The role of Sprouty4 in development and cancer and functional consequences of Sprouty interacting with Caveolin-1

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1 Zusammenfassung

Wachstumsfaktoren binden an der Zelloberfläche and Rezeptoren, sogenannte Rezeptor Tyrosine Kinasen und induzieren damit intrazelluläre Signalkaskaden die schlussendlich darüber entscheiden, ob sich eine Zelle teilt, differenziert, wandert oder durch Zelltod oder Seneszenz eliminiert wird. Um die normale Entwicklung eines Organismus zu garantieren und um zu verhindern, dass eine Zelle zu einer Krebszelle wird, müssen diese Signalkaskaden sehr genau reguliert werden. Sprouty (Spry) gehört zu einer Familie von Proteinen, die für diese Regulation mitverantwortlich sind. In Säugetieren gibt es vier Isoformen. Da Spry Proteine Signalwege von vielen verschiedenen Wachstumsfaktoren kontrollieren, habe sie ein weites Spektrum an Funktionen innerhalb eines Organismus. Abhängig vom Wachstumsfaktor, der Spry-Isoform, aber auch vom zellulären Kontext kann die Aktivität von Spry Proteinen einen Signalweg sowohl hemmen als auch begünstigen. In dieser Arbeit untersuche ich die Konsequenz der Interaktion von Spry mit Caveolin-1 auf die Regulation von FGF2 und EGF Signalwegen. Ich zeige, dass diese Konsequenzen je nach Spry-Isoform unterschiedlich sind. Wir vermuten deshalb, dass Caveolin-1 die unterschiedlichen Funktionen und Aktivitäten der vier Spry-Isoformen mitbestimmt. Ausserdem nehmen wir an, dass das Expressionsmuster von Caveolin-1 dazu beitragen kann, die Funktion von Spry auf den Zellkontext abzustimmen.

In einem zweiten Teil meiner Studien analysiere ich die Aufgabe von Spry4 in der Entwicklung des Pankreas in der Maus. Im Pankreas sind endokrine Zellen in den sogenannten Langerhans'schen Inseln organisiert, die im exokrinen Teil des Pankreas verteilt sind. Die Insulin-sekretierenden β Zellen werden umgeben von einem Ring aus α, δ und PP Zellen die jeweils Glucagon, Somatostatin und Pankreaspolypeptid produzieren. Immunohistochemische und Immunofluoreszenz-Analysen haben gezeigt, dass endogenes Spry4 spezifisch in den α Zellen exprimiert wird. Um die Funktion von Spry4 in der Entwicklung des Pankreas weiter zu untersuchen, haben wir Spry4 mit Hilfe eines Doxycycline-induzierbaren Promoters spezifisch in den β Zellen überexprimiert. Ich zeige in meiner Arbeit, dass durch dieses veränderte Expressionsmuster von Spry4 die Bildung der Langerhans'schen Inseln beeinträchtigt wird. Mit Hilfe der Zelllinie PANC-1 habe ich den Prozess der Pankreasentwicklung *in vitro* untersucht. Ich fand, dass Spry4 die Lokalisation von PTP1B verändert, dabei wahrscheinlich Signalwege des Adhäsionsmoleküls Integrin beeinflusst und somit zu einer Hemmung von Migration und Adhäsion der PANC-1 Zellen führt.

Schliesslich habe ich auch die Rolle von Spry4 in der Tumorentstehung im Rip1Tag2 Mausmodell untersucht. In diesen Mäusen führt die Expression eines viralen Onkogens (large T-Antigen) zu β Zell-Karzinogenese. Die Analyse dieser Mäuse ergab, dass die Expression von Spry4 in β Zellen die Tumorentstehung und –entwicklung kaum beeinflusst. In isolierten β Tumorzellen finden wir, dass der p42/44 ERK Signalweg konstitutiv aktiviert ist. Wir nehmen daher an, dass die Transformation mit large T-Antigen eiene Punkt innerhalb des Signalweges aktiviert der nach dem Schritt liegt and dem Spry normalerweise in den Signalweg eingreift und somit Spry4 in diesen tumorzellen ineffektiv ist.

2 Summary

Binding of growth factors to receptor tyrosine kinases (RTKs) triggers intracellular signaling pathways that ultimately determine cell fate. The coordinated action of both positive and negative modulators is essential for spatial and temporal control of these pathways to avoid developmental defects and malignant transformation. Sprouty (Spry) proteins are a family of ligand-induced regulators of RTK-mediated intracellular cascades. In mammals, there are four isoforms. Their activity targets signal transduction induced by various growth factors, suggesting that their activities have broad biological consequences. Spry proteins have the capacity to attenuate or potentiate different pathways depending on the Spry isoform, the growth factor and the cellular context. In this study I investigate the functional consequences of Spry interacting with Caveolin-1. I demonstrate that in the context of FGF2 and EGF signaling, Caveolin-1 differentially modulates the function of the four isoforms. This suggests that Caveolin-1 might contribute to the divergence among the four isoforms and, depending on its own expression pattern, could contribute to the cell context-dependency of Spry proteins.

In a second part of my studies I assess the role of mSpry4 in murine pancreas development. Analyzing this organ for the presence of Spry proteins, I detect very specific expression of mSpry4 in one population of endocrine cells, namely in the α cells. In the pancreas, different endocrine cell types are organized within islets of Langerhans. The insulin-secreting β cells occupy the core of the islets and are surrounded by α, δ and PP cells, secreting glucagon, somatostatin and pancreatic polypeptide, respectively. Using a doxycycline-inducible transgenic system I conditionally express mSpry4 in the insulin-producing β cells and demonstrate that ectopic mSpry4 interferes with proper segregation of the endocrine cells. Employing the endocrine precursor cell line PANC-1 as an *in vitro* model for islet formation I find that mSpry4 inhibits migration and adhesion of these cells, probably by interfering with the localization PTP1B, a mediator of integrin signaling.

Furthermore, the role of mSpry4 in tumorigenesis was assessed using the well established Rip1Tag2 mouse model. In these mice, transgenic expression of large T antigen results in β cell carcinogenesis. We find that expression of mSpry4 in β cells has a moderate effect on tumor formation. Experiments with isolated tumor cells imply that transformation of β cells by means of large T antigen results in constitutive activation of p42/44 ERK signaling by a mechanism that renders mSpry4 ineffective as an inhibitor of this pathway.

3 General Introduction

3.1 Receptor tyrosine kinase (RTK) signaling

RTKs activate signal transduction pathways that control a variety of processes in multicellular organisms such as proliferation, differentiation, migration and survival (for review see (1)). RTKs contain an extracellular ligand binding domain which is connected to the cytoplasmic tail by a single transmembrane helix. The cytoplasmic domain contains a conserved protein tyrosine kinase core and additional regulatory sequences that are subjected to autophosphorylation and phosphorylation by heterologous protein kinases. Epidermal growth factor (EGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and hepatocyte growth factor (HGF) are some of the ligands among many others that activate RTKs. Binding of these growth factors to their respective RTKs results in dimerization and tyrosine autophosphorylation of the receptors. The phosphorylated tyrosines in turn provide docking sites for adaptor molecules that bind through Src-homology 2 (SH2) domains or phosphotyrosine-binding (PTB) domains. Despite the structural diversity among RTKs, most of them activate broadly overlapping sets of signaling pathways (for review see (1)).

One of the adaptor molecules that binds to activated RTKs is growth-factor receptor-bound-2 (Grb2) (**Figure 3-1**, yellow). Grb2 recruits the nucleotide-exchange factor Son-of-sevenless (Sos), which subsequently triggers the exchange of GDP for GTP in the small GTPase Ras. In this GTP-bound form, Ras activates the protein kinase Raf resulting in the phosphorylation of p42/44 extracellular regulated kinase (p42/44 ERK) through mitogen-activated protein kinase (MAPK)/ERK kinase (MEK). Thus a variety of downstream effectors are phosphorylated, including transcription factors in the nucleus (for review see (1)). In addition, the p42/44 ERK pathway can be activated in a Ras–independent manner (**Figure 3-1**, orange) where autophosphorylated RTKs recruit and activate an isoenzyme of phospholipase C (PLCγ) (2,3). PLCγ induces hydrolysis of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] resulting in the release of Ca^{2+} from intracellular stores. Elevated levels of cytosolic Ca^{2+} activate a specific isoform of protein kinase C (PKCδ) which in turn activates Raf leading to phosphorylation of p42/44 ERK by MEK.

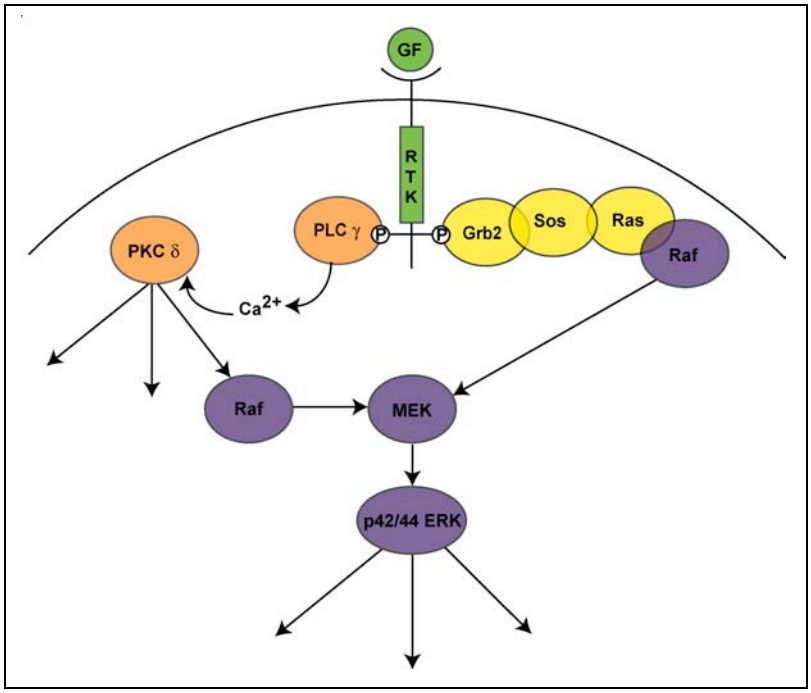

Figure 3-1. RTK signaling pathways. *Activation of RTKs results in phosphorylation of p42/44 ERK through different downstream signaling pathways: Ras dependent (yellow) or Ras independent (orange) activation of Raf.*

3.2 The Sprouty proteins

To ensure a physiologically appropriate biological outcome, the precise spatial and temporal regulation of RTK signaling pathways is crucial. An effective mechanism of modulation is the evolution of negative-feedback loops, *i.e.*, the induction of negative regulators by the same pathway that will eventually be inhibited. The Sprouty (Spry) proteins represent one such family of negative regulators of RTK signaling (for review see (4-9)).

3.2.1 dSpry

Spry was identified in *Drosophila* as a novel antagonist of the FGF signaling pathway during trachea development (10). It was first suggested that dSpry is released into the extracellular environment, competes with FGF ligand for binding to its receptor and prevent neighbouring cells from branching. Later, it was shown that dSpry is also implicated in eye and wing development by inhibiting signaling of the EGFR (11,12). However, there was no further evidence for the model of dSpry as a secreted factor acting on neighboring cells. In general, subsequent work indicated that dSpry is an intracellular protein and acts cell autonomously interfering with the signaling pathway downstream of RTK activation.

dSpry is a 63kDa protein with a unique 124 residue cysteine-rich C-terminal region the so-called 'Spry-domain', lacking any recognizable protein-protein interaction domains (10). The protein localizes to the inner surface of the plasma membrane and intercepts the Ras-Raf-p42/44 ERK pathway by interacting with Gab1, a GTPase-activating protein and Drk, the *Drosophila* homologue of the adaptor molecule Grb2 (11).

3.2.2 Homologues of dSpry

Homologues of dSpry, by virtue of their sequence similarity within the cysteine-rich domain, have been identified in the genomes of the African clawed frog (*Xenopus laevis*), zebrafish, chickens, mice (mSpry), humans (hSpry), and other organisms (13-17). The mammalian genome contains four *SPRY* genes *(SPRY1-SPRY4)* encoding proteins of 32-34 kDa (14,16,18,19). Similarity to the *Drosophila* protein is restricted primarily to the cysteine-rich C-terminal domain, the Spry domain (

Figure 3-2). This domain is necessary for the function of Spry proteins and for plasma membrane interaction in response to growth factor stimulation (18,20-24). Also, they contain a highly conserved motif that mediates binding to Raf1, the Raf1-binding domain (RBD) (25). Previously we have shown, that Caveolin-1 (Cav-1) binds to the C-terminal domain, thereby targeting Spry to membrane microdomains (18,26). The N-terminal domains of the Spry proteins are highly variable except for the presence of a conserved tyrosine residue (Y53 in mSpry1) which becomes tyrosine phosphorylated in response to growth factor stimulation. This tyrosine is important for Spry inhibition of RTK signaling and mutation to an alanin or a phenylalanin results in a dominant-negative Spry that represses the activity of the wild-type protein (27-29).

3.2.3 Sprouty-related proteins: Spred

Spry-related proteins with an EVH1 domain (Spreds) are a family of proteins that bear structural and functional similarities to Spry (

Figure 3-2) (21,30-33). Three Spred genes are present in the mammalian genome all containing a C-terminal Spry-like cysteine-rich domain (Spry-domain). In addition, Spred proteins have an amino-terminal ENA/Vasodilator-stimulated phosphoprotein (VASP) homology-1 (EVH1) domain known to interact with the actin cytoskeleton (21,33,34). The third structure found in Spred proteins, the central c-Kit binding domain (KBD) is only present in Spred1 and Spred2 (31). Similar to Spry, Spred

proteins are targeted to the plasma membrane by their cysteine-rich domain and undergo tyrosine phosphorylation in response to stimulation by several growth factors (31,33). They attenuate signaling through the Ras-Raf-p42/44 ERK pathway by suppressing Raf activation (33). The biochemical mechanisms by which Spreds inhibit Raf as well as the signaling context of their activity are different compared to Spry proteins (for review see (4)). Therefore Spred and Spry might provide complementary modalities for the modulation of RTK signaling.

A physiological function for Spred1 and Spred2 proteins was reported in bone morphogenesis and hematopoiesis. However, knockout mouse models revealed that neither Spred1 nor Spred2 are necessary for fertility and development of mice (35,36). The specific function of Spred3, which is expressed exclusively in the brain, is unknown. (for review see (4,8)).

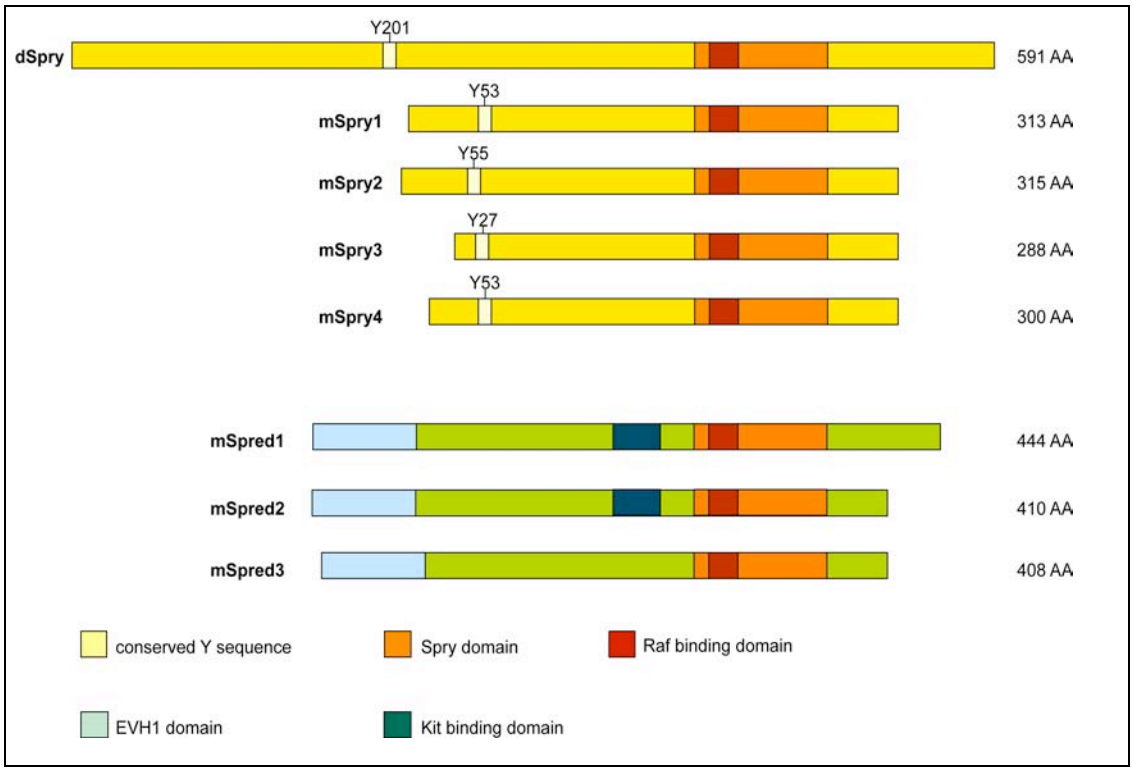

Figure 3-2. Structure of Spry and Spred proteins.
Schematic representation of Drosophila Spry and murine Spry and Spred isoforms. The figures and features are not drawn to scale. All eight proteins share the conserved Spry domain and the Raf binding domain. In addition, Spry isoforms have a conserved N-terminal tyrosine (Y). The EVH1 and Kit binding domain is only present in Spred but not in Spry proteins.

3.3 Spry protein interactions and their functional consequences

Similar to Spry proteins in *Drosophila*, all four mammalian isoforms attenuate the activation of the RTK-p42/44 ERK pathway induced by various growth factors including FGF, VEGF, HGF, PDGF, insulin, nerve growth factor and glial cell-line-derived neurotrophic growth factor (GDNF) (25,28,29,37-42). In addition, recent data demonstrate that Spry also effects T cell antigen receptor signaling (43). However, the points at which Sprys intercept RTK signaling pathways are still controversial; a number of experiments in different cell types suggest that Sprys may function downstream of RTKs but upstream of Ras (*see chapter 3.3.1*). In contrast, other studies demonstrate that Spry blocks FGFR and VEGFR signaling at the level of Raf (*see chapter 3.3.2*). Surprisingly, in some cases Spry proteins fail to repress EGF signaling or even potentiate the activation of this pathway (*see chapter 3.3.3*) suggesting that in vertebrates Spry proteins are not simply general inhibitors of RTK signaling but rather selective modulators of different RTK pathways (for review see (5,6,8,9)). In addition, there is growing evidence that Spry influences cell motility and cell spreading through a mechanism that acts independently of the RTK-Raf-p42/44 ERK pathway (*see chapter 3.3.4*).

Multiple mechanisms of Spry acting on RTK signaling might exist depending on the cellular context, the identity of the activating growth factor or the Spry isoform (7,9,26). However, major insights in the molecular basis of Spry function have been gained by investigating Spry interactions with other signaling molecules.

