shape became apparent: many of the plated βTifl/fl cells formed filopodia-like protrusions (Figure 9A, left panel) some of them longer than the cell/cell clusters diameter (Figure 9A). In contrast, βTiΔ cells did not seem to out-grow cell protrusions and generally looked rather unhealthy under this growth conditions (Figure 9A, right panel. Quantification of this effect by counting cells with or without protrusion in a defined volume revealed that more than 70% of the counted βTifl/fl cells developed protrusions, whereas not a single of the β_1-integrin deleted cells showed formation of these structures. One week after plating, all βTiΔ cells died, whereas β_1-integrin expressing βTifl/fl cells were still alive, forming protrusions and proliferating.

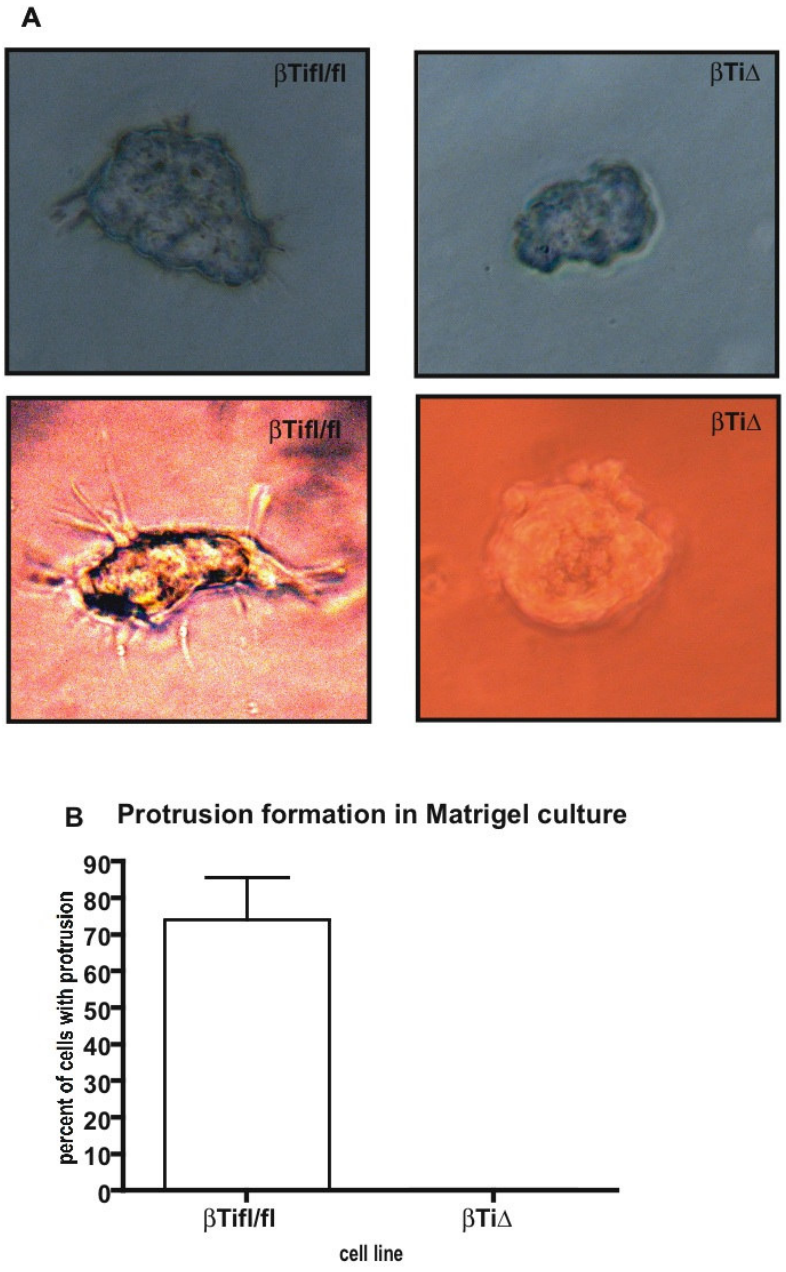

Figure 9: Growth of βTifl/fl and βTiΔ cells in matrigel

A: Pictures of cells two days after seeding them in a 3D matrigel culture. β_1-integrin expressing βTifl/fl cells (left panel) develop filopodia or neurite-like protrusions, in contrast, βTiΔ (right panel) cells do not produce these structures and do not seem to proliferate. B: Quantification of protrusion formation of βTifl/fl and βTiΔ cells.