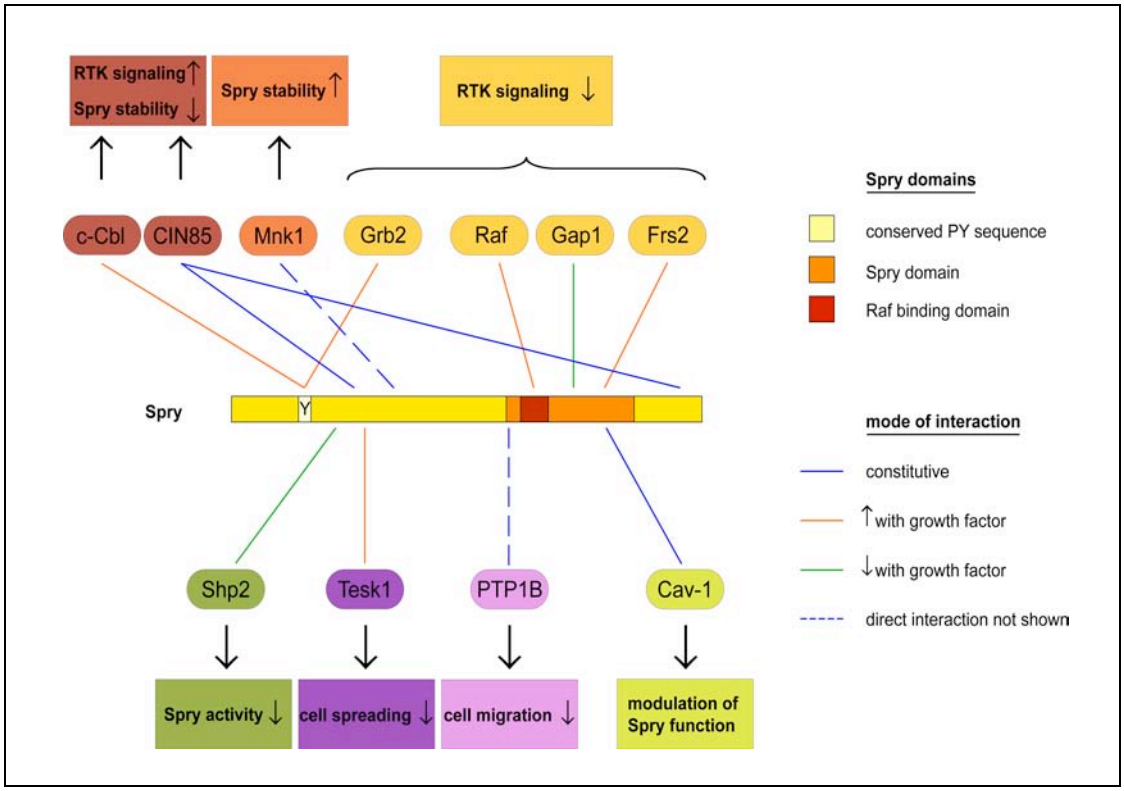

Figure 3-4. Spry interacting proteins.
Proteins that interact with Spry physically (solid arrows) or functionally (broken arrows). Functional consequences of the interactions are indicated in the boxed area. c-Cbl, ubiquitin ligase; CIN85, Cbl interacting protein of 85kDa, Mnk1, MAPK interacting kinase-1; Grb2, growth-factor-receptor bound-2; Gap1, GTPase activating protein-1; Frs2, FGFR substrate-2; Shp2, Src-homology-2-domain-containing protein tyrosine phosphatase-2; Tesk1, testis specific protein kinase 1; PTP1B, protein-tyrosine phosphatase-1B; Cav-1, Caveolin-1

3.3.1 Inhibition of Ras activation: Grb2, FRS2, Gap1

From several studies it was concluded that Spry acts upstream of Ras because Spry cannot inhibit p42/44 ERK phosphorylation induced by activated Ras (27,44-46). Spry was shown to associate with three different signaling molecules that act upstream of Ras activation. One of these proteins is Grb2 (27,45,47). Grb2 binds the phosphorylated form of the conserved N-terminal tyrosine of Spry via its SH2 domain. As a consequence of this interaction, the Grb2-Sos complex is not recruited to the activated RTK (48) (**Figure 3-5 A**).

In addition, Spry is able to bind to the adaptor protein FGFR-substrate-2 (Frs2) which links the activated receptor to the Grb2-Sos complex (27,28,48). Similar to the interaction with Grb2, the association of Spry with Frs2 is increased by FGF stimulation of

the cell and might inhibit the recruitment of the Grb2-Sos complex to the activated receptor (**Figure 3-5 B**). The involvement of the docking protein Frs2 to link the activated receptor to the Grb2-Sos complex is specific for FGF signaling (49). This might provide a possible explanation as to why Sprys have different effects on FGF as compared to EGF signaling. However, the functional consequence of this interaction is still under debate (27,28,48).

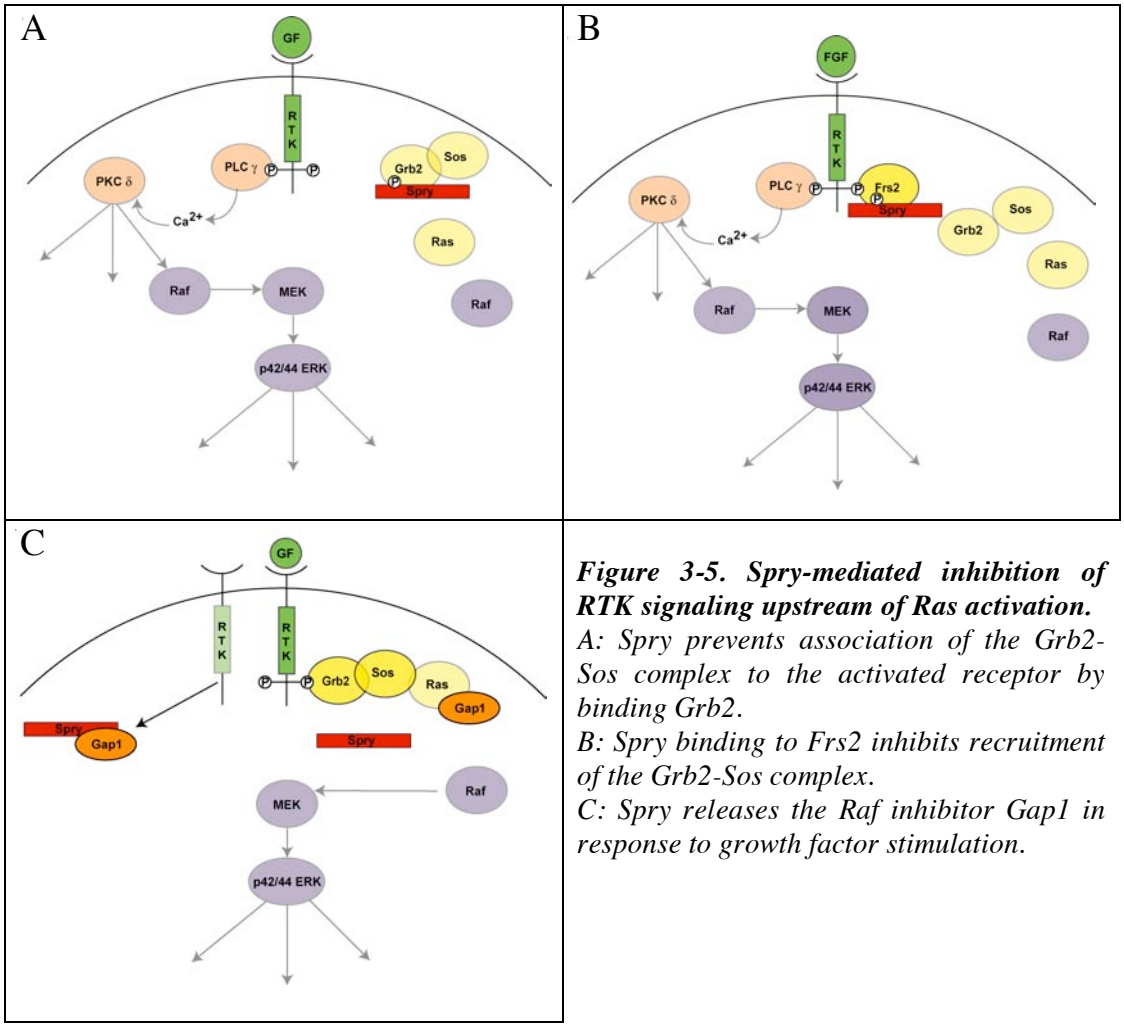

Figure 3-5. Spry-mediated inhibition of RTK signaling upstream of Ras activation.
A: Spry prevents association of the Grb2-Sos complex to the activated receptor by binding Grb2.
B: Spry binding to Frs2 inhibits recruitment of the Grb2-Sos complex.
C: Spry releases the Raf inhibitor Gap1 in response to growth factor stimulation.

The third Spry interacting protein that functions upstream of Ras is the GTPase activating protein Gap1 (11,48). Gap1 belongs to a family of Ras regulators which inactivate Ras by binding to the GTP-bound form and enhancing the hydrolysis of the bound nucleotide back to GDP (50). In contrast to Grb2 and Frs2, the interaction of Spry with Gap1 is decreased after FGF stimulation (48). This implies that Spry might interfere with Ras activity by releasing the Ras inhibitor, Gap1. However, it remains to be determined whether the reversible interaction of Spry with Gap1 contributes directly to the negative regulation the Ras-Raf-p42/44 ERK pathway (**Figure 3-5 C**).

3.3.2 Inhibition of the RTK-PLCγ-Raf1-p42/44 ERK pathway: Raf1

Yussof and coworkers suggested that Spry2 is able to inhibit FGF-mediated signal transduction downstream of Ras at the level of Raf1 (51). Later, it was shown that Spry4 inhibits VEGF-induced, Ras-independent activation of Raf1 (25). VEGF signaling results in p42/44 ERK phosphorylation through the PLCγ-PKCδ pathway, but not through Ras (25,52). Spry proteins directly interact with Raf1 through a conserved region of approximately 70 residues in the C-terminal cysteine-rich Spry domain (25,48). This interaction blocks the activation of Raf1 by PKCδ, but has no effect on the Ras-dependent activation of Raf1 (25) (**Figure 3-6**). Unlike Spry binding to Grb2, association of Spry with Raf1 is independent of the conserved N-terminal tyrosine. As a consequence, mutants of Spry4 lacking this conserved tyrosine are ineffective in the context of FGF signaling but retain the ability to repress VEGF signaling (25). However, studying Spry function on T cell antigen receptor signaling provided evidence that Spry not only inhibits this Ca^{2+}-dependent pathway by binding to Raf but also by preventing PLCγ activation (43). This implies that the different pathways used by RTKs to activate p42/44 ERK can be differentially regulated by Spry proteins.

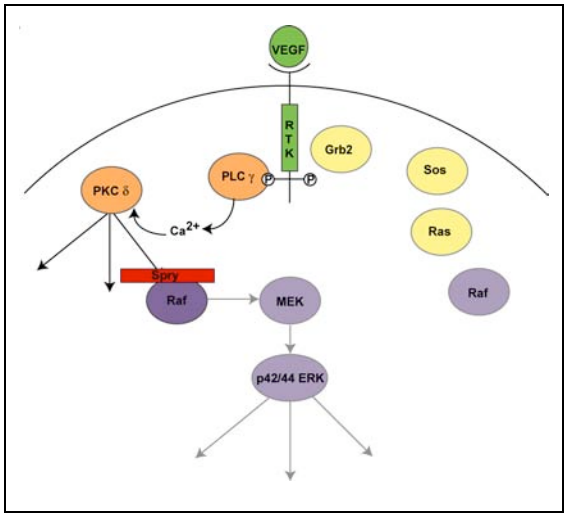

Figure 3-6. Spry inhibits VEGFR signaling by binding to Raf.
Spry binds to Raf and thereby inhibits PKCδ-mediated activation of Raf.

3.3.3 Potentiation of EGFR signaling: c-Cbl

A number of experiments in different cell types revealed that the presence of Spry proteins potentiate rather than attenuate EGF signaling (18,20,23,25,44,53). An explanation for this might be the association of Spry with the E3 ubiquitin ligase c-Cbl (Casitas B-lineage lymphoma). The phosphorylated form of the conserved N-terminal tyrosine of the Spry proteins interacts with the RING (really interesting new gene) finger

domain of c-Cbl (28,54). As a consequence of this interaction, Spry proteins become polyubiquitylated and are targeted for degradation (*see chapter 3.4.6*). However, c-Cbl is also involved in RTK regulation, transducing the signal as a multivalent adaptor protein. In addition, c-Cbl terminates signaling via endocytosis/ubiquitylation of the receptor through its function as a E3 ubiquitin ligase (for review see (55,56)). EGFR, similar to other receptors such as PDGFR, colony stimulating factor 1 receptor, FGFR, and HGFR, is ubiquitylated and targeted for endocytosis upon interaction with c-Cbl (for review see (55)).

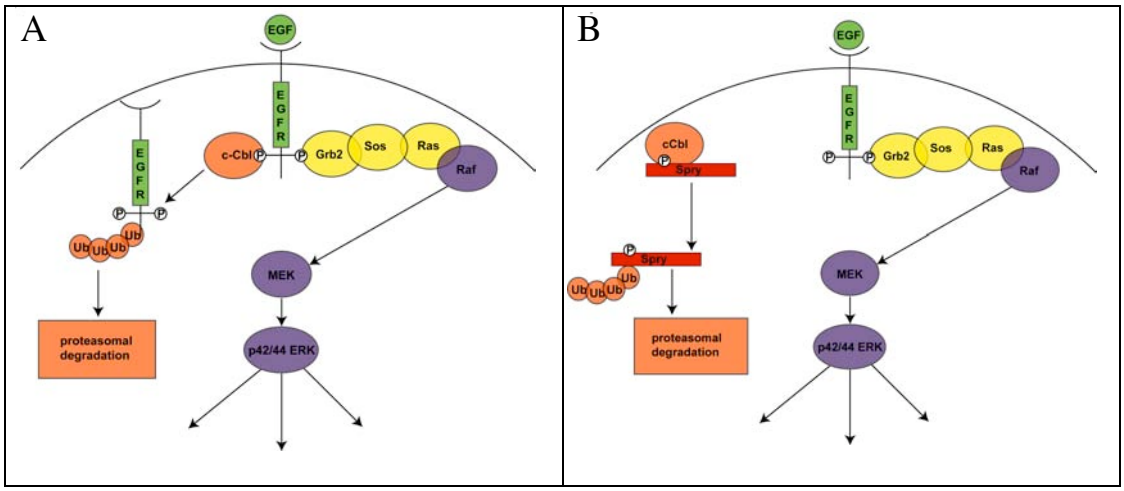

Figure 3-7. Potentiation of EGFR signaling.
A: The ubiquitin ligase c-Cbl mediates internalization and proteasomal degradation of activated RTKs.
B: Spry sequesters c-Cbl away from activated receptors resulting in Spry ubiquitylation and degradation, a decrease of RTK internalization and prolonged signaling.

The widely accepted model of Spry acting on EGFR signaling is that the complex formation of Spry with c-Cbl sequesters the E3 ligase away from the activated receptor. As a consequence, EGFR endocytosisis is inhibited and downstream signaling pathways, such as p42/44 ERK activation remain in an activated state (23,44,53-57). Recently, Haglund and co-workers could show that Spry not only interacts with c-Cbl but also with the Cbl-interacting protein of 85kDa (CIN85). This allows Spry to block CIN85-mediated clustering of Cbl molecules, a step that is important for stabilization of Cbl-EGFR interactions and efficient ubiquitylation and downregulation of EGFR (57,58). It remains unclear why Spry upregulates EGF signaling but downregulates FGF signaling since, in both systems c-Cbl mediates receptor degradation and Spry forms a phosphorylation-dependent complex with c-Cbl (59,60).

3.3.4 Spry influences cell motility and cell spreading: interaction with PTP1B and Tesk1

(i) Protein-tyrosine phophatase-1B (PTP1B)

Spry proteins can inhibit migration by a mechanism that may act independently of the RTK-p42/44 ERK pathway by affecting the localization of PTP1B (61). PTP1B inhibits cell migration and adhesion by interfering with integrin signaling (62). However, conflicting models still exist as to how PTP1B exactly exerts its function on cell migration (62-65). Yigzaw *et al*. proposed that Spry2 expression leads to a relocalization and thereby to an enhanced activation of PTP1B which in turn dephosphorylates and inhibits the Rac1 activator p130Cas (61). To date, the molecular mechanism of this Spry-mediated PTP1B relocalization is not known and no direct interaction between the Spry and PTP1B has been shown.

(ii) Testicular protein kinase 1 (Tesk1)

Tesk1 is a serine/threonine kinase, with the structure composed of an N-terminal protein kinase domain and a C-terminal proline rich region (66). Despite its name, Tesk1 is expressed in various tissues and cell lines (67,68). Experimental evidence suggest that Tesk1 plays an important role in integrin-mediated actin remodelling and cell spreading (69,70). Spry4 associates with Tesk1 through the C-terminal cysteine-rich Spry region (40,71). The interaction of the two proteins increases upon growth factor stimulation, but phosphorylation of the conserved N-terminal tyrosine is not essential for binding. Spry represses the kinase activity of Tesk1 and thereby inhibits integrin-mediated cell spreading (71).

3.4 Regulation of Spry activity

In negative feedback loops, certain stimuli induce the transcription of inhibitors and therefore, such inhibitors are unable to act on early phases of signaling (for review see (72,73)). In contrast, Spry is be involved in a more rapid feedback mechanism: Spry proteins are present at early phases of signaling and are control at multiple levels, including gene expression, localization, posttranslational modification, stability, and modulation of Spry function.

3.4.1 Expression

FGFR activity induces the expression of dSpry (10). Similar, a close spatial and temporal interdependence between RTK signaling and Spry gene expression exists in various mammalian embryonic tissues (for review see (8)). In isolated cell lines, Spry gene expression is induced in response to RTK activation by different growth factors (18,74), emphasizing that Spry is involved in a negative feedback mechanism. Yet, how, when and where this negative feedback loop is engaged remains to be determined. The fact that Spry1 is a direct target of the transcription factor Wilms'-tumor–suppressor-gene (WT1) provides initial insights into the transcriptional control mechanisms of Spry expression (38).

3.4.2 Localization

Mutations that disrupt Spry localization abolish its function as an inhibitor of RTK signaling (22). Spry are cytosolic proteins that rapidly translocate to a peripheral membrane location upon stimulation by different growth factors (for review see (5,7,8)). Two regions in the C-terminal Spry domain are essential for Spry translocation to the membrane (22). Moreover a highly conserved arginine at position 252 of hSpry2 has been identified that binds to [PtdIns(4,5)P_2] and thereby targets the protein to membrane ruffles. It has been postulated that a mutation of this arginine (R252D) results in the inability of hSpry2 to bind to [PtdIns(4,5)P_2], and abolishes translocation of the protein to the membrane. As a consequence, hSpry2-R252D is not able to attenuate FGF signaling (75). However, recently we demonstrated that in COS7 cells a mutation of this conserved arginine did not affect the localization of mSpry2 and mSpry3 whereas the inhibitory effect on the ERK42/44 pathway was still abrogated (26). The importance of Spry translocation might be due to the fact that several Spry interacting proteins (*i.e.* Grb2, Frs2, and Raf1) are located at the plasma membrane. Movement of Spry to and from the plasma membrane might quantitatively or qualitatively control its inhibitory activity (for review see (9)).

3.4.3 Post-translational modifications: palmitoylation and phosphorylation

Membrane localization is crucial for Spry function. However, there is no apparent transmembrane domain in the primary sequence of Spry but the protein is anchored to membranes by palmitoylation of cysteine residues in the C-terminus (18). In response to RTK signaling Spry proteins become phosphorylated at serine (*see chapter 3.4.6*) (18,76), as well as tyrosine residues (18,20,23,27,28,44,53). Growth factor-induced tyrosine

phosphorylation is mediated by a Src-like kinase (28,47,77). Spry proteins contain several tyrosine residues, five of which are conserved among the mammalian family members (28). However, a key residue is the highly conserved N-terminal tyrosine (Y53 in mSpry1) which is essential for Spry to inhibit FGF-stimulated, but not VEGF-stimulated p42/44 ERK activation (20,23,27,28,44,53). Mutation of the conserved N-terminal tyrosine residues generates dominant-negative Spry proteins. This dominant-negative effect might result from sequestration of the wild-type protein through hetero- and homodimerization or altered interaction with Spry-binding partners (27-29).