4.3.5. β1-integrin expression, but not NCAM is necessary for metastasis formation

We next asked whether cell lines carrying the β_1-integrin or NCAM deletion were able to grow and form tumors and metastases *in vivo*. Equal amounts of tumor cell suspensions of βTifl/fl, βTiΔ , βTN2 and βT2 cells were injected subcutaneously into immune-competent C57 mice. After five weeks, mice were sacrificed and tumor incidence, tumor size and tumor volumes were determined (Figure 10A and 10B). Tumors were formed in 12,5% of all βT2 injected sites and in 37,5% of all βTifl/fl injected sites. The average size of tumors from βT2 cells was 293,35 +/- 129.4 mm^3 as compared to 174,66 +/- 53.11 mm^3 for

tumors arising from βTifl/fl cells. In contrast, βTiΔ and βTN2 cells did not give rise to any tumors.

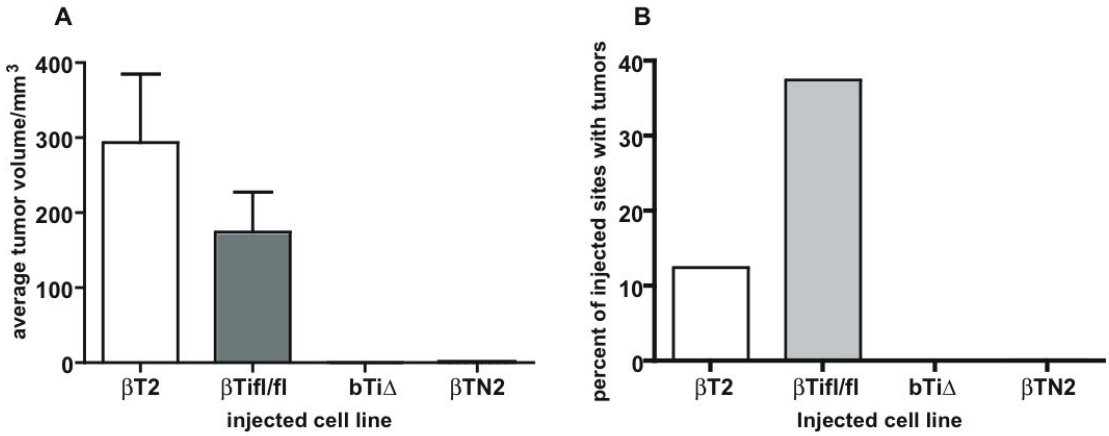

Figure 10: Ectopic transplantation of β tumor cell lines

A: Average volumes of tumors arising from either βT2 or βTifl/fl cells (see text and Materials and Methods). B: tumor incidence in C57 mice injected with the indicated cell lines. 16 sites were injected, the percetage of sites with tumors is displayed.

To assess the metastatic potential of the cell lines, we injected equal amounts of cells into the tail vein of athymic nude mice. After four weeks of incubation, mice were sacrificed. Pancreata, lungs and livers of all mice were isolated, organs were fixed in PFA and sections were cut through the whole organs. As shown in Table 3, by immunopathological investigation by H&E staining, metastases to either the lungs and/or the livers were identified in all β tumor cell lines except for βTiΔ, lacking β_1-integrin expression.