3.4.4 Hetero- and homodimerization

Through their cysteine-rich C-terminal domain Spry proteins form homo- and hetero-oligomers (27,29,78). Ozaki and co-workers provide evidence that co-expression of different Spry isoforms increases the inhibitory potential of single Spry proteins on FGF2 signaling. The four Spry isoforms have highly variable N-terminal domain, through which each Spry protein can interact with specific signaling molecules (78). Thus, the formation of hetero-oligomers might facilitate cooperative activity making oligomers more efficient in sequestering different players of RTK signaling pathways compared to the single isoform. The formation of homo- and hetero-oligomers is independent of growth factor stimulation and of the conserved N-terminal tyrosine residue. Consequently, Spry proteins can form oligomers with Spry proteins bearing a mutation in their N-terminal tyrosine forming a nonfunctional complex, potentially explaining the dominant negative effect of these mutated Spry forms (27-29).

3.4.5 Attenuation of Spry activity: Shp2

Spry binds to SH2 domain-containing phosphatase 2 (Shp2), but is released once its N-terminal tyrosine is phosphorylated in response to RTK signaling (48,79,80). Shp proteins are a small, highly conserved subfamily of protein-tyrosine phosphatases (PTP). They function as positive regulators of RTK signaling pathways. Initially, it was suggested that Shp2, by binding to Grb2, might act as an adaptor protein to recruit the Grb2-Sos complex to the activated RTK (for review see (49,81)). Therefore, by sequestering Shp2, Spry might interfere with complex formation of RTK signaling effectors (48).

However, several studies have shown that mutation of Shp2 in the putative Grb2 binding site did not interfere with the function of the protein in mediating p42/44 ERK

activation (82,83). In contrast, several lines of evidence indicate that the phosphatase activity of Shp2 is required for RTK signaling (for review see (81)). Therefore, a second model where Spry is a target of Shp2 phosphatase activity might be more attractive. It was suggested that Shp2 dephosphorylates FGF-induced phosphotyrosines on Spry which results in the dissociation of Spry from Grb2 (79,80). This implies that Shp2 acts as a positive regulator in RTK signaling by dephosphorylating and inactivating Spry.

3.4.6 Protein stability: Serine versus tyrosine phosphorylation

Growth factor-mediated serine phosphorylation of Spry proteins depends on the concerted activity of both the ERK and the p38 mitogen-activated protein kinase (MAPK) cascade and is mediated by the MAPK-interacting kinase 1 (Mnk1) (76). DaSilva and co-workers demonstrated that serine phosphorylation of Spry stabilizes the protein by negatively regulating tyrosine phosphorylation and c-Cbl binding of Spry. As mentioned before (*see chapter 3.3.3)* Spry proteins associate with the E3 ubiquitin ligase c-Cbl upon tyrosine phosphorylation (20,23,28). This interaction results in polyubiquitylation of Spry and targets the protein for degradation by the 26S proteasome (20). Similarly, Spry2 accumulates to higher levels and inhibits FGFR signaling more efficiently in c-Cbl-null mouse embryonic fibrolasts (MEFs) than in control MEFs (28). Thus, c-Cbl acts as an important regulator by ubiquitylating active Spry which limits Spry function for a defined period after receptor engagement. Together, these observations demonstrate that the balance between serine and tyrosine phosphorylation of Spry dictates the stability of the protein and thereby the temporal properties of the feedback regulation of RTK signaling.

3.4.7 Modulation of Spry function: Caveolin-1

Spry proteins bind to Caveolin-1, the main structural component of caveolae via its conserved C-terminal domain (18,26). The interaction with Caveolin-1 modulates Spry function as an inhibitor of RTK signaling. Interestingly, while all four Spry isoforms associate with Caveolin-1, the functional consequence of the interaction differs among the four Spry family members (26) (*see chapter 5*).

3.5 Biological consequences of Spry expression

Spry proteins are key negative regulators that limit the strength, duration and range of activation of RTKs, thus allowing for controlled growth and differentiation. Still, the identification of the signaling pathways regulated by the Spry proteins *in vivo* remains a major challenge. In *Drosophila Spry* mutants, ectopic secondary branching of the tracheal system is induced owing to a change in tracheal cell fate (10). For the mammalian homologues, similar regulatory functions in branching morphogenesis have been observed: in mice, ectopic expression of mSpry4 inhibits branching morphogenesis of the respiratory system (84) and mSpry1 is important for kidney formation by regulating branching of the ureteric bud from the Wolffian duct as seen in the mSpry1 null mouse (37,85). Also, ectopic expression of mSpry4 in endothelial cells of cultured mouse embryos inhibits branching and sprouting of small vessels resulting in abnormal embryonic development (46). These data indicate that the regulation of branching morphogenesis by Spry proteins has been conserved through evolution from *Drosophila* to mammals. However, whereas FGF is the key mediator for the formation of the tracheal system in *Drosophila* and the respiratory system in mice, GDNF regulates branching of the ureteric bud. This emphasizes that Spry function is not specific for a particular signaling pathway and it is conceivable that Spry proteins have the ability to integrate diverse signaling pathways during development and homeostasis.

The importance of correct Spry function is strengthened by examining the role of Spry in human cancers. The expression of Spry1 and Spry2 is significantly downregulated in breast (79% and 96%, respectively) and prostate (40% for Spry1) cancer specimens, as well as in melanoma cell lines (86-88). Loss of Spry expression may be a late event in the progression of cancer. For instance, low-grade prostate cancers retain Spry2 expression, whereas metastatic cancers often fail to express Spry2 (88).

In summary, it is now widely accepted that Spry proteins exert crucial functions in RTK-dependent cellular homeostasis. However, differences among the four isoforms, functional consequences of the interaction with other proteins, and context-dependent regulation of Spry activity are open questions that still need to be addressed. Also, further investigation into the function of Spry proteins might enhance the understanding of the molecular links between the dysregulation of RTK signaling and human disease.

4 Material and Methods

Cell culture

All reagents used for cell culture and all chemicals were obtained from Sigma/Fluka (Basel, Switzerland) unless otherwise noted. All cells were maintained at 37°C with 5% CO_2. Human embryonic kidney cells (HEK293T), human breast carcinoma cells (T47D) and human pancreatic PANC-1 cells were obtained from the American Tissue Culture Collection (ATCC; Manassas, VA). The following protocol was used for the generation of tumor cell lines: Rip1Tag2 mice positive for one or both of the transgenes (Rip1rtTA and tet(O)$_7$mSpry4) were sacrificed at the age of 12 weeks. Pancreatic tumors were isolated using a dissecting microscope and tumor cell lines were established as described previously (89). HEK293T, PANC-1 and tumor cell lines were cultured in high glucose (4.5 mg/l) DMEM, containing 10% fetal bovine serum and 2mM glutamine. T47D cells were cultured in RPMI 1640 with the same supplements as above. Doxycycline (1μg/ml) was added to normal media to induce mSpry4 expression in tumor cells.

Transient Transfections and Adenoviral Vectors

HEK293T cells were transiently transfected using PerFectin (Gene Therapy Systems, San Diego, CA, USA) or Metafectene (Biontex, Munich, Germany). For co-transfections, the ratio of two plasmids was 1:1 and was normalized using empty vectors. Transfection with pcDNA3.1/EGFP was used to monitor the transfection efficiency. Adenoviral constructs encoding the mSpry cDNAs (AdmSpry4) and the firefly luciferase cDNA (AdLite) were generated as described previously (90). Amplification of the virus was carried out in HEK293 cells and virus particles were purified from cell lysates using cesium chloride gradients and gel filtration (90). Viral quantities were based on protein content using the conversion of 1 mg viral protein/3.4 x 10^{12} virus particles. For viral infection of T47D and PANC-1 cells, culture medium was replaced with starvation medium (RPMI 1640 or DMEM, 2 mM glutamine) containing 2,500 virus particles per cell. After 5h, the medium was replaced with fresh growth medium and cells were allowed to recover for 5h before overnight starvation and stimulation experiments.

Proliferation Assay

At t_0, 1 x 10^5 β tumor cells were seeded onto 24-well plates. Every 24 h, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, was added to the cells at 1mg/ml and incubated at 37°C for 90 min. Cells were solubilized in 500 µl solubilization buffer (95% isopropanol, 5% formic acid). Absorption of the solution was determined at 570 nm.

Migration Assay

PANC-1 cells (3 x 10^4/well) were seeded onto 8-µm pore size Transwell membranes (BD Biosciences) and incubated with media without sera or media supplemented with either 10% FCS or FGF2 (100 ng/ml). When the cells were trypsinized to induce migration, cells were first incubated in media containing 10% FCS for 2.5 h to allow the cells to adhere. Next, cells were exposed to 0.05% trypsin at room temperature for 60-120 s to loosen, but not detach the cells from their extracellular matrix. Following trypsinization, cells were incubated for an additional 4.5 h at 37°C in DME/F12 medium containing 17.5 mM glucose, 1% BSA and insulin-transferrin-selenium (GIBCO), 0.05% gelatin and 25mM HEPES (pH7.2). FGF2 (100 ng/ml) was diluted in media and added to the lower chamber. Then, cells on top of the membranes were removed with sterile cotton swabs and membranes were fixed with fresh 4% paraformaldehyde (15 min at room temperature), stained with crystal violet (0.5% in 20% methanol, 20 min at room temperature). Labeled nuclei of cells trapped in the membrane were counted using a 200 x magnification on a Zeiss microscope (Zeiss, Feldbach, Switzerland).

Adhesion Assay

Matrix adhesion assays were performed as previously described (91). 96-well plates were left uncoated or coated with 5 µg/cm^2 of mouse collagen IV (BD Biosciences) according to the manufacturers recommendations. Next, PANC-1 cells (5 x 10^4 cells /well) were seeded and incubated for 2.5 h. Nonadherent cells were removed by washing with phosphate buffered saline (PBS), while adherent cells were fixed for 20 min with 25% glutaraldehyde, stained with crystal violet and solubilized with 10% acetic acid. Absorbance was measured at 595 nm.

Stimulation experiments

After viral infection, cells were allowed to recover for approximately 5 h followed by overnight starvation and stimulation with growth factors the following day. In the case of β tumor cells, doxycycline treatment (see above) for 2 days was used to induce mSpry4 expression. Then, β tumor cells were incubated overnight in starvation medium. Following starvation, cells were stimulated for 30 min by addition of either recombinant human FGF2 (50 ng/ml), EGF (50 ng/ml), HGF (20 ng/ml) or PDGF (30 ng/ml) (Catalys AG/Promega, Wallisellen, Switzerland or Sigma/Fluka).

Immunoblotting

Cells were lysed for 30 min on ice in lysis buffer (1% Triton X-100, 160 mM NaCl, 20 mM Tris pH 8.0, 2 mM Na_3VO_4, 10 mM NaF and a 1:200 dilution of stock protease inhibitor cocktail for mammalian cells (Sigma/Fluka)). Tumor tissue samples were lysed in tissue lysis buffer (0.5% Triton X-100, 100 mM NaCl, 2.5 mM EDTA,10 mM Tris pH 8.0). Protein concentration was determined using a modified Bradford protocol (Bio-Rad Protein Assay; BioRad Laboratories, Reinach, Switzerland). Equal amounts of protein were diluted in sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (10% glycerol, 2% SDS, 65 mM Tris, 1 mg/100 ml bromophenolblue, 1% beta-mercaptoethanol) and resolved by 12% SDS-PAGE (92). SDS-PAGE gels were transferred to polyvinylidene fluoride (PVDF) (Milipore, Volketswil, Switzerland) by semi-dry transfer in Towbin's buffer (20% methanol, 25mM Tris, 192 mM glycine), blocked with either 4% BSA or 5% skim milk powder in Tris-buffered saline with 0.05% Tween 20 (TBST). Primary and secondary antibodies were diluted in 4% BSA or 5% skim milk powder in TBST. The following primary antibodies were used: mouse monoclonal anti-diphosphorylated p42/44 ERK (Sigma/Fluka), rabbit polyclonal anti-p42/44 ERK (Sigma/Fluka), goat polylclonal anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-Caveolin-1 (BD Biosciences), mouse monoclonal anti-GFP (Roche Diagnostics), mouse monoclonal anti-phosphotyrosine (BD Biosciences), polyclonal rabbit anti-FGFR2 (Santa Cruz Biotechnology, Santa Cruz, CA) and mouse monoclonal anti-PTP1B (BD Biosciences). Expression of mSpry4 was analyzed using rabbit sera against mSpry4 peptide as previously described (26). The following secondary antibodies conjugated to horseradish peroxidase were used: goat anti-mouse IgG (Sigma/Fluka or Jackson Immunoresearch, Cambridgeshire, UK), rabbit anti-goat IgG (Sigma/Fluka), donkey anti-rabbit IgG (Amersham or Jackson Immunoresearch). Detected antibodies were

visualized using enhanced chemiluminescence (GE Health Sciences/Amersham Biosciences, Otelfingen, Switzerland or Interchim, Montluçon, France). In some cases, immunoblots were also visualized and quantitated using the Odyssey Imager (Li-Cor Biotechnology, Bad Homburg, Germany). In these cases, the following secondary antibody conjugates were used: goat anti-mouse Alexa 680 (Invitrogen) and goat anti-rabbit IRDye 800 (Rockland Immunochemicals, Gilberstsville, PA).

Biotinylation

Cell-surface proteins were biotinylated as described previously (93,94). Briefly, following growth factor stimulation, HEK293T cells were placed on ice and washed with cold borate butter (10mM boric acid, 154mM NaCl, 7.2mM KCl, 1.8mM $CaCl_2$, pH 9.0). Cells were incubated with 0.5 mg/ml Sulfo-NHS-SS-Biotin (Pierce, Rockford, Il) in borate buffer for 15 min on ice. The cells were then rinsed with cold quenching buffer (192mM glycine, 25mM Tris, pH 8.3) to remove any unreacted biotin. The cells were lysed for 20 min in RIPA buffer (1% deoxycholic acid, 1% Triton X-100, 0.1% SDS, 150mM NaCl, 1mM EDTA, 10mM Tris-Cl, pH 8.3) containing a 1:200 dilution of stock protease inhibitor cocktail for mammalian cells (Sigma/Fluka)). An aliquot of the lysate was saved for immunoblotting. Lysates were incubated with 0.1ml /ml lysate ImmunoPure immobilized streptavidin beads (Pierce, Rockford, Il) for 1h at 4°C to bind the biotinylated proteins. The supernatant was removed and the streptavidin beads were washed with RIPA buffer. Then, 2x SDS-PAGE loading buffer (10% glycerol, 2% SDS, 0.065M Tris, 1 mg/100 ml bromophenolblue, 1% beta-mercaptoethanol) was added to the beads and incubated for 1h at room temperature to cleave the disulphide bond in the biotinylating reagent and to release the captured proteins. Samples were subsequently analysed by SDS-PAGE and immunoblot.

Immunoprecipitation

Cells were lysed for 30 min on ice in lysis buffer (1% Triton X-100, 160 mM NaCl, 20 mM Tris pH 8.0, 2 mM Na_3VO_4, 10 mM NaF and a 1:200 dilution of stock protease inhibitor cocktail for mammalian cells. Protein concentration was determined using a modified Bradford protocol (Bio-Rad Protein Assay; BioRad Laboratories, Reinach, Switzerland). For immunoprecipitations, equal amounts of lysates were incubated overnight (4°C on rotator) with either preimmune sera or sera specific for mSpry2. Protein G-Sepharose beads (Sigma) (10% v/v in lysis buffer) were added to each tube and allowed to incubate with the immune complexes for at least 1h (4°C on rotator). Immunoprecipitate-

bead complexes were washed three times in cold lysis buffer and an equal volume of 2x SDS-PAGE loading buffer was added to the washed beads followed by boiling of the samples.

Immunofluorescence Experiments

PANC-1 cells were plated on glass coverslips or on coverslips coated with 5 μg/cm^2 of mouse collagen IV (BD Bioscience). After 24h, cells were fixed using 4% paraformaldehyde for 15 min at 37°C. Cells were permeabilized with cold 0.1% Triton X-100 in PBS for 10 min at 4°C. Then, cells were blocked in 4% goat serum in PBS for 1 hour at room temperature. Anti-PTP1B antibody (BD Biosciences) was diluted 1:100 in 4% goat serum and incubated overnight at 4°C. Alexa 488-conjugated anti mouse IgG and Alexa 568-conjugated anti-Phalloidin antibodies (Molecular Probes) were diluted 1:200 in 4% goat serum. Nuclei were stained with 1μg/ml 6-diamidino-2-phenylindole (Sigma) for 10 min at room temperature.

Transgenic mice

Rip1rtTA transgenic mice have been previously generated according to standard procedures (95,96). The mice express the reverse tetracycline transactivator (rtTA) under the control of the rat insulin promoter (Rip1). Rip1rtTA mice were crossed to transgenic $(tetO)_7$mSpry4 mice where the expression of murine Spry4 is driven by a promoter consisting of the rtTA binding element $(tetO)_7$ linked to an inactive cytomegalovirus minimal promoter $(tetO)_7$mSpry4. Generation of $(tetO)_7$mSpry4 mice has been described previously (84). Double transgenic Rip1rtTA;tet$(O)_7$mSpry4 females were crossed with Rip1Tag2 males to generate triple-transgenic Rip1Tag2;Rip1rtTA;tet$(O)_7$ mSpry4 offsprings. Generation and phenotypic characterization of Rip1Tag2 mice has been described previously (97). All mouse lines were kept in a strict C57Bl/6 background. The PCR primers used for genotyping were: Rip1rtTA 5'-CATCTCAATGGCTAAGGCGTC-3' and 5'-GACCAGCTACAGTCGGAAACC-3'; $(tetO)_7$mSpry4: 5'-CACCGGGACCGATCCAGC-3' and 5'-GAAGTGCTGCTACTGCTGCTTA-3' Rip1Tag2: 5'-GGACAAACCACAACTAGAATGGCAG-3' and 5' -CAGAGCAGAATTGTGGA

GTGG-3'. All mouse experiments performed were in accordance with the guidelines of the Swiss Federal Veterinary Office and the regulations of the Cantonal Veterinary Office of Basel-Stadt.

Transgene induction experiments

Transgenic mSpry4 expression was induced by addition of doxycycline (1 mg/ml,) to the drinking water in bottles protected from exposure to light. The water was replaced every second day. To express mSpry4 during embryogenesis, pregnant females were treated with doxycycline. Gestation was dated by detection of the vaginal plug. Double transgenic offspring Rip1rtTA;tet(O)$_7$mSpry4 and single transgenic control littermates were sacrificed at 2 or 4 weeks, respectively. Triple transgenic tumor mice (Rip1Tag2;Rip1rtTA;tet(O)$_7$ mSpry4) were treated with doxycycline for 2 to 3 weeks and sacrificed at 7, 10, or 12 weeks of age. Tumor incidence was determined macroscopically and tumor volumes were calculated from the tumor diameter by assuming a spherical shape of the tumors.

β-tumor cell injection into nude mice

Athymic nude-FoxN1 nu/nu mice were obtained from Harlan (Harlan Sprague Dawley, Inc., Indianapolis). β tumor cells ($1x10^6$) positive for the transgenes Rip1rtTA;(tetO)$_7$mSpry4 were injected into both flanks of the mice. Mice were treated with 1 mg/ml doxycycline (1 mg/ml) in their drinking water from the day of injection or left untreated. After 6 weeks mice were sacrificed and tumor volume was measured.