Table III: Intravenous injection of β-tumor cell lines

Cell line	Number of injected sites	Number of mice with metastases	Site of metastasis
βT2	**5**	**4**	**lung/liver**
βTifl/fl	**5**	**3**	**lung**
βTN2	**5**	**4**	**lung/liver**
βTiΔ	**5**	**0**	**-**

These results indicate that depletion of NCAM and therefore β_1-integrin activity in βTN2 cells diminishes tumor formation but does not interfere with metastasis formation. However, total loss of β_1-integrin function results in an incapability of βTiΔ cells to form tumors and metastases *in vivo* indicating a crucial role of β_1-integrin in these processes.

4.4. Discussion

4.4.1. β_1-integrin function is required for proper islet development

We show here that deletion of β_1-integrin in the β cells of pancreatic islets of Langerhans leads to disturbance in proper islet-cell segregation. Interestingly, a similar phenotype was observed in NCAM-deficient islets (Esni et al., 1999) and islet expressing a dominant negative version of E-cadherin (Dahl et al., 1996), indicating that both cell adhesion molecules play an important role in proper cell sorting. Previously, our *in vitro* studies showed that NCAM dependent FGFR signaling leads to β_1-integrin activation (Cavallaro et al., 2001). Therefore, one might speculate that this signaling pathway is involved in the sorting process during islet development.

4.4.2. β_1-integrin outside-in signaling but not NCAM mediated inside-out signaling is required for metastasis formation

Tissue disaggregation and lymphangiogenesis, have been shown to be sufficient on their own to induce the formation of lymph node metastases. Interestingly, in RCre;RT2;$\beta_1^{fl/fl}$ mice, tumor cell clusters were found disseminated into lymphatic vessels. However, the fate of the disseminated tumor clusters in RCre;RT2;$\beta_1^{fl/fl}$ mice is not clear. They are not able to form metastases, since none could be observed in RCre;RT2;$\beta_1^{fl/fl}$ mice, neither in the local lymph nodes, nor in other organs. In addition RCre;RT2;$\beta_1^{fl/fl}$ tumor-derived cells do not form metastasis when injected into nude mice. This phenomenon does not seem to be linked to the adhesive defects of β_1-integrin-deleted cells, since βTN2 cells, which are deficient for β_1-integrin mediated adhesion, can still metastasize. Rather, the proliferation deficiency observed in the primary tumor and in the RCre;RT2;$\beta_1^{fl/fl}$ tumor-derived cells might be the main reason for the incapability to grow metastasis. However, we cannot exclude that the clusters are not circulating at all since these cells are very difficult to detect in the circulatory system.

Integrins have been shown to be bi-directional signaling molecules (Hynes, 2002). Inside-out signals activate integrin-mediated adhesion to ligands such as collagen, laminin and others. On the other hand, outside-in integrin signaling results in cellular responses such as proliferation, survival or apoptosis. βT2 cells have very high NCAM levels and hence show high levels of β_1-integrin activation. βTN2 cells have lost NCAM-dependent, inside-out activation of β_1-integrin but still express the protein, allowing reception of outside-in signals. In the contrary, βTiΔ cells lost both integrin functions. Using these established cell lines in adhesion, proliferation and transplantation assays allowed for the discrimination of the role of adhesive (inside-out) and growth promoting (outside-in) integrin functions. The cell-line-derived data are summarized in Table 4.

Table IV: Summary of data derived from experiments on β tumor cell lines

	NCAM exp	Col IV stim.	2D	3D	s.c.	i.v.
βT2	+++	+	+	+ *	+	+
βTifl/fl	+	+	+	+	+	+
βTN2	-	+***	+	+ *	-	+
βTiΔ	+	-	-/+	-	-	-

*NCAM exp, NCAM expression levels of cell lines, +++: high levels, +, low levels, - no expression; Col IV stim., adhesion to collagen IV stimulatable by NCAM transfection, +: stimulatable, -: not stimulateble; 2D, growth of cell lines in 2D cultures, +: cells growing; -/+: cells grow slower; 3D, growth of cell lines in matrigel, +: cells grow, -: cells cannot grow; s.c., tumor growth after subcutaneous injection, +: tumors are formed, -: no tumor formation; i.v., metastasis formation after intravenous injection, +: metastases formed, -: no metastases formed; * data not shown; *** shown in (Cavallaro et al., 2001)*