Histology and immunohistochemistry

Pancreata were fixed overnight at 4°C in 4% paraformaldehyde in PBS before processing and embedding in paraffin. Five-micron thick sections were cut for immunohistochemical analysis. For bromodeoxyuridine (BrdU) labeling, mice were injected intraperitoneal with 100 μg BrdU per gram of body weight 2 hours before tumor harvesting. Rabbit sera against mSpry4 peptides were generated as previously described (26) and used for immunohistochemical analysis of mSpry4 expression. Further, the following antibodies were used for immunohistochemistry: guinea pig anti-insulin (DakoCytomation, Glostrup, Denmark), goat anti- human glucagon (Santa Cruz Biotechnology, Santa Cruz, CA), biotinylated mouse anti-BrdU (Zymed, South San Francisco, CA). Antibody specificity was confirmed by incubating pancreatic sections without primary antibody or with preimmune sera, respectively. Apoptotic cells were visualized with the *in situ* Cell Death Detection kit for peroxidase (Roche, Rotkreuz, Switzerland). All biotinylated secondary antibodies (Vector, Burlingame, CA) were used at a 1:200 dilution, and positive staining was visualized with the ABC horseradish peroxidase kit (Vector, Burlingame, CA) and 3-amino-9-

ethylcarbazole substrate kit for peroxidase (Vector, Burlingame, CA) according to the manufacturer's instructions. For the analysis of tissue morphology, slides were counterstained with hematoxylin. For immunofluorescence analysis, Alexa Fluor 568- and Alexa Fluor 488-labeled secondary antibodies diluted 1:200 were used (Invitrogen, Basel, Switzerland). For nuclear counterstaing DAPI was used. For quantitation of proliferation (BrdU incorporation), and apoptosis (terminal deoxynucleotidyl transferase-mediated nick end labeling, TUNEL reaction), the numbers of nuclei staining positive for BrdU or the TUNEL reaction were determined in 10 comparable fields per section at 400 x magnification, and the mean of positive cells ± standard deviation/field was calculated. Islet area was determined by measuring the area of insulin positive cells with ImageJ software (ImageJ, National Institutes of Health, USA).

Statistical analysis

Statistical analyses were performed and graphs were generated with GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA).

5 Sprouty and Caveolin-1

5.1 Introduction

5.1.1 Caveolae

Caveolae are 50-100nm flask-shaped invaginations of the plasma membrane with a regular shape and size (for review see (98)). In contrast to other known membrane domains, like clathrin-coated structures, these membrane microdomains appear to be uncoated. Caveolae share a characteristic lipid composition with other lipid rafts, namely an enrichment in sphingolipids, such as sphinogmyelin, ceramide, gangliosides and cholesterol. However, while the overall biochemical composition of lipid rafts and caveolae is thought to overlap, these microdomains are not completely equivalent. Several proteins have been shown to preferentially localize to either caveolae or lipid rafts, respectively (for reviews see (98-101)). Cholesterol is an integral component for the formation and maintenance of caveolae and is necessary to stabilize oligomerization of Caveolin-1, the major structural protein of caveolae (102). The expression of Caveolin-1 is sufficient and necessary to drive the formation of morphologically identifiable caveolae (103). Caveolae are thought to function in transcytosis and endocytosis. Also, caveolae play a pivotal role in cholesterol homeostasis of the cell. Still, the relationship between the unique, highly conserved structure of caveolae and their physiological function requires further investigation (for reviews see (98-101)).

5.1.2 Caveolin-1

The first molecular marker of caveolae to be discovered was Caveolin-1 (102). The family of Caveolin proteins consists of three isoforms, Caveolin-1, Caveolin-2 and Caveolin-3 (for reviews see (98-100)). Caveolin proteins are highly expressed in terminally differentiated or quiescent cells. Caveolin-1 and Caveolin-2 have similar tissue distribution being present in most cell types with the exception of skeletal muscle fibers and cardiac myocytes. Caveolin-3 is limited to skeletal muscle, the diaphragm, and the heart. Caveolin-1 and Caveolin-3 (but not Caveolin-2) are palmitoylated on three cysteines in the C-terminal region, but the palmitoylation is not necessary for Caveolin targeting to caveolae.

Caveolin-1 is a 22 kDa protein that is phosphorylated on serine/threonine sites (104) and on tyrosines by c-Src (105,106) and possibly other non-receptor tyrosine kinases

(107). Caveolin-1 forms high molecular mass oligomers of ~400kDa via on oligomerization domain located in the N-terminal half of the protein (**Figure 5-1**). The protein is integrated into the membrane by a 33-residue central hydrophobic region which is postulated to form a hairpin into the lipid bilayer (intramembrane loop) (105,108,109). The flanking N- and C-terminal domains are cytoplasmic. Interestingly, the intramembrane domain is not essential for membrane localization, but rather two adjacent regions in the flanking N- and C-terminal regions bind to membranes with high affinity (110,111). These regions are now referred to as the membrane attachment domain (112) (for reviews see (98-100)). Another feature of the Caveolin-1 proteins is a 'scaffolding domain' (residues 82-101) that interacts with different RTK and other signal transduction molecules (113,114). The interaction of Caveolin-1 with various components of signaling pathways suggests that Caveolin-1 might play a role in the modulation of important celluar processes (for reviews see (99,115)).

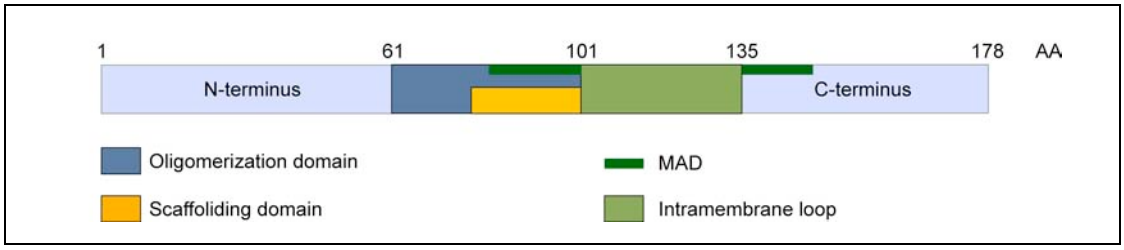

Figure 5-1. A schematic representation of Caveolin-1.
Caveolin proteins are integrated into the plasma membrane via their intramembrane loop whereas the N- and C-termini remain cytoplasmic. The scaffolding domain is crucial for the interaction of Caveolin-1 with various signal transduction molecules. The membrane attachement domain is essential for membrane localization of Caveolin. Numbers denote amino acids (AA).

5.1.3 Caveolae, Caveolin-1 and signaling

The most important role attributed to caveolae may be their function in signal transduction. Many signaling molecules localize to caveolae. This compartmentalization within the membrane might allow efficient and rapid coupling of activated receptors to downstream effectors and could facilitate cross-talk between different signaling pathways (for reviews see (115-117)).

Many of the proteins in caveolae have been shown to associate with Caveolin-1. Thus Caveolin-1 acts as a scaffolding protein by further concentrating proteins within caveolae. Also, it has been shown that the interaction with Caveolin-1 regulates the activity of proteins. GTPase activity of hetero-trimeric G-proteins, kinase activity of Src tyrosine kinases, EGFR kinase and PKC are functionally suppressed by binding to Caveolin-1

(113,118-122). Similarly, disruption of caveolae by either cholesterol depletion or siRNA Caveolin-1 results in p42/44 ERK hyperactivation (123,124).

A number of experiments suggest that Caveolin-1 functions as kinase inhibitor by recognizing a conserved Caveolin-binding motif that is present within the catalytic domain of many known kinases and other Caveolin-interacting proteins (113,118,125). Engelman and coworkers could show that co-expression with Caveolin-1 dramatically inhibits signaling from EGFR but also from effectors further downstream in the pathway like Raf and p42/44 ERK. Their *in vitro* results suggest, that Caveolin-1 directly inhibits the kinase activity of purified p42/44 ERK (114).

In contrast to the negative feedback loop described for Spry proteins, a reciprocal negative regulation exists between p42/44 ERK activation and Caveolin-1 expression, *i.e*. up-regulation of Caveolin-1 protein expression down-modulates p42/44 ERK activity and up-regulation of p42/44 ERK activity down-regulates Caveolin-1 mRNA and protein expression (114,126).

5.1.4 Physiological roles of the Caveolin proteins

Caveolin-1 and Caveolin-3, but not Caveolin-2 null mice show a complete loss of caveolae in all tissues and cell types that normally express the proteins (for review see (99)). However, all of the Caveolin-deficient mouse models generated (Caveolin-1 null, Caveolin-2 null, Caveolin-3 null, and Caveolin-1/3 double knockout mice) are viable and fertile. The physiological consequences of Caveolin-1 loss in knockout mice are multiple, including defects in insulin-mediated lipogenesis, abnormal cardiac function, urinary bladder dysfunction, deregulated Ca^{2+} homeostasis, defects in angiogenesis and microvascular hyperpermablilty (127-129). Some of these defects might be attributed to a dysregulation of signaling pathways in the absence of Caveolin-1. For example, isolated cardiac fibroblasts from Caveolin-1 null mice show hyperactivation of the p42/44 ERK pathway (130) and cultured MEFs derived from the same mice show a marked increase in growth rate, compared with matched control wild-type MEFs (103).

5.1.5 Caveolin-1 and cancer

Caveolin-1 has been proposed to act as a tumor suppressor protein, inhibiting the functional signaling activity of several proto-oncogenes and consequently disrupting the process of cellular transformation (for review see (99)). For instance, Caveolin-1 protein levels are downregulated in cells transformed with several activated oncogenes such as v-Abl, Bcr-abl, and H-Ras. Also, the ability of transformed cells to grow in soft agar, a hallmark of cellular transformation is abrogated by the reintroduction of Caveolin-1 (126,131). However, as Caveolin-1 null mice do not develop spontaneous tumors, the loss of Caveolin-1 alone appears insufficient to induce cell transformation *in vivo*. Loss of Caveolin-1 potentiates the process of cell transformation when combined with a transforming agent (a carcinogen or a tumor-prone genetic background) (for review see (99)).

The human Caveolin-1 gene was mapped to a locus on human chromosome 7q13.1 that is commonly deleted in a variety of human cancers (132). In addition, investigations of Caveolin-1 in human tumors has revealed sporadic mutations and changes in expression levels. However, Caveolin-1 expression levels can be reduced, unchanged or upregulated, depending on the tumor cell type. Caveolin-1 downregulation is typical of ovarian, lung and mammary carcinomas whereas it is consistently upregulated in bladder, esophagus, and prostate carcinomas. Interestingly, it was found that Caveolin-1 levels are increased during metastasis even in cancers where the protein is downregulated in the primary tumor. These contrasting roles for Caveolin-1 in tumor progression may be partly explained by the observation that Caveolin-1 has several peptide domains with opposing functions (for reviews see (99,133)).

5.1.6 Spry/Caveolin-1 protein interaction

Our lab found a strong colocalization of Spry proteins with Caveolin-1 in human endothelial cells, using Caveolin-1 as a marker of membrane localization. Consistent with this results, co-immunoprecipitation and biochemical fractionation revealed an association of endogenous Caveolin-1 with mSpry1 and with mSpry2 (18).

Subsequently, we have shown a constitutive interaction of Caveolin-1 with all four Spry isoforms (26). Biochemical experiments, including GST pull-down binding assays and peptide arrays confirmed a direct physical association of the two proteins. Spry proteins interact with two distinct domains of Caveolin-1, namely with the central oligomerization

domain and the C-terminus of Caveolin-1. Moreover, we have shown that Spry proteins associate with Caveolin-1 via their highly conserved cysteine-rich C termini. Using specific point mutants a conserved arginine was identified as being essential for the Spry/Caveolin-1 interaction. However, despite the mutants being unable to bind to Caveolin-1, they still translocate to the membrane in response to growth factor stimulation suggesting that the association with Caveolin-1 is not required for Spry localization. The mutant Spry proteins at the membrane are non-functional when considering attenuation of RTK signaling, as has been previously demonstrated (26).

5.2 Results

5.2.1 Inhibitory activity of Caveolin-1 in T47D cells depends on cell density

Given that all members of the mSpry family bind to Caveolin-1 (26), we set out to characterize the consequence of this interaction. Therefore, we used the human breast cancer cell line T47D, lacking Caveolin-1 expression due to silencing of the Caveolin-1 promoter by methylation (134). T47D cell clones expressing Caveolin-1 (T47D-Cav-1) or EGFP (T47D-EGFP) were generated by stable transfection (26). These cell lines provided an experimental system to examine mSpry acting on EGF and FGF2 signaling in the presence or absence of Caveolin-1.

Localization of Caveolin-1 depends on cell confluency. Endogenous and exogenous Caveolin-1 redistributes to cell-cell contacts as cells become more confluent (135,136). It was suggested that Caveolin-1 plays an important role mediating cell contact inhibition by moving to cell-cell contacts where it attenuates signaling through the Ras-p42/44 ERK cascade (135,136). We therefore expected that the inhibitory activity of Caveolin-1 on FGF2 and EGF signaling might vary with different cell densities, and we analyzed signaling in T47D-Cav-1 and T47D-EGFP cells under sparse and confluent growth conditions (**Figure *5-2***).

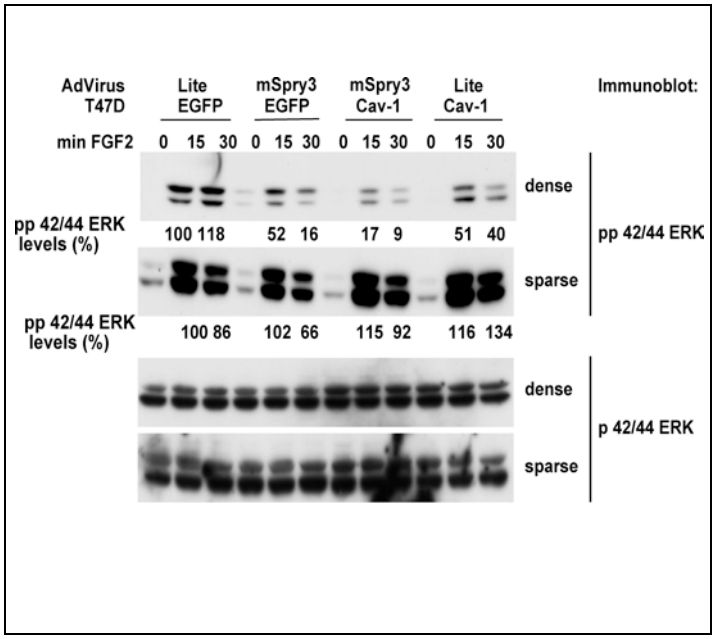

Figure 5-2. Caveolin-1 function depends on cell density.
T47D-Cav-1 and T47D-EGFP cells were plated at low and high cell densities and stimulated with FGF2 for the times indicated. Under dense growing conditions, levels of phosphorylated p42/44 ERK were reduced in T47D-Cav-1 cells when compared to T47D-EGFP control cells. In contrast, Caveolin-1 expression in sparsely growing cells potentiated FGF2 mediated p42/44 ERK activatio.

Indeed, Caveolin-1 only inhibited FGF2-mediated p42/44 ERK activation at higher cell densities. In contrast, in sparsely seeded T47D-Cav-1 cells, FGF2 signaling was not attenuated but rather potentiated compared to signaling in T47D-EGFP. Comparable results were found for EGF signaling (data not shown). These data confirm that Caveolin-1 is a modulator of different RTK signaling pathways and acts in a cell density-dependent manner.

5.2.2 Functional consequence of Spry/Caveolin-1 interaction on FGF2 signaling

To analyze Spry function on FGF2 signaling in the presence or absence of Caveolin-1, each stably transfected cell line was infected with adenoviruses encoding either the mSpry cDNAs (AdmSpry1-4) or the firefly luciferase cDNA (AdLite). First, experiments were performed with cells growing at high cell densities (

Figure 5-3). In T47D-EGFP cells, *i.e.* in the absence of Caveolin-1, FGF2 mediated p42/44 ERK activation was decreased by all four mSpry isoforms. mSpry2 and mSpry3 were more efficient than mSpry1 and mSpry4. In T47D-Cav-1 cells mSpry1 and mSpry3 cooperated with the inhibitory activity of Caveolin-1. In contrast, mSpry2 and mSpry4 were not able to decrease the levels of phosphorylated p42/44 ERK compared to the levels in T47D-Cav-1 cells infected with control virus. These data suggest that the presence of Caveolin-1 impedes the inhibitory function of mSpry2 and mSpry4 but has no effect on the activity of mSpry1 and mSpry3 (**Table 5-1**). We conclude that Caveolin-1 differentially modifies the inhibitory function of the four mSpry isoforms.

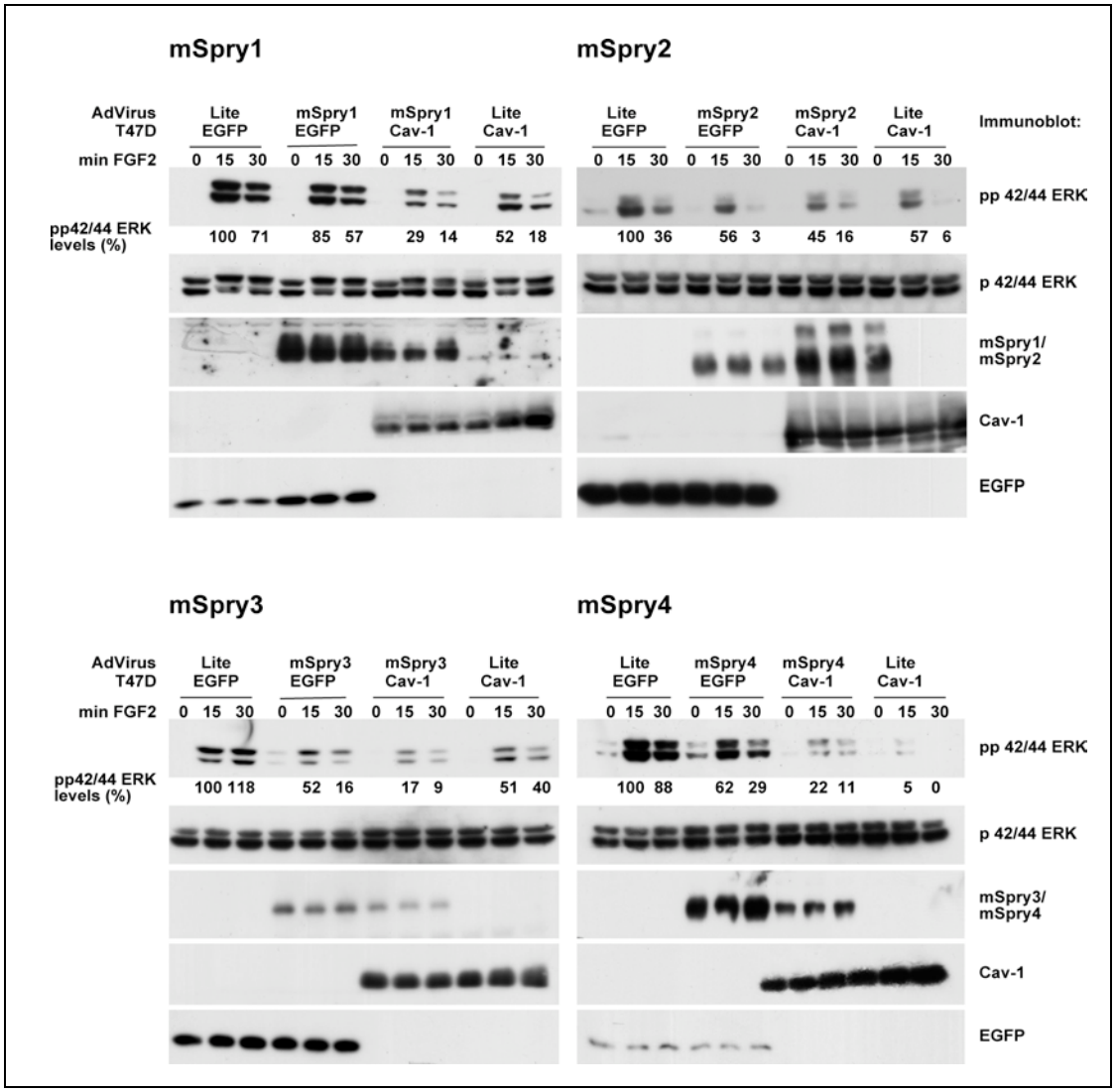

Figure 5-3. Spry differentially inhibits FGF2 signaling in the presence of Caveolin-1. *T47D-Cav-1 and T47D-EGFP cells plated at high cell density were infected with AdmSpry1, AdmSpry2, AdmSpry3, or AdmSpry4 or control virus AdLite. Cells were stimulated with FGF2 for the times indicated. In T47D-EGFP cells, all four Spry isoforms inhibited FGF2-mediated p42/44 ERK activation. In T47D-Cav-1 cells, mSpry1 and mSpry3 cooperate with the inhibitory function of Caveolin-1. In contrast, mSpry2 and mSpry4 in T47D-Cav-1 cells do not attenuate p42/44 ERK phosphorylation when compared to control transfected T47D-Cav-1 cells. See also* ***Table 5-1****.*

Considering the density-dependent function of Caveolin-1 discussed before, all experiments were also performed under sparse growing conditions (

Figure 5-5). In sparsely growing T47D-EGFP cells all four isoforms attenuate p42/44 ERK activation. Similar to what was experienced in dense cells, the presence of Caveolin-1 abrogated the inhibitory function of mSpry2 and mSpry4 without affecting

mSpry1 and mSpry3 activity. These observations imply that Spry activity and functional consequences of the mSpry/Caveolin-1 interaction are independent of cell density.