Deletion of β_1-integrin clearly reduces the proliferative capability of cells *in vitro* as well as *in vivo*, which we have shown in 2D and 3D growth assays and subcutaneous transplantation experiments. Interestingly, βTN2 cells that have lost NCAM dependent inside-out activation cannot form tumors when injected subcutaneously, but are still capable of metatasis formation when injected intravenously into nude mice. In contrast, βTiΔ cells that can receive neither inside-out nor outside-in signals, do not grow tumors or metastasize at all upon transplantation. This suggests that expression of β_1-integrin is required to transduce outside-in signals that allow cells to survive and proliferate at distant sites.

One therefore might speculate that tumor cells that have reduced adhesive properties due to disturbances in the inside-out signaling to β_1-integrin (such as for example RT2;NCAM-/- tumors) disseminate and therefore more easily enter routes for metastasis formation. Since they still express β_1-integrin, the protein is available to transduce pro-proliferative outside-in signals. In contrast, in RCre;RT2;$b_1^{fl/fl}$ mice β_1-integrin expression is lost in 40% of the tumor cells. These cells disseminate but, lacking β_1-integrin, cannot receive outside-in signals that allow them to proliferate and eventually metastasize. This hypothesis could be tested by interfering with β_1-integrin function in RT2;NCAM-/- tumors. We are currently intercrossing RCre;RT2; $\beta_1^{fl/fl}$ and RT2;NCAM-/- mouse strains. If our hypothesis holds true, we would expect no more metastases to be formed.

4.4.3. Loss of β_1-integrin reduces tumor burden potentially by inducing senescence

We found that tumor volumes, but not tumor incidence are reduced in β_1-integrin depleted tumors, which might be an indication that β_1-integrin function is required for later stages of tumor progression. When cells are properly attached to the right ECM, they receive signals allowing them to survive and proliferate. In contrast, dislocation of cells, or total loss

of ECM anchorage induces anoikis, driving cells into apoptosis. The main mediators of these signaling processes are integrins, communicating and influencing cell behavior via outside-in signaling. Hence we suspected that the reduced tumor burden in β_1-integrin-deleted tumors might have been due to the induction of anoikis. However, the number of apoptotic cells and proliferating cells even decreased in RCre;RT2;$\beta_1^{fl/fl}$ tumors. This result suggests that anoikis is not induced in the tumors. Rather the general metabolic activity seems to be reduced. Such a change in metabolic activity can be associated with quiescence, senescence and tumor dormancy. Senescence-associated β-Galactosidase (SA-β-Gal) is expressed in senescent cells and is therefore frequently used as a marker. It can be specifically detected at pH 6 via histochemistry (Dimri et al., 1995). In contrast, bacterial β-Galactosidase is most active at pH7.5. Since the $\beta_1^{fl/fl}$ mice are constructed in a way that, upon efficient recombination, a bacterial β-Galactosidase reporter comes under the control of the β_1-integrin promoter, and because bacterial β-Galactosidase also displays activity at pH 6, we could not test for SA-β-Gal in these mice. However, we recently obtained an alternative mouse line with conditional β_1-integrin alleles designed in a different way, not carrying a β-Galactosidase reporter (β_1EII mice). We have intercrossed these mice to the RT2 tumor model giving rise to RCre,RT2;β_1EII mice. Importantly, when we investigated sections of tumors derived from RCre;RT2;β_1EII mice, we could detect positive staining for SA-β-Gal (data not shown). This suggests that deletion of β_1-integrin in RT2 tumors reduces tumor burden by inducing senescence. We are currently investigating the expression of other senescence-associated markers, such as the formation of heterochromatin protein-1γ (HP-1γ) containing heterochromatin foci, in β_1-integrin-deficient tumors. To our knowledge, this is the first time that integrin function is associated with senescence.

Interestingly, outside-in signals from integrins are mainly transduced via the focal adhesion kinase (FAK) and it has been shown that inhibition of FAK signaling in human carcinoma cell lines induces quiescence *in vivo*. This suggest that FAK activity might be reduced in RCre;RT2;$\beta_1^{fl/fl}$ tumors and in β_1-integrin deleted cell lines. It will be of importance to investigate the quality of the focal complexes in our cellular and mouse systems in the future.

Targeting β_1-integrin function in tumors might induce senescence and interfere with metastasis formation, thus β_1-integrin inactivating antibodies might represent a novel approach for cancer treatment. Consistent with this notion, studies on cell lines confirmed their potential efficacy since blocking β_1-integrin could revert the malignant phenotype of a breast cancer cell line (Weaver et al.,1997).

4.4.4. Loss of β_1-integrin induces tumor cell cluster dissemination but not lymphangiogenesis

It has been shown that increased tumor lymphangiogenesis correlates positively with the incidence of metastases (Cao, 2005). Among the factors that contribute to

lymphangiogenesis are VEGF-C and VEGF-D. Transgenic expression of these factors induces lymphangiogenesis and metastasis in the Rip1Tag2 tumor model (Mandriota et al., 2001, Kopfstein *et al*., submitted). Interestingly, in RT2;NCAM-/- tumors these two ligands were found up-regulated. RT2;NCAM-/- tumors also show severe tissue disaggregation and the appearance of large lacunae, filled with hemorrhagic fluid, potentially leading to increased interstitial fluid pressure. One controversial theory hypothesizes that tumor associated lymphangiogenesis is induced to reduce the tumors interstitial fluid pressure. Since the observed tissue disaggregation is potentially caused by the reduced capability of β cells to adhere to ECM substrates, we hypothesized that the inactivation of β_1-integrin might lie upstream of the induction of lymphangiogenesis in RT2;NCAM-/- mice. However, our results show that β_1-integrin deletion does not lead to tumor lymphangiogenesis. Thus, it seems that the increased lymphangiogenesis observed in RT2;NCAM-/- mice is not directly due to a defect in the β_1-integrin function.

A recent report linked the appearance of hemorrhagic lacunae and the concomitant tissue disaggregation phenotype in NCAM-/- tumors to decreased pericyte recruitment to endothelial cells. In this study, it was demonstrated that loss of NCAM in β-cells negatively influenced pericyte-endothelial cell-cell interactions, which results in increased blood vessel leakage. Furthermore, pericyte deficiency per se caused haematogenous spreading of tumor cells and metastasis formation (Xian et al., 2006).

Deletion of β_1-integrin in the RT2 model did result in a disaggregation phenotype, yet distinct to that observed in NCAM-deficient tumors. In RCre;RT2;$\beta_1^{fl/fl}$ mice, tumor architecture per se was not altered, but clusters of tumor cells disseminated into lymphatic structures were found. Disseminated tumor cells are also found in NCAM-deficient mice, suggesting that in RT2;NCAM-/- tumors two different mechanisms are employed, impaired pericyte recruitment and loss of cell matrix adhesion. Both of them result in tumor cell dissemination. In the first one, loss of NCAM in β cells seems to induce disaggregation indirectly by affecting blood vessel stability. In the second one, intrinsic adhesive properties of β cells are affected upon NCAM ablation as we could show by the study of the loss of β_1-integrin activation and function. This leads to the tumor cell cluster dissemination described in RCre;RT2;$\beta_1^{fl/fl}$ mice and we think this reflects an additional, alternative pathway leading to tumor dissemination in NCAM-negative tumors. Still, it is not clear how the disseminated cells in RCre;RT2;$\beta_1^{fl/fl}$ mice enter the lymphatics. Lymphatic vessels are structurally different to blood vessels and are thought to be more permeable, which may be an explanation
.