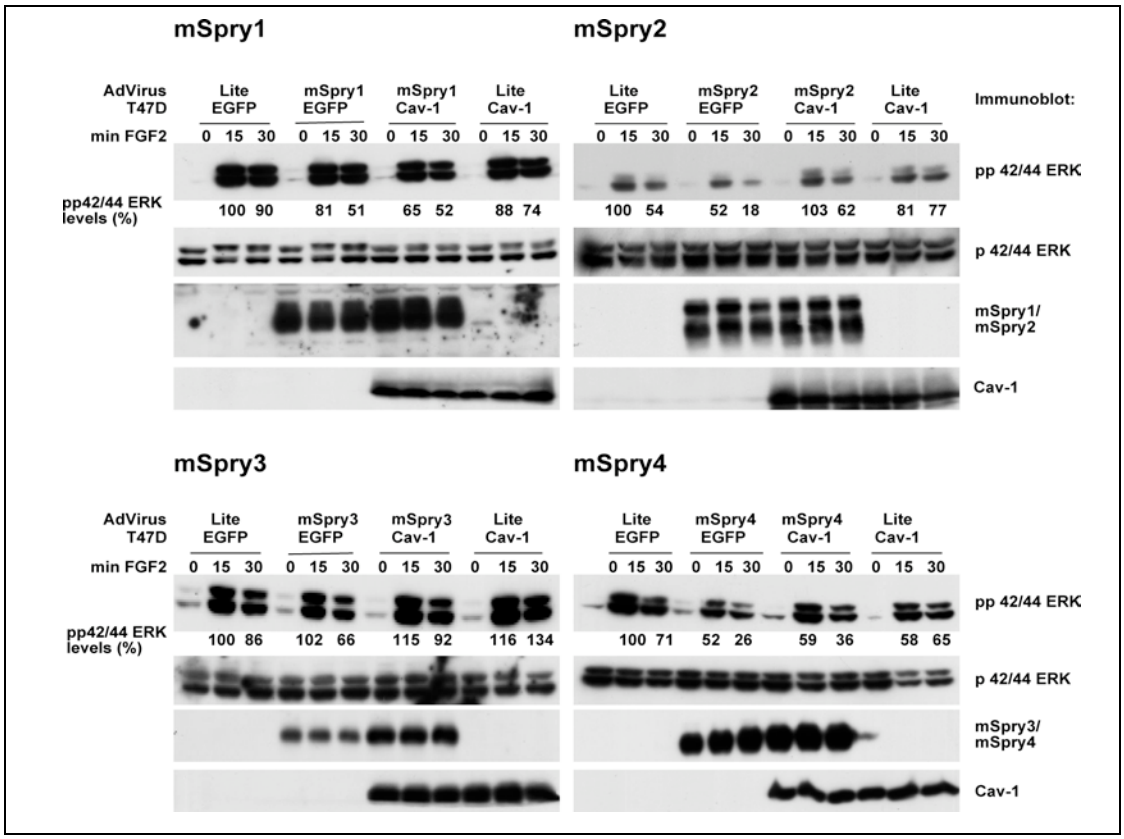

Figure 5-5. Consequences of mSpry/Caveolin-1 on FGF2 signaling in sparse cells. *T47D-Cav-1 and T47D-EGFP cells growing under sparse conditions were infected with AdmSpry1, AdmSpry2, AdmSpry3, or AdmSpry4 or control virus AdLite. Cells were stimulated with FGF2 for the times indicated. In T47D-EGFP cells, all four Spry isoforms inhibited FGF2-mediated p42/44 ERK activation. The presence of Caveolin-1 abrogated the inhibitory function of mSpry2 and mSpry4 but had no effect on mSpry1 and mSpry3 activity. See also* ***Table 5-1****.*

5.2.3 Functional consequence of Spry/Caveolin-1 interaction on EGF signaling

Next, the functional consequence of Spry/Caveolin-1 interaction was studied in the context of EGF-mediated p42/44 ERK activation. In confluent cells, we found that in the absence of Caveolin-1 (T47D-EGFP) mSpry2 and mSpry3 inhibited EGF-mediated p42/44 ERK activation whereas mSpry1 and mSpry4 did not affect pp42/44 ERK levels (**Figure 5-7**). Surprisingly, a potentiation of EGF signaling, as has been previously reported (18,20,23,25,44,53), was not observed for any of the four isoforms in T47D cells. Similar to what was noted for FGF2 signaling, expression of mSpry2 in T47D-Cav-1 cells did not

decrease the levels of p42/44 ERK phosphorylation when compared to control infected T47D-Cav-1 cells, indicating that mSpry2 and Cav-1 do not cooperate. In contrast, mSpry3 mediated inhibition was effective in both T47D-Cav-1 and in T47D-EGFP cells (**Table 5-1**).

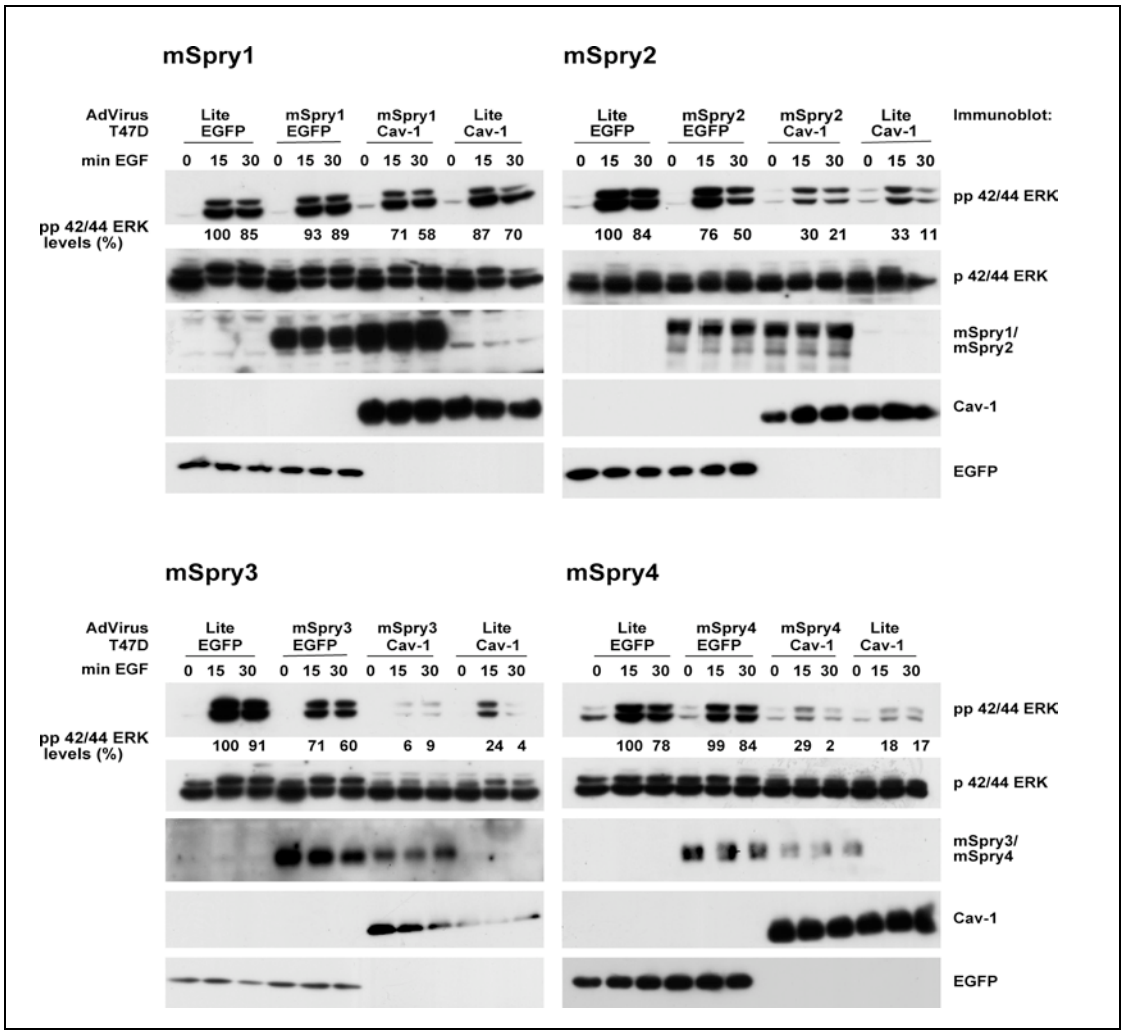

Figure 5-7. EGF signaling is differentially inhibited by Spry depending on Caveolin-1. *T47D-Cav-1 and T47D-EGFP cells growing under dense conditions were infected with AdmSpry1, AdmSpry2, AdmSpry3, or AdmSpry4 or control virus AdLite. Cells were stimulated with EGF for the times indicated. In T47D-EGFP cells, mSpry2 and mSpry3 inhibited EGF-mediated p42/44 ERK activation, mSpry1 and mSpry4 did not affect EGF signaling. In T47D-Cav-1 cells, the presence of mSpry2 did not decrease p42/44 ERK levels compared to control infected T47D-Cav-1 cells. mSpry3 was an efficient inhibitor in T47D-EGFP and in T47D-Cav-1 cells . See also* ***Table 5-1****.*

Again, experiments were repeated in sparsely growing cells. In agreement with results of the FGF2 stimulation experiments, the activity of all four Spry isoforms and the functional consequence of the Spry/Caveolin-1 interaction is independent of cell density (data not shown).

To summarize the results above we conclude that: (i) Caveolin-1 is acting in a cell density-dependent manner, (ii) Caveolin-1 differentially regulates the activity of the four mSpry isoforms, (iii) functional consequences of mSpry/Caveolin-1 interaction are not specific for FGF2 or EGF signaling and are cell density-independent (**Table 5-1**).

Table 5-1 Effect of mSpry on FGF2 and EGF-mediated p42/44 ERK phosphorylation in presence or absence of Caveolin-1.

	FGF2				EGF			
mSpry	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
T47D-EGFP	+	++	++	+	-	+	+	-
T47D-Cav-1	+	-	++	-	-	-	+	-

\+ *inhibition*
++ *strong inhibition*
\- *no effect*

5.2.4 Receptor internalization

Spry proteins are bimodal regulators of RTK signaling. In addition to their inhibitory function, they also act to potentiate growth factor mediated p42/44 ERK activation by interfering with internalization of activated RTKs (23,44,53-57). Up until now, this stimulatory function of Spry was only described in the context of EGF signaling. However, other RTKs like FGFR are internalized by c-Cbl-mediated ubiquitylation similar to EGFR (59,60). The underlying mechanisms that determine the stimulatory versus the inhibitory effects of Spry are not known. A tightly regulated balance between these two functions of the Spry proteins may dictate the final functional consequence on RTK signaling. We wondered whether the interaction with Caveolin-1 might be implicated in this regulation of bimodal Spry function.

Experiments in T47D-EGFP and T47D-Cav-1 cells showed that mSpry2 and mSpry4 do not cooperate with Caveolin-1 to inhibit EGF or FGF2 signaling. We hypothesized that Caveolin-1 changes Spry function as an inhibitor of receptor internalization. Therefore, the effect of mSpry2 and mSpry4 on levels of cell surface EGFR and FGFR2 were investigated in the absence or presence of Caveolin-1. For these studies,

HEK293T cells were used as mSpry2 was reported to potentiate EGF signaling in this cell line (23,47). HEK293T cells, having no or very low amounts of endogenous Caveolin-1, were transiently co-transfected with plasmids encoding Caveolin-1 or EGFP as control and plasmids encoding mSpry2 or mSpry4. To separate cell surface EGFR and FGFR2 from the cytosolic fraction of the receptors, cell surface proteins were biotinylated prior to lysis of the cells and then separated from the total lysate using streptavidin beads.

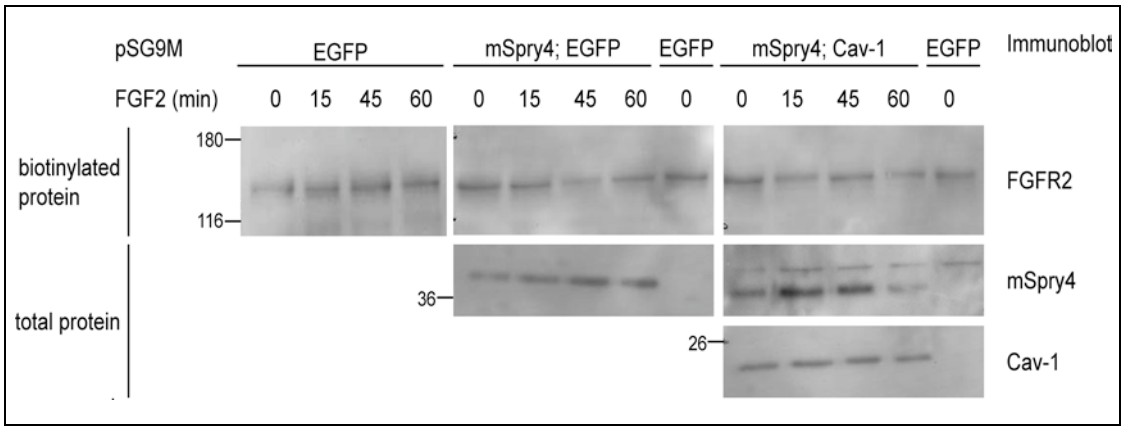

Figure 5-8. FGFR2 at the cell surface in HEK293T cell.
HEK293T cells were co-transfected with different combinations of vector encoding mSpry4, Caveolin-1 and EGFP as indicated. Cells were stimulated with FGF2 for the times indicated, cell surface protein was biotinylated and separated from total cell lysate using streptavidin beads. Levels of cell surface FGFR2 did not change in response to FGF2 stimulation and the presence of mSpry4 or mSpry4 together with Caveolin-1 did not influence the amount of receptor at the membrane,

First, we analyzed levels of cell surface EGFR after stimulation with EGF in the presence or absence of mSpry2. In unstimulated, control transfected HEK293T cells, the amount of biotinylated receptor was very low and we were not able to detect changes in cell surface EGFR levels after EGF stimulation and/or in the presence of mSpry2 (data not shown). Therefore, we decided to analyze changes in FGFR2 levels in response to FGF2 stimulation in the presence of absence of mSpry4. In control transfected 293T cells ($pEGFPN_2$), stimulation with FGF2 had no effect on the amount of FGFR2 which was present at the membrane (**Figure 5-8**). mSpry4 expressing HEK293T cells had levels of cell surface receptor which were comparable to the levels in control transfected cells and again, growth factor stimulation did not affect the amount of biotinylated receptor. Similar, co-expression of Caveolin-1 together with mSpry4 did not influence cell surface receptor levels.

Taken together, up to 60 minutes after EGF or FGF2 treatment we were not able to observe receptor internalization in HEK293T. Similar, the presence of mSpry4 or

mSpry4 together with Caveolin-1 had no effect on the amount of FGFR present at the cell membrane. However, given the very low levels of biotinylated receptor detectable by immunoblotting, the method we employed might not be sensitive enough to investigate changes in levels of cell surface receptors in this cell line.

5.2.5 Sprouty protein stability

Next, we wondered whether Caveolin-1 might modulate mSpry function by affecting protein stability. Proteins are either degraded via the lysosomal or the proteasomal pathway (for review see (137)). For proteasomal degradation proteins are tagged by polyubiquitin chains which are attached to the protein by sequential reactions of three classes of enzymes; ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2) and ubiquitin ligases (E3). Spry2 interacts with the ubiquitin ligase c-Cbl resulting in ubiquitylation and proteasomal degradation of Spry2 (20,23). To analyze whether the presence of Caveolin-1 has an impact on c-Cbl-mediated degradation of Spry we blocked proteasomal degradation by using the proteasome inhibitor N-Acteyl-Leu-Leu-Nle-CHO (ALLN). Expression of mSpry2 and mSpry4 was induced by adenoviral infection of T47D-EGFP and T47D-Cav-1 cells.

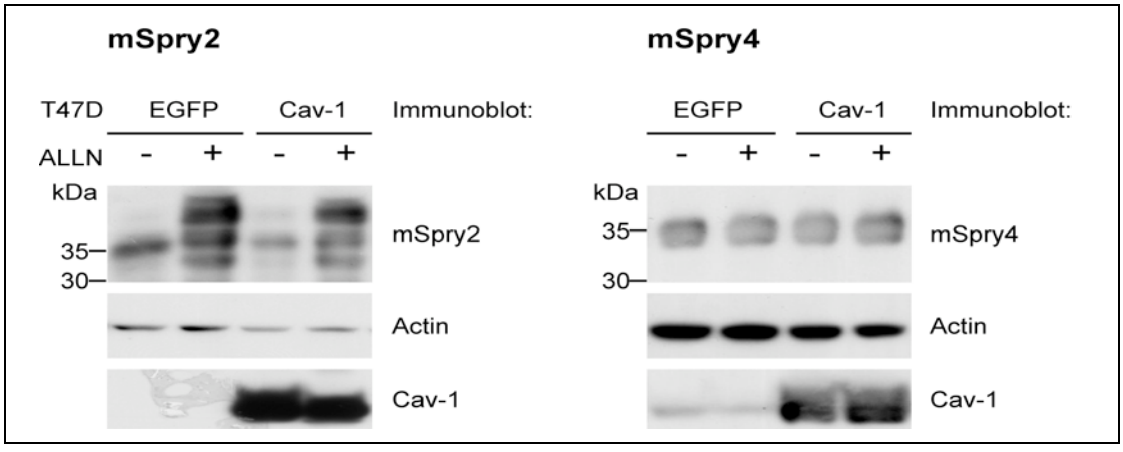

Figure 5-9. Caveolin-1 does not affect the protein stability of mSpry2 or mSpry4.
T47D-EGFP and T47D-Cav-1 cells were infected with AdmSpry2 and AdmSpry4 and treated with the proteasome inhibitor ALLN for 4h. mSpry2 but not mSpry4 was stabilized by ALLN. Caveolin-1 had no impact on the protein levels of mSpry2 or mSpry4.

Treatment of T47D-EGFP cells with ALLN for 4 hours increased protein levels of mSpry2 indicating that normally the protein is degraded via the proteasomal pathway (**Figure 5-9**). As was previously demonstrated (47,76), in ALLN-treated cells,

mSpry2 showed a different electrophoretic moblilty compared to mSpry2 in untreated cells. According to previous publications, these differentially migrating bands represent variable states of serine-phosphorylation resulting in mSpry2 proteins with the apparent molecular masses of 35, 42 and 45 kDa (76). However, Caveolin-1 expression had no effect on mSpry2 levels. These results demonstrate that Caveolin-1 does not modulate mSpry2 function by interfering with the proteasomal degradation of mSpry2. Therefore, we assume, that the presence of Caveolin-1 has no effect on the interaction of mSpry2 with c-Cbl. Further, we looked at the stability of mSpry4. Surprisingly, treatment with ALLN had no effect on mSpry4 levels indicating that in contrast to mSpry2, mSpry4 is not degraded via the proteasome. This is in agreement with previous publications showing that mSpry4 does not interact with c-Cbl (28,54). Again, the presence of Cavoelin-1 had no effect on the protein levels mSpry4. Together, these data show that the turnover of mSpry isoforms is governed by different mechanisms. However, for both mSpry2 as well as for mSpry4 we could not detect differences in protein levels in the presence or absence of Caveolin-1. We conclude that Caveolin-1 does not modulate mSpry function by affecting its protein stability.

5.2.6 Tyrosine phosphorylation of mSpry2

We hypothesized that Caveolin-1 might regulate Spry activity by interfering with its tyrosine phosphorylation. Spry proteins are activated in response to growth factor stimulation by Src kinase-mediated tyrosine phosphorylation (20,23,27,28,44,53). Multiple evidence shows that tyrosine phosphorylation is important for Spry acting as an inhibitor of FGF signaling as well as for the Spry/c-Cbl interaction. T47D-EGFP and T47D-Cav-1 cells were infected with AdmSpry2 and stimulated with FGF2 for 15min. As expected, FGF2 stimulation resulted in an increase in tyrosine phosphorylation of proteins (**Figure 5-10**). mSpry2 protein was isolated from total cell lysates by immunoprecipitation using anti-mSpry2 sera which revealed a remarkable FGF2-dependent tyrosine phosphorylation of mSpry2. Immunoprecipitation using preimmune sera demonstrates that tyrosine phosphorylation is specific for mSpry2. However, results were similar in T47D-Cav-1 as compared to T47D-EGFP cells indicating that Caveolin-1 does not seem to modulate mSpry2 function by interfering with tyrosine phosphorylation.

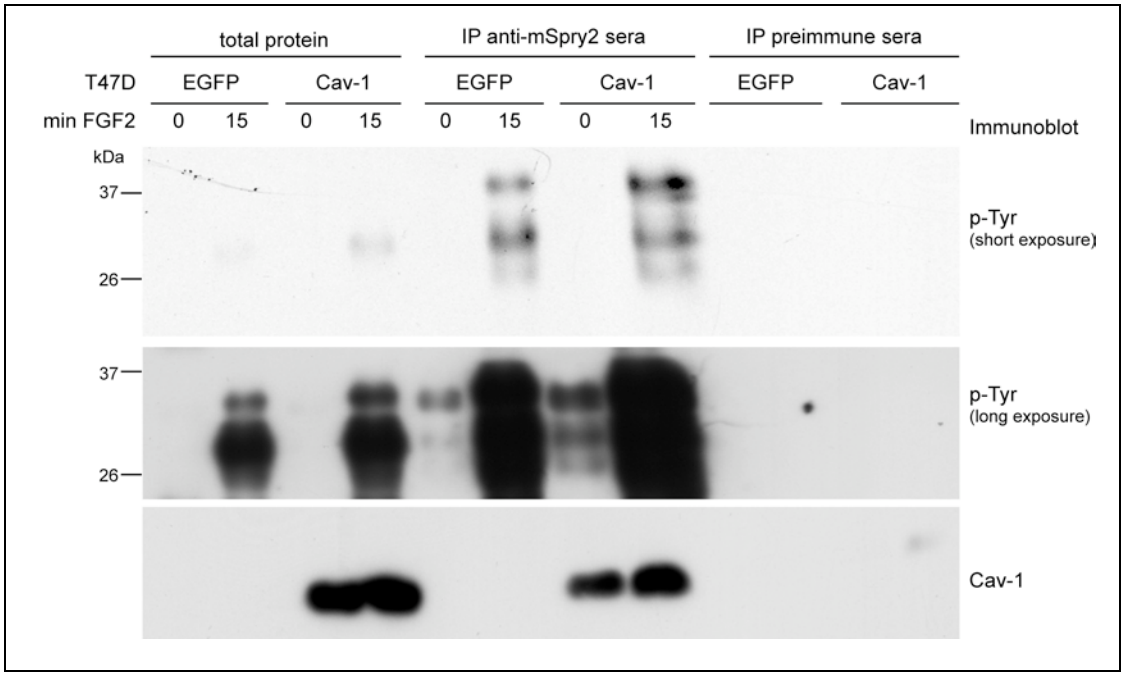

Figure 5-10 FGF2-mediated tyrosine phosphorylation is not affected by Caveolin-1
T47D-EGFP and T47D-Cav-1 cells were infected with AdmSpry2 and stimulated with FGF2 for 15 min. IP using anti-mSpry2 sera revealed FGF2-mediated tyrosine phosphorylation of mSpry2 in the absence or presence of Caveolin-1.

5.3 Discussion

5.3.1 Caveolin-1 in FGF2 and EGF signaling

Our results show that ectopic Caveolin-1 inhibits FGF2 as well as EGF signaling in densely growing cells. This is in agreement with previous publications suggesting that Caveolin-1 plays an important role in contact inhibition, moving to cell-cell contacts upon cell confluency (136). In this context, Caveolin-1 acts as an inhibitor of RTK signaling by binding to the catalytic domain of many known kinases via its scaffolding domain (113,118,125). However, when cells are growing under sparse conditions, we find that the presence of Caveolin-1 rather potentiates FGF2 and EGF signaling. Indeed, as discussed in the introduction, there is also evidence that Caveolin-1 might facilitate RTK signaling by concentrating key players of these pathways in caveolae. For example, isolation of caveolae from unstimulated cells showed that the molecular machinery that links PDGFR to p42/44 ERK activation is preorganized in caveolae of quiescent cells (138). Further, disassembly of caveolae by filipin treatment of endothelial cells prevents propagation of PDGF-induced signaling cascade (101). Also, by interfering with caveolae formation, a dominant-negative mutant of Caveolin-1 blocks H-Ras-dependent Raf activation (119).

The bimodal function of Caveolin-1 might be regulated by differential localization of Caveolin-1. In sparsely growing cells, Caveolin-1 localized in caveolae exerts a stimulatory function on RTK signaling. At cell confluency, the distribution of Caveolin-1 changes from a uniform punctate distribution over the entire cell surface and becomes localized primarily to areas of cell-cell contacts (136). There it exerts its inhibitory function on different signaling molecules to regulate cell contact inhibition. However, the effect of Caveolin-1 on RTK signaling does not differ between FGF2- and EGF-mediated p42/44 ERK activation. This similarity is not surprising since for both FGFR and EGFR most of the downstream effectors were shown to localize within caveolae and are therefore affected by the presence of Caveolin-1. Also, most of the proteins that have been shown to interact and to be inhibited by Caveolin-1 are implicated in both pathways.

5.3.2 Spry in FGF2 and EGF signaling in the absence of Caveolin-1

We demonstrate that all four Spry isoforms inhibit FGF2-mediated p42/44 ERK activation in T47D cells. mSpry2 and mSpry3 are strong inhibitors of FGF2 signaling whereas the effects of mSpry1 and mSpry4 are rather modest. Similar results showing that

mSpry2, in comparison to mSpry1 and mSpry4, causes a more profound inhibition of the Ras-p42/44 ERK pathway were previously described (51,139). It was postulated that Spry inhibits FGF2 signaling by sequestering Grb2 via the conserved tyrosine, present on all four Spry isoforms (27). However, recently, Lao and coworkers suggested that Spry binds to Grb2 via a C-terminal proline-rich sequence that is found exclusively on Spry2 (139). This implies that only Spry2 is able to inhibit FGF2 signaling by sequestration of Grb2. The inhibitory activity observed for the other isoforms might be due to an interaction with other signaling molecules like Frs2 or Sos (19,28,78).

In the context of EGF stimulation we find that in the T47D-EGFP cell line mSpry2 and mSpry3 but not mSpry1 and mSpry4 disrupt p42/44 ERK phosphorylation. It was rather surprising that none of the isoforms resulted in a potentiation of EGF signaling as previously reported (18,23,44,47,53), further indicating that the effect of Spry on EGF signaling is strongly cell- context dependent. For example, Spry1 acts as an inhibitor of the EGF pathway in C2C12 cells but has no effect on the same pathway in HeLa cells (27). It is now widely accepted that Spry potentiation of EGF signaling is mediated by Spry interaction with c-Cbl, thereby competing away c-Cbl from activated EGFR and preventing EGFR ubiquitylation and degradation (20,23,53,140). Interestingly, although all four Spry isoforms contain a putative c-Cbl-binding motif, Spry3 and Spry4 do not interfere with EGFR downregulation (53). A molecular explanation for these functional differences between Spry isoforms was provided by the finding that the Cbl-interacting protein of 85 kDa (CIN85) is necessary for complex formation of Spry and c-Cbl (57). Indeed, sequence analysis revealed that Spry1 and Spry2 but not Spry3 and Spry4 contain a CIN85-binding motif.

Another open question that remains to be investigated is, why Spry proteins have the capacity to upregulate EGF but not FGF signaling. In both systems, c-Cbl mediates receptor degradation (59,60). Also, the interaction of Sprys with c-Cbl is induced by EGF as well as by FGF stimulation of the cell (28). Differential phosphorylation of the Spry proteins in response to EGF and FGF might be possible mechanisms of regulation. For instance, it was shown that only FGF stimulation results in a significant phosphorylation of a specific tyrosine residue (Tyr 227 in mSpry2) in the C-terminus (47). Mason and coworkers revealed that each Spry family member is selectively tyrosine phosphorylated with different kinetics by a unique cohort of growth factors, suggesting that tyrosine phosphorylation not only mediates growth factor specificity but also dictates functional variability between the four isoforms (28). In brief, Spry1 is phosphorylated after FGF and PDGF stimulation, Spry2 is phosphorylated following EGF and FGF stimulation. Spry4 is not phosphorylated following

exposure to any of these growth factors. In contrast, insulin stimulation induces tyrosine phosphorylation of mSpry4 (141).

In summary, Spry proteins have an intrinsic complexity that results from their bimodal functionality, their strong cell context-dependent activity, and the large number of isoforms with differential consequences on RTK signaling. Our data demonstrate that in the context of T47D cells, Spry activity is restricted to an attenuating effect on both FGF2 and EGF signaling and that the inhibitory potential differs among the Spry isoforms.

5.3.3 Functional consequences of Spry interacting with Caveolin-1

To decipher the complexity of Spry function, it is important to find Spry-associating proteins and to reveal functional consequences of their interaction. Recently, our lab demonstrated that all four Spry isoforms interact with Caveolin-1 by direct and constitutive binding (26). Here, we investigated the effect of different Spry isoforms on FGF2 and EGF mediated p42/44 ERK activation in the absence or presence of Caveolin-1. First of all, our results demonstrate that Caveolin-1 is not necessary for Spry function but rather fine-tunes Spry activity. Thereby, this modulation differs between the four Spry isoforms, adding another level of complexity to Spry function.

Comparing the consequences of Spry/Caveolin-1 interaction on FGF2 signaling we repeatedly observe, under dense and sparse growing conditions, the same pattern: mSpry1 and mSpry3 inhibit FGF2 signaling to comparable extents in the presence or absence of Caveolin-1. In contrast, in T47D-Cav-1 cells, the presence of mSpry2 and mSpry4 does not decrease the levels in p42/44 ERK activation when compared to control infected T47D-Cav-1 cells. For EGF signaling, we observe an inhibition by mSpry2 and mSpry3. Similar to the FGF2 pathway, mSpry3 acts as a cooperative inhibitor with Caveolin-1, whereas mSpry2 has no additive effect on EGF signaling in T47D-Cav-1 cells compared to control infected cells. From these results we assume that Caveolin-1 abrogates the inhibitory function of mSpry2 and mSpry4 in EGF and FGF2 signaling.

What are the mechanisms that underlie the Caveolin-1 mediated modulation of Spry function? Here we report that Spry stability as well as tyrosine phosphorylation in response to FGF2 stimulation are not affected by the presence of Caveolin-1. Similarly, there is evidence implying that Caveolin-1 is not modulating Spry function by localizing it to the membrane; recently, we have shown that mutation of a conserved C-terminal arginine (residue 252 in hSpry2) impairs Spry/Caveolin-1 interaction but Spry does not affect Spry translocation to the cell membrane in response to growth factor stimulation (26). Therefore,

further investigations are necessary to reveal the mechanism of how Caveolin-1 modulates Spry function. For example, the interaction with Caveolin-1 could influence Spry association with other proteins like Grb2 or Frs2, thereby interfering with the inhibitory potential of the Spry isoforms.

Here we demonstrate that in the context of FGF2 and EGF signaling mSpry2 and mSpry4 do not cooperate with the inhibitory activity of Caveolin-1. In contrast, mSpry1 and mSpry3 attenuate FGF- or EGF-mediated p42/44 ERK phosphorylation to similar extents in T47D-EGFP and T47D-Cav-1 cells. This suggests that Caveolin-1 is one of the factors implicated in the divergence of the different isoforms. Also, being ubiquitously, yet variably expressed, Caveolin-1 might play an important role in the cell-context specificity of Spry proteins. In conclusion, cells with a highly variable repertoire of Spry and Caveolin-1 expression have the possibility to specifically tailor RTK signaling for an appropriate physiological outcome.

6 mSpry4 in the developing pancreas

6.1 Introduction

6.1.1 Expression of Spry isoforms in mice

In adult tissues, Spry1, Spry2 and Spry4 are widely expressed (14,16,19), whereas Spry3 expression is restricted to brain and testis (16,40). However, the functional consequences of Spry activity and regulation of Spry expression in adult tissues have not been extensively characterized. During development and organogenesis the expression pattern of Spry strongly overlaps with known sites of RTK signaling (16). For example, in the developing zebrafish, Spry4 expression colocalizes with and is dependent on FGF8 and FGF3 signaling where it antagonizes pathways activated by FGFR1 (142,143). In mammals, embryonic tissues, such as brain, heart, gut, lung, and muscle show a close spatial and temporal interdependence between FGF signaling and Spry gene expression (16,144). Detailed analysis of mSpry expression during mouse embryogenesis revealed that mSpry1, mSpry2 and mSpry4 although often co-expressed have a distinct expression pattern. Their presence in both epithelial and mesenchymal tissues suggests a role in the epithleial-mesenchymal interactions that govern organogenesis (145).

6.1.2 In vivo studies of Spry isoforms

FGF pathways are implicated in the development of many branched organs (146-149). For example, in *Drosophila,* branchless (Bnl) which is a homolog of mammalian FGF is indispensable to development of the tracheal system (150). Spry was identified in *Drosophila* by its ability to act as an inhibitor of Bnl signaling limiting its activity to the presumptive tip cell (10). As a consequence, excessive secondary branching of the tracheal system is inhibited. dSpry expression is induced by the Bnl pathway. Thus, Bnl and dSpry comprise a negative feedback circuit that is finely tuned to allow only short range signaling by Bnl.

The respiratory system of vertebrates shows highly divergent morphology compared to the tracheal system in *Drosophila*. However, some aspects of the molecular mechanisms underlying trachea formation appear to be conserved among vertebrates and invertebrates (for review see (151)). In parallel to the Bnl function in tracheal development, FGF10 plays a crucial role for lung development in mammals (152). Thereby FGF10 is

produced by the peripheral mesenchymal cells and acts on distal bud epithelium as a chemoattractant (152,153). Spry1 and Spry2 are expressed in epithelial and in mesenchymal cells of the developing lung (154). They were shown to have a pivotal role regulating branching morphogenesis by acting as a feedback inhibitors of FGF signaling (19,84,144,154,155).

It is becoming more and more evident that Spry is an important regulator of branching morphogenesis in many different developing organs by controlling bidirectional signaling between different tissues like the mesenchyme and the epithelium; (i) Spry2 is involved in the coordination of GDNF and FGF mediated signaling between mesenchyme and epithelium that controls ureteric bud initiation during kidney development (38,156), (ii) in tooth development, Spry2 and Spry4 restrict mesenchymal-epithlial FGF signaling and thereby prevent formation of teeth in a region that is normally toothless (157), (iii) Spry2 is involved in male sex organogenesis controlling FGF-mediated mesonephric cell migration (158), (iv) Spry2 modulates villous branching in the placenta (159), (v) ectopic Spry4 expression inhibits angiogenesis in mice (39), and (vi) the differential expression pattern of Spry1 and Spry2 in various stages of breast development suggest a regulatory potential of the proteins on branching of epithelial ducts (87).

Further, it was shown that Spry2 is a negative regulator of GDNF in the development of enteric nerve cells (40,42). Also, Spry2 regulates FGF-dependent cell fate transformation in the auditory epithelium, and when ablated results in hearing loss (41,46). Further, in chicken, constitutive expression of Spry2 and Spry4 results in the repression of FGF-mediated limb development (14,16). Together these *in vivo* studies demonstrate that Spry proteins are implicated in the development of many different organs by interfering with various signaling pathways.

6.1.3 Pancreas development

Development of the pancreas initiates in the anterior midgut region of the endoderm epithelium. As development proceeds, endocrine precursor cells delaminate from the pancreatic epithelium, migrate into the surrounding mesenchyme and aggregate into islets of Langerhans; a process that requires cell sorting, cell migration and cell reaggregation (160,161). However, it is not until several weeks after birth that the pancreas fully matures and islets of Langerhans are definitively established with their distinctive cellular architecture: non β cells (α cells producing glucagon, δ cells producing somatostatin, and PP cells producing pancreatic polypeptide) in the periphery and β cells secreting insulin

in the center. Studies of pancreas development in mice and rats have revealed an important role for FGF signaling in the development of the pancreas (142,147,162-166). In adult mice, FGFR1 and 2 together with the ligands FGF1, FGF2, FGF4, FGF5, FGF7 and FGF10 are exclusively expressed in β cells of the endocrine pancreas, indicating that FGF signaling may also have a role in differentiated β cells. Indeed, expression of dnFGFR1 in β cells affects the organization of endocrine cells within the islets of Langerhans, reduces the number of β cells and disturbs glucose homeostasis (163). Considering the importance of FGF signaling in pancreatic development we assumed that Spry proteins as modulators of the pathway, might be implicated in organogenesis of the pancreas.

6.2 Results

6.2.1 mSpry4 is endogenously expressed in α cells of the murine pancreas

We aimed to investigate a possible role of Spry in the development of the murine pancreas. To determine if endogenous mSpry proteins are found in the murine pancreas, sections from pancreata of adult C57Bl/6 mice were stained for all four mSpry isoforms with the corresponding anti-mSpry sera. No expression was detectable for mSpry1, mSpry2 or mSpry3 (data not shown). In contrast, we found endogenous mSpry4 in a subset of cells in the islets of Langerhans (**Figure 6-1 A**). Stainings using preimmune sera as a negative control confirmed that the signal for mSpry4 protein is specific (**Figure 6-1 B**). The localization of these mSpry4-positive cells in the outer rim of the islet was reminiscent of the localization of α cells. Immunofluorescence co-staining using antibodies against glucagon as a marker for α cells and anti-mSpry4 sera confirmed that endogenous mSpry4 was exclusively expressed in the α cells of the islets of Langerhans (**Figure 6-1 C-E**).

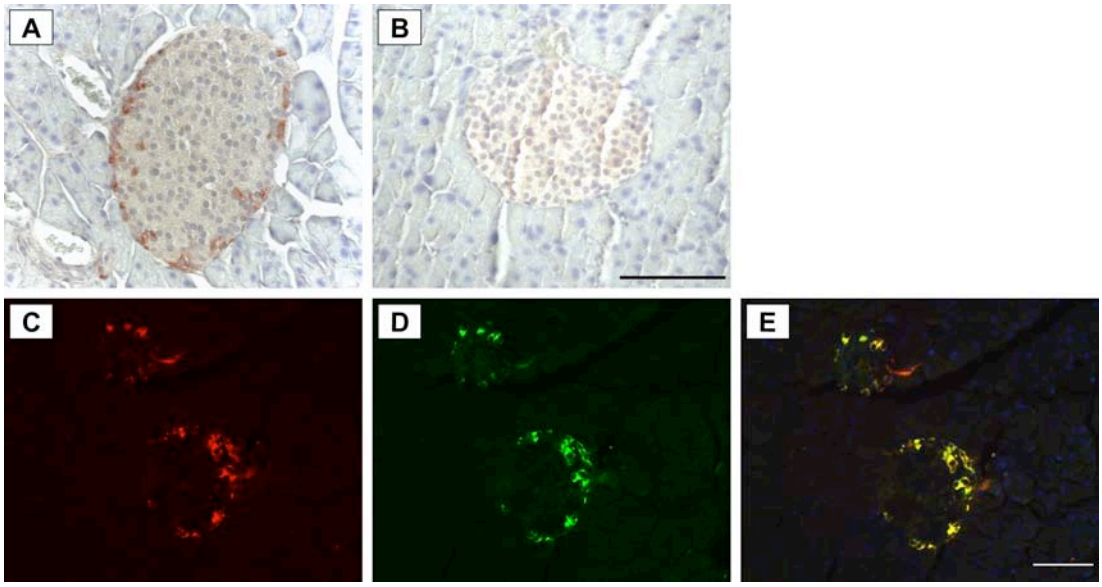

Figure 6-1. Endogenous mSpry4 in α cells of pancreata of adult C57Bl/6 mice.
Expression of mSpry4 was investigated in paraffin sections of pancreata of adult C57Bl/6 mice by immunohistochemical staining using anti-mSpry4 sera (A, brown) or preimmune sera as a control (B). Immunofluorescence staining for the α cell marker glucagon (C) and mSpry4 (D) revealed that mSpry4 and glucagon are co-expressed in α cells of the islets of Langerhans (E). Scale bars: 100μm

6.2.2 Ectopic expression of mSpry4 in β cells perturbs the organization of the islets of Langerhans

To address the question if this exclusive expression of endogenous mSpry4 in α cells plays a role in islet morphology, mSpry4 was ectopically expressed in β cells. Transgenic mice having mSpry4 under the control of the doxycycline-responsive element promoter ($(tetO)_7$mSpry4) (84) were crossed to transgenic mice in which the rat insulin promoter (Rip1) controls synthesis of the reverse doxycycline transactivator (Rip1rtTA). Ectopic mSpry4 protein was readily detected in β cells of double-transgenic Rip1rtTA;$(tetO)_7$mSpry4 mice treated with doxycycline for two weeks (**Figure 6-2 A**). In single transgenic littermates, endogenous mSpry4 expression in α but not in β cells as previously shown for nontransgenic C57Bl/6 mice was observed (**Figure 6-2** and data not shown). Surprisingly, double transgenic Rip1rtTA; $(tetO)_7$mSpry4 mice that were not treated with doxycycline still exhibited weak expression of mSpry4 in β cells demonstrating the leakiness of the doxycycline-inducible promoter (**Figure 6-2 B**).

Development of the endocrine pancreas starts when pancreatic precursor cells in the duct epithelium invade the surrounding mesenchyme (167,168). At around E17.5 the first islets are found in their final cellular orientation, *i.e.*, β cells in the center and non-β cells in the periphery (169,170). To study the effects of ectopic mSpry4 expression on developing pancreata, pregnant females were treated with doxycycline to induce mSpry4 production in β cells of embryos.The inducibility of the transgene was analyzed in pancreata of embryos at E19.5, using immunofluorescence co-stainings for mSpry4 and for the β cell marker insulin. In single-transgenic control animals we observed cells expressing endogenous mSpry4 (**Figure 6-2 C**) surrounding insulin-positive β cells (**Figure 6-2 D, merge E**). In agreement with the staining pattern of mSpry4 we observed in adult C57Bl/6 mice (**Figure 6-1**), co-staining for mSpry4 and glucagon confirmed that these cells represent the α cells of the developing pancreas (data not shown). In pancreata of double-transgenic animals, the number of cells positive for mSpry4 was clearly increased (**Figure 6-2 F**). However, there were less insulin-positive cells than expected and insulin expression levels were decreased compared to control littermates (**Figure 6-2 G**). In fact, only a small number of β cells co-expressed mSpry4 and insulin, and within these, insulin-staining levels were weaker (**Figure 6-2 H-K arrow**). From these results we conclude that the expression of the transgene can be induced during embryogenesis.

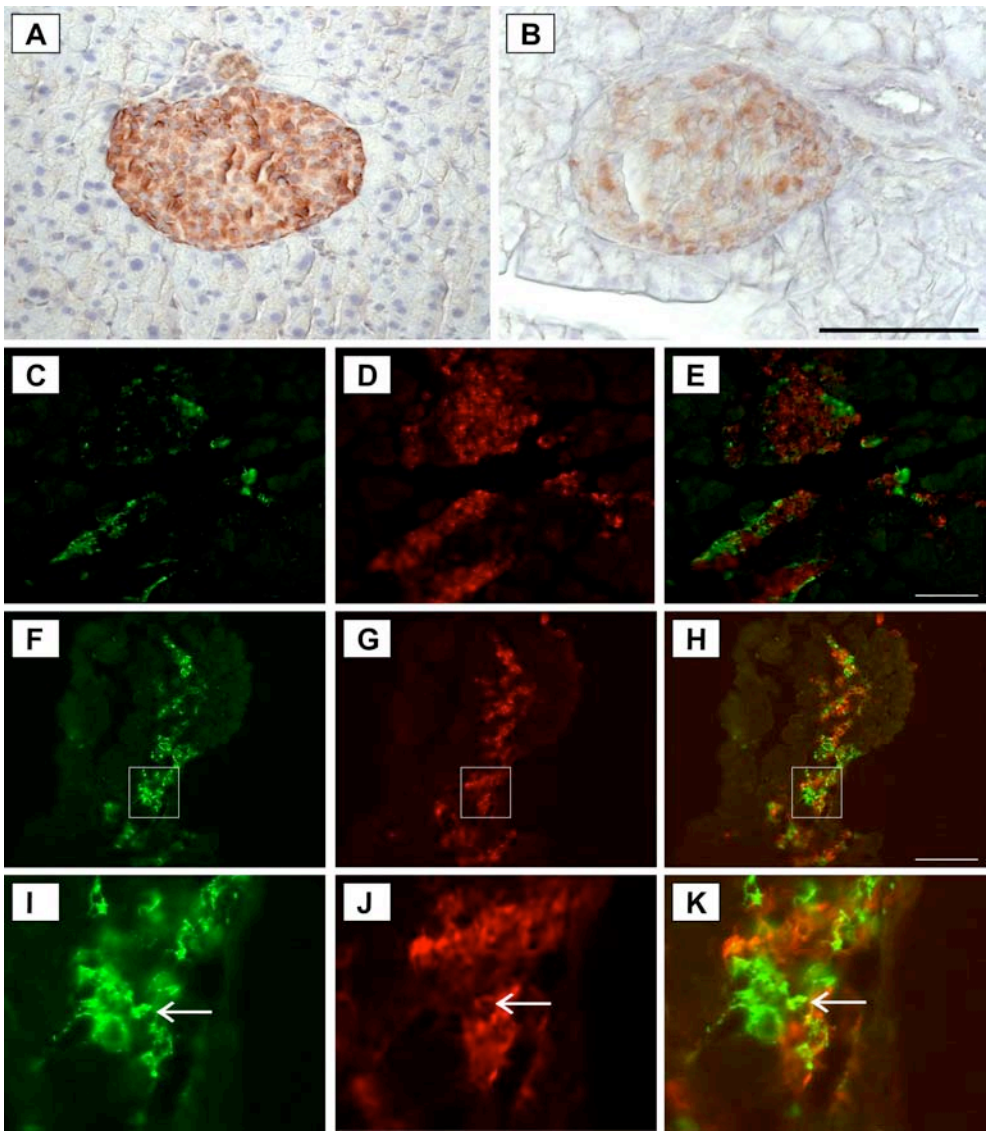

Figure 6-2. Transgenic mSpry4 expression in β cells.
Pancreata from adult Rip1rtTA;(tetO)$_7$mSpry4 mice, treated with doxycycline for 2 weeks (A) or left unreated (B) were analyzed by immunohistochemical staining on paraffin sections using anti-mSpry4 sera (A-B). Pregnant females were treated with doxycycline from day of conception to induce mSpry4 expression in developing pancreata of single or non-transgenic control offsprings (C-E) and Rip1rtTA;(tetO)$_7$mSpry4 littermates (F-H). Pancreata of embryos at E19.5 were analyzed by immunofluorescent co-staining using anti-mSpry4 sera (C,F green) and antibodies against the β cell marker insulin (D,G, red). Merge pictures (E,H). I-K: Increased magnification of areas indicated in F-H. Arrows indicate cells expressing mSpry4 and low levels of insulin. Scale bars: 100μm

The lack of co-stainings of mSpry4 and insulin suggested that mSpry4 in β cells has an inhibitory effect on insulin expression and/or influences β cell identity. To test this hypothesis, we co-stained for insulin and glucagon to analyze β and α cell distribution in the developing pancreas at E19.5 of Rip1rtTA; $(tetO)_7$mSpry4 embryos and of single transgenic control littermates. In control embryos a large part of the endocrine pancreas was already organized into normal islets of Langerhans with glucagon-expressing α cells surrounding insulin-positive β cells (**Figure 6-2 A**). Such well structured islets were not observed in Rip1rtTA; $(tetO)_7$mSpry4 mice (**Figure 6-2 B**). Furthermore, the average islet size in the double transgenic embryos was significantly reduced compared to control littermates, whereas the number of α cells per islet area was increased (**Table 6-1**). These results indicate that the expression of mSpry4 in β cells during development dramatically changes islet morphology.

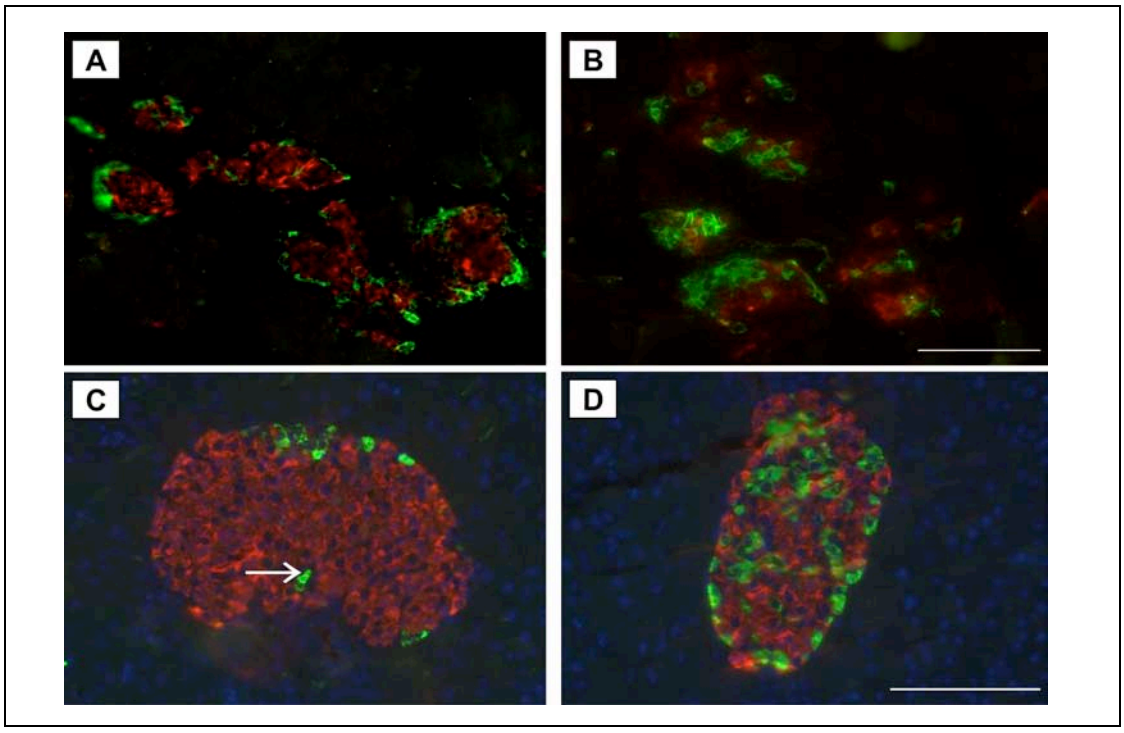

Figure 6-3. Cell sorting phenotype in pancreatic islets expressing ectopic mSpry4 in β cells.
Islet architecture was analyzed in pancreata of embryos at E19.5 (A, B) and of 4 week old mice (C,D) using immunofluorescent co-staining with antibodies against glucagon (green) and insulin (red). Single or non-transgenic control mice (A, C) and Rip1rtTA;tetOPmSpry4 littermates (B, D) were treated with doxycycline throughout development. C, arrow: a cell localized centrally to the two most peripheral cell layers. Scale bars: 100mm

Next, I investigated if this difference in islet architecture is still present at later stages of pancreas development. Therefore, analysis of islet morphology was performed in pancreata of older animals: In 2 week old mice (PN 14) the process of cell segregation is still ongoing whereas in 4 week old pups (PN 28) islet formation should be complete (170). Pregnant females were treated with doxycycline from day of conception to induce mSpry4 expression throughout development in Rip1rtTA; $(tetO)_7$mSpry4 progeny. Single transgenic littermates were again used as controls. In agreement with the results of E19.5 pancreata, a significant reduction in islet size in mSpry4-expressing embryos was observed at the age of two weeks (**Table 6-1**). The difference between Rip1rtTA; $(tetO)_7$mSpry4 and control littermates was also present at the age of 4 weeks but it was not significant. The proportion of α cells per islet area was significantly increased at both timepoints. To determine whether mSpry4 has any influence on islet cell type segregation I quantified the number of missorted α cells, *i.e.*, α cells that are localized centrally to the two most peripheral cell layers of the islet. Missorted α cells can be found in islets of 2 week old mice and to a lesser extent also in those of 4 week old control mice (**Figure 6-3 C arrow, Table 6-1**). However, in both experimental groups analyzed the number of missorted α cells was significantly increased in Rip1rtTA; $(tetO)_7$mSpry4 mice (**Figure 6-3 D**, **Table 6-1**). From these data I conclude that the presence of mSpry4 in β cells does not result in a delay of pancreas development but in aberrant islet formation that is not rescued in adult pancreata.

Table 6-1 Disturbed islet architecture by mSpry4 expression in β cells

age Dox treatment	**E19.5** E_0 - $E_{19.5}$		**2 weeks** E_0 - PN_{14}		**4 weeks** E_0 - PN_{28}	
	ctrl [a]	**mSpry4** [b]	**ctrl** [a]	**mSpry4** [b]	**ctrl** [a]	**mSpry4** [b]
islet area (μm^2)	3924 ± 316	2930 ± 267	6636 ± 503	4731 ± 412	9766 ± 1042	7617 ± 718
p value[c]	0.0004		0.0185		0.0788	
α cells per area (per 1000μm^2)	3.91 ± 0.19	5.1 ± 0.31	5.48 ± 0.26	6.97 ± 0.39	2.76 ± 0.25	4.22 ± 0.57
p value[c]	0.0053		0.0037		0.0151	
missorted α cells / islet area (1000μm^2)	N/A		1.5 ± 0.13	2.94 ± 0.26	0.86 ± 0.12	1.92 ± 0.3
p value[c]			0.0001		0.0003	
	n=121 N=9	n=132 N=13	n=117 N=6	n=64 N=7	n=54 N=6	n=64 N=12

a: Rip1rtTA or $(tetO)_7$mSpry4
b: Rip1rtTA;$(tetO)_7$mSpry4
c: p value of mSpry4 vs ctrl , analyzed by a two-tailed Mann-Whitney test
Values represent mean±SD
n=number of islets, N=number of mice
N/A=not applicable

Next, I assessed whether this phenotype we observed in Rip1rtTA; $(tetO)_7$mSpry4 mice could be initiated by inducing mSpry4 expression later in pancreas development. Therefore, mice were treated with doxycycline only after birth and then the animals were sacrificed at 4 weeks. Islet architecture in these mice was comparable to 4 week old Rip1rtTA;$(tetO)_7$mSpry4 mice treated with doxycycline throughout development, namely, a significant increase in the amount of total α cells and in the number of missorted α cells (data not shown). This suggested that expression of mSpry4 in β cells not only interfered with islet formation but also had an effect on maintenance of islet organization in established islets. However, it is possible that due to the leakiness of the doxycycline-inducible promoter mentioned above (see **Figure 6-2 B**), Rip1rtTA;$(tetO)_7$mSpry4 mice weakly expressed transgenic mSpry4 throughout development. Low levels of mSpry4 during embryogenesis could be responsible for this phenotype in islet architecture. Together, these results demonstrate that transgenic expression of mSpry4 in β cells strongly affects islet formation during pancreas development.

6.2.3 mSpry4 inhibits migration and adhesion of PANC-1 cells without affecting RTK mediated p42/44 ERK activation

Human pancreatic PANC-1 cells have been reported to be a useful model to study early stages of islet formation (171-173). When grown in normal serum-containing medium, PANC-1 cells form an epitheloid, adherent monolayer. However, in defined serum-free media (SFM) the cells start to form islet-like cell aggregates that differentiate into cells expressing markers of the endocrine pancreas, *i.e.* insulin, glucagon or somatostatin (171). This process is strongly dependent on FGF signaling, whereby FGF2 expressed by PANC-1 cells, serves as a chemoattractant to induce migration and aggregation of these cells into islet-like cell aggregates (171). Thus, we considered PANC-1 cells to be suitable to assess mSpry4 function on islet formation on a cellular level. PANC-1 cells were infected with adenovirus encoding mSpry4 (AdmSpry4) or as a control luciferase (AdLite). Adenoviral-driven expression of mSpry4 was confirmed by Western blot (data not shown).

To assess whether mSpry4 can interfere with cell migration of PANC-1 cells, transwell migration assays were performed. In SFM, PANC-1 cells did not migrate (data not shown). The presence of 10% FCS in the media induced migration of PANC-1 cells which was significantly inhibited by the expression of mSpry4 (**Figure 6-4**). Next, we assessed the inhibitory effect of mSpry4 on directed cell movement, *i.e.* migration of cells towards chemokines. In this case, only media of the lower chamber was supplemented with either 10% FCS or with 100 ng/ml FGF2 respectively. As previously reported, FGF2 in the lower chamber serves as a strong chemoattractant for PANC-1 cells (171). The inhibitory effect of mSpry4 on FCS- and FGF2-driven directed cell migration was comparable to what we observed for undirected movement in serum-containing media (**Figure 6-4**).

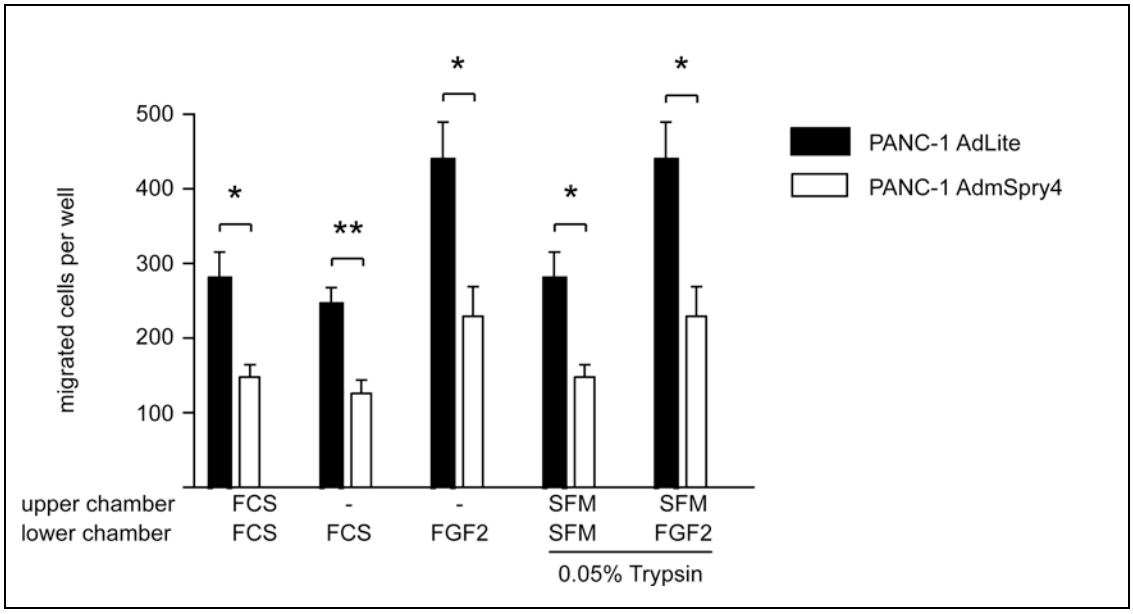

Figure 6-4. mSpry4 inhibits migration of PANC-1 cells.
*PANC-1 cells were infected with AdmSpry4 or control virus (AdLite) and seeded on 8µm pore transwell. Media in upper and/or lower chamber was supplemented with 10% FCS or 100 ng/ml FGF2. To use trypsinization as a stimulus for migration, cells were allowed to adhere for 3 hours to the transwell membrane and then exposed for 1min to low levels of trypsin followed by incubated for another 4h in serum free medium (SFM) alone or with 100ng/ml FGF2 in the lower chamber. Mean ± standard deviation are representative of four replicates. Graph shown is representative of 2 independent experiments. Statistical analysis: student's t test, two-tailed (* p value <0.05; ** p value <0.01).*

In their work, Hardikar and co-workers showed that the formation of islet-like cell aggregates was initiated by brief exposure of PANC-1 cells to low concentrations of trypsin in order to loosen the cells from their substratum followed by incubation in SFM (171). The authors postulated that PANC-1 cells secreting FGF2 cluster together by paracine chemotaxis (171). To test if mSpry4 could inhibit this paracrine chemotactic movement, migration of PANC-1 cells was assessed in SFM after trypsinization. Again, the capacity of PANC-1 cells to migrate under these conditions was significantly reduced by the presence of mSpry4 (**Figure 6-4**). These data imply that mSpry4 is a general inhibitor of PANC-1 movement that does not discriminate between variable triggers nor between chemokinesis (nondirectional / random movement) and chemotaxis.

Cell-cell and cell-matrix adhesion also plays an important role in cell type segregation (173) and thus may affect the formation of islets of Langerhans. Mice that are deficient for the cell adhesion molecules N-CAM or β_1-integrin show a phenotype in islet formation that is very similar to the phenotype we observed in mice overexpressing mSpry4 in β cells ((169); Kren et al, unpublished data). Therefore, we analyzed the adhesion

properties of PANC-1 cells infected either with AdmSpry4 or AdLite to uncoated versus collagen IV-coated tissue culture plates. Indeed, the expression of mSpry4 strongly inhibited adhesion of PANC-1 cells to both substrates (**Figure 6-5**).

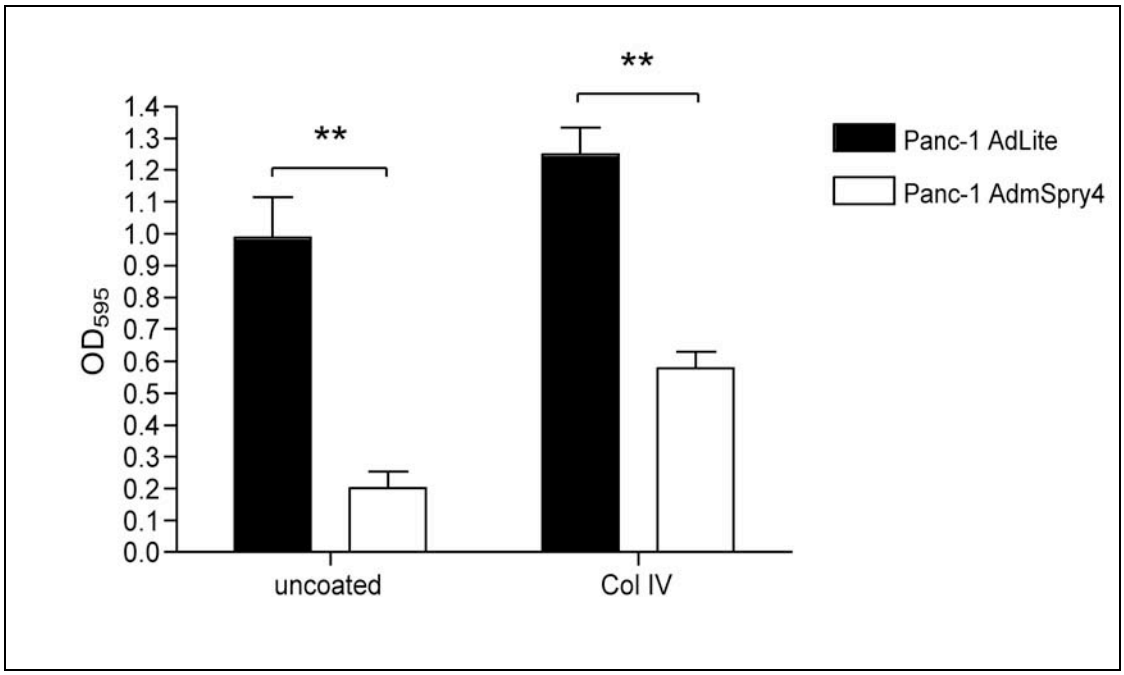

Figure 6-5. mSpry4 inhibits adhesion of PANC-1 cells.
*PANC-1 cells were infected with AdmSpry4 or with control virus (AdLite) and seeded on collagen IV or on uncoated tissue culture dishes. After 2.5 hours, unattached cells were washed off with PBS. Attached cells were fixed, stained with crystal violet, lysed and absorbance was measured at OD_{595}. Mean ± standard deviation are representative of four replicates. Graph shown is representative of 2 independent experiments. Statistical analysis: Student's t test, two-tailed (** p value <0.01).*

Next, we checked whether the inhibitory effects of mSpry4 on migration and adhesion are achieved by attenuation of p42/44 ERK activation by different RTKs. PANC-1 cells were again infected with AdmSpry4 or AdLite, starved overnight and stimulated with different growth factors. Surprisingly, mSpry4 had no effect on pp42/44 ERK levels for any of the activated signaling pathways tested (**Figure 6-6**). These data suggest that mSpry4 inhibits adhesion and migration of PANC-1 cells by a mechanism independent of the RTK signaling pathway.

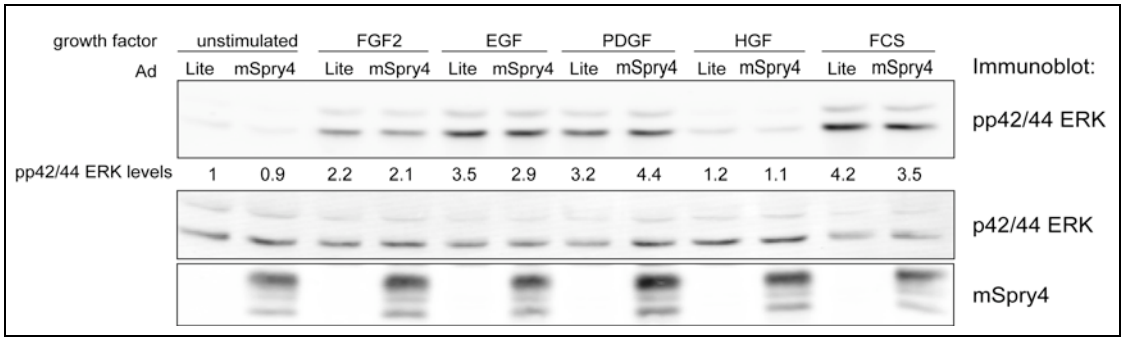

Figure 6-6. mSpry4 does not affect signaling by different RTK in PANC-1 cells.
PANC-1 cells were infected with AdmSpry4 or control virus (AdLite), starved overnight and then stimulated for 30 min with the growth factors indicated or incubated in media supplemented with 10% FCS. Lysates were resolved by SDS-PAGE and resulting immunoblots were sequentially probed with the indicated antibodies. The immunoblot shown is representative of 3 independent experiments.

6.2.4 mSpry4 expression interferes with the localization of PTP1B

Spry proteins have also been reported to be inhibitors of cell migration (24,61,174). Protein-tyrosine phosphatase-1B (PTP1B), a protein involved in integrin signaling (62,64), is a mediator of the anti-migratory actions of Spry2 (61). Therefore, PTP1B levels were analyzed in lysates of PANC-1 cells infected with AdmSpry4 or AdLite. Expression of mSpry4 in these cells led to an increase in the levels of PTP1B in the soluble fraction of cell lysates (**Figure 6-7**). Concomitantly, in the Triton X-100 insoluble cell fraction, PTP1B levels were reduced when mSpry4 is present, suggesting that mSpry4 interferes with the cellular localization of PTP1B.

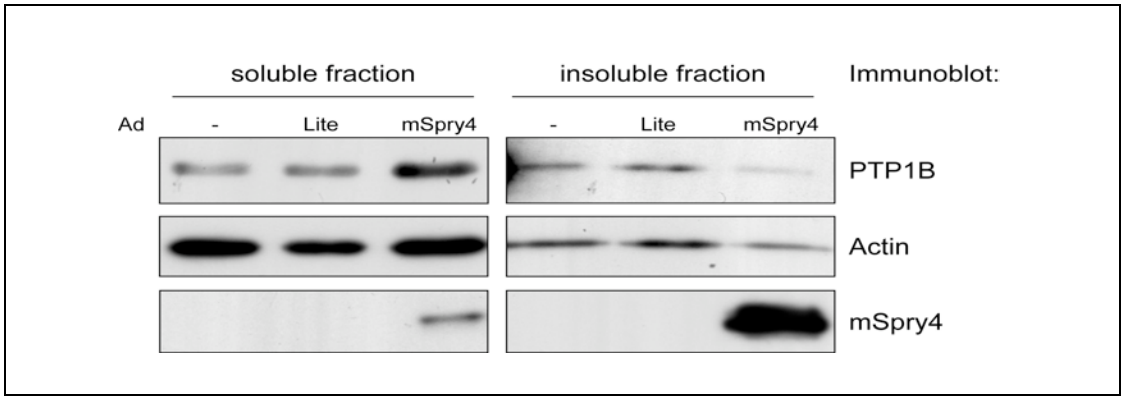

Figure 6-7. mSpry4 influences the compartmentalization of PTP1B in PANC-1 cells.
Lysates of PANC-1 cells infected with control virus (AdLite) or with AdmSpry4. Lysates were resolved by SDS-PAGE and resulting immunoblots were sequentially probed with the indicated antibodies.

To further characterize PTP1B subcellular localization, we performed immunofluorescence stainings of cells grown on uncoated coverslips or on coverslips coated with collagen IV. The staining pattern for PTP1B in PANC-1 cells infected with AdLite was very similar to what was observed previously (175,176), namely a localization typical for proteins that are associated with the endoplasmatic reticulum (**Figure 6-8 A**).

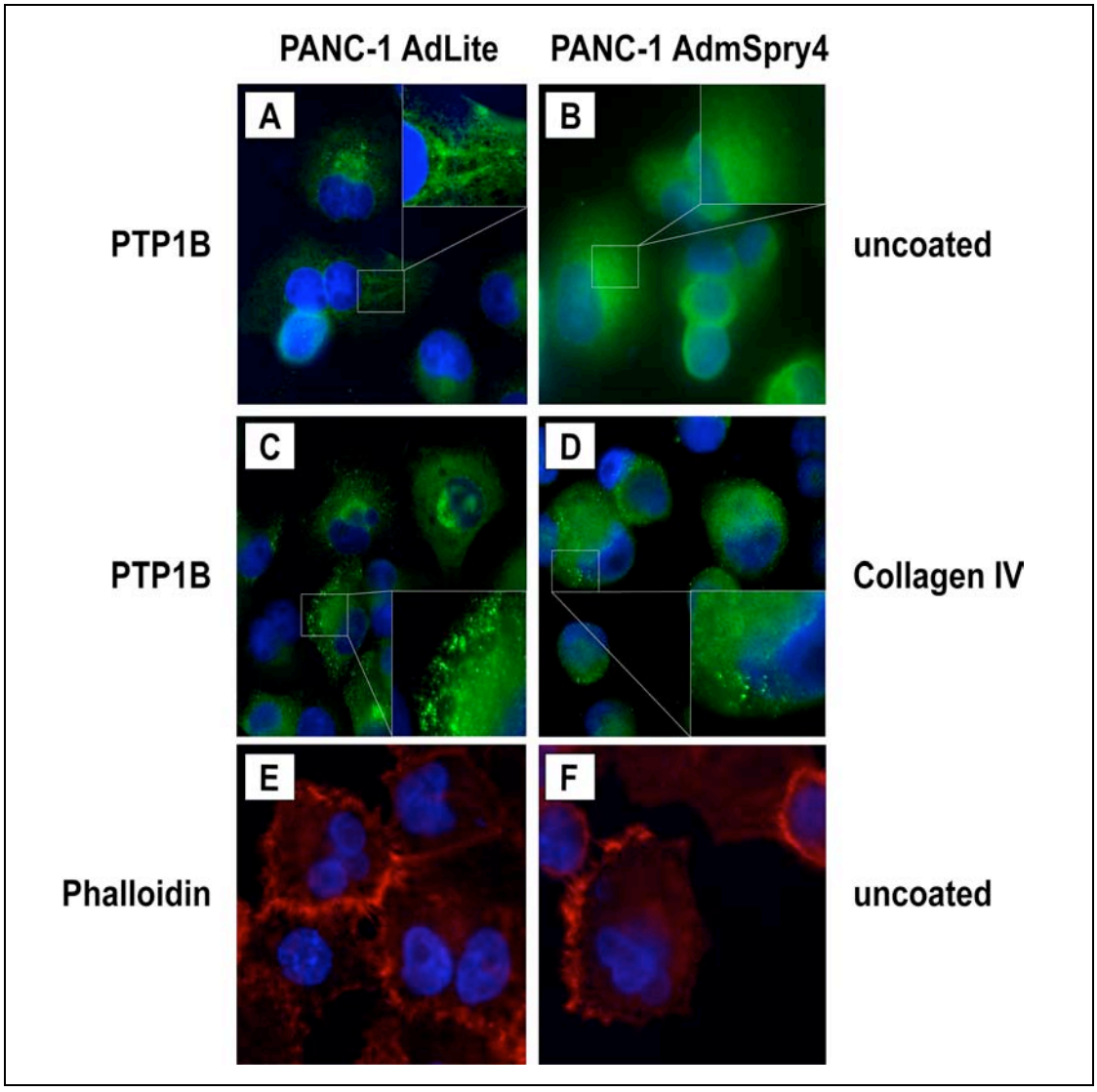

Figure 6-8. Changes of PTP1B localization in PANC-1 cells by the expression of mSpry4. *PANC-1 cells were infected with control virus AdLite (A,C,E) or with AdmSpry4 (B,D,F) and grown on uncoated glass coverslips (A-B, E-F) or on coverslips coated with collagen IV (C-D). The localization of PTP1B was analyzed by immunofluorescence staining using anti-PTP1B antibody (A-D). The actin cytoskeleton was visulaized using phalloidin staining (E, F).*

In contrast, in mSpry4-expressing cells PTP1B was distributed throughout the cytosol (**Figure 6-8 B**). When grown on collagen IV, control cells showed a concentration of PTP1B at punctate structures in the margins of the cells (**Figure 6-8 C**), wich were previously identified as cell matrix adhesion sites using confocal microscopy and the markers vinculin and paxillin (176). Some PTP1B-containing cell matrix adhesion sites were also found in AdmSpry4-infected cells, however, the expression of mSpry4 mostly perturbed the localization of PTP1B (**Figure 6-8 D**). To exclude the possibility that the effect of mSpry4 on PTP1B localization was due to general changes in cytoskeletal architecture, actin was stained using phalloidin. No apparent changes in cell organization were found in mSpry4-expressing cells compared to control cells. (**Figure 6-8 E, F**).

Here I showed that mSpry4 was able to attenuate migration and adhesion of PANC-1 cells independently of p42/44 ERK activation. These results and previous publications suggest that mSpry4 perturbs the correct localization of PTP1B, thereby interfering with integrin signaling (24,174). Applying the PANC-1 cell system as a model for islet formation, we hypothesize that this inhibitory function of mSpry might play an important role in migration and sorting of endocrine cells during pancreas development *in vivo*.

6.3 Discussion

In this section of the thesis I investigate the impact of transgenic mSpry4 expression in β cells during pancreas development. I show that endogenous mSpry4 is exclusively expressed in α cells of the developing as well as of the adult pancreas. Perturbing this specific pattern by inducing transgenic mSpry4 expression in β cells of the islets of Langerhans I observe a severe phenotype in islet morphology namely, smaller islets, a higher number of total α cells per islet area and defect in α and β cell sorting (**Figure 6-3**, **Table 6-1**). The phenotype caused by mSpry4 misexpression may be explained in several ways. mSpry4 could be implicated in cell fate decisions by inhibiting β cell and favoring α cell differentiation. The expression of dominant negative FGFR1 (dnFGFR1) in β cells results in a net loss of insulin-positive cells without increasing β-cell apoptosis (163). Hart *et al*. propose an autocrine loop of FGF signaling that might be involved in terminal differentiation and/or maturation of β cells. Our stainings of pancreata of Rip1rtTA; $(tetO)_7$mSpry4 mice reveal that the mSpry4 transgene results in lower or abrogated insulin levels (**Figure 6-2**). We hypothesize that mSpry4 might interfere with FGF-mediated β cell maturation. However, in dnFGFR1 neonates neither islet organization nor the number of α cells are affected, indicating that inhibition of FGFR1 signaling is not sufficient to explain the phenotype observed in Rip1rtTA; $(tetO)_7$mSpry4 embryos and newborns.

Also, mSpry4 could affect migration and aggregation of endocrine cells during development. Induction of precursor cell migration via FGFRs has been suggested to occur during pancreas development in rats (162,165) where mSpry4 might act as an inhibitor of FGFR-mediated ERK activation. However, our experiments with PANC-1 cells suggest that mSpry4 inhibits cell movement induced by various stimuli and with a mechanism that is independent of the p42/44 ERK pathway (**Figure 6-4**, **Figure 6-6**). It was reported that Spry proteins are able to affect cell migration by inhibiting the activation of the small GTPase Rac1 (39,158,174). Yigzaw *et al*. proposed that mSpry2 expression leads to a relocalization and thereby to an enhanced activation of PTP1B which in turn dephosphorylates and inhibits the Rac1 activator p130Cas (61). In PANC-1 cells, I observe a shift in PTP1B localization from structures resembling the endoplasmatic reticulum and focal adhesion sites to the cytosol in the presence of mSpry4 (**Figure 6-8**). PTP1B inhibits cell migration and adhesion by interfering with integrin signaling but conflicting models still exist for the exact mechanism of PTP1B function (62-64). However, it is interesting to note that mice lacking the β_1 integrin gene in β cells show a phenotype in islet formation that is very similar to what

I describe in this report (Kren *et al*. unpublished data), suggesting that integrin signaling plays an important role in cell segregation during islet formation.

A similar cell sorting phenotype of the endocrine pancreas was previously described in mice lacking the cell