Taken together, our results support our previous findings that loss of β_1-integrin function in β cells reduces their cell-matrix adhesion and causes tumor cell dissemination. Our data furthermore show that the inactivation of β_1-integrin does not lie upstream of the induction of lymphangiogenesis since RCre;RT2;$\beta_1^{fl/fl}$ tumors do not display increased lymphatic vessel coverage. Further experiments are needed to unravel the pathways and mechanisms leading to the induction of lymphangiogenesis and lymph node metastasis in NCAM deleted tumors.

5. General discussion

In this work we provide evidence that NCAM binds to all members of the FGFR family as well as to PDGFRα and PDGFRβ. Based on the observation that RTKs lacking Ig domains in their extracellular domains cannot bind to NCAM, we suggest that the presence of an Ig domain is a requisite for NCAM binding. If this holds true, one might expect to identify additional, if not all Ig-domain RTKs, as NCAM binding partners.

So far, the role of NCAM in modulating RTK signaling has been mainly studied with FGFR signaling in neurons, and its effects were primarily thought to be pro-stimulatory (Povlsen et al., 2003). Here, we broaden NCAMs "place of action" to additional RTKs in non-neuronal tissues, such as endocrine and fibroblastic cells. Importantly, we furthermore identify a novel way of action for NCAM, since we demonstrate that it can exert also inhibitory functions on RTKs. In many human cancers, both a reduction or an increase of NCAM levels are correlated with increased malignancy which might be a reflection of NCAMs dual role in modulating RTK signaling.

The Rip1Tag2 tumor model is one example where loss of NCAM correlates with increased malignancy. NCAM-deficiency in these tumors leads to tissue disaggregation, induction of lymphangiogenesis and the formation of metastases (Perl et al., 1999; Crnic et al., 2004; Cavallaro et al., 2001). One target of NCAM signaling in β tumor cells is β_1-integrin. We show here that loss of β_1-integrin is sufficient to induce tumor cell dissemination. In contrast, lymphangiogenesis induced by NCAM-deficiency is independent of β_1-integrin function and probably relies on an alternative NCAM dependent pathway. Induction of lymphangiogenesis alone has been shown to induce metastasis (Mandriota et al., 2001), however, interfering with lymphangiogenesis in NCAM depleted tumor mice only partially blocked the formation of metastases (Crnic et al., 2004), indicating that an additional mechanism contributes to the metastatic phenotype. This additional feature might be tissue disaggregation that results from impaired pericyte recruitment to blood vessel endothelial cells in NCAM knock out tumors (Xian et al., 2006), since lack of pericyte recruitment also resulted in metastasis formation. Importantly, tissue disaggregation induced by blocking β_1-integrin function did not give rise to metastases. Furthermore, tumor sizes were reduced in β_1-integrin deleted tumors, probably due to the induction of senescence. These findings suggest β_1-integrin to be a more promising target for the intervention with metastasis formation in NCAM deficient tumors.

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7. Curriculum Vitae

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Education

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1999-2001	Diploma thesis research at the Department of Molecular Genetics, University of Vienna, Group Prof. Karl Kuchler,
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1998	Laboratory Student in the Lab of Prof. Michael Duchene, Department for Pathophysiology, General Hospital Vienna
1997	Student at Leicester University, UK, Program in Biochemistry
1994-2000	Studies at the University of Vienna, Program in Biochemistry
1986-1994	Gymnasim BG7BRG Mössingerstrasse, Klagenfurt, Austria, Matura
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Conferences

Oral presentations

2003	Spetses Summer School 2003 "Molecular Mechanisms in Homeostasis and Disease"

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2004	USGEB Meeting, Fribourg, Switzerland
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Publications

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Kren A, Mamnun YM, Bauer BE, Schuller C, Wolfger H, Hatzixanthis K, Mollapour M, Gregori C, Piper P, Kuchler K. War1p, a novel transcription factor controlling weak acid stress response in yeast. Mol Cell Biol. 2003 Mar;23(5):1775-85.

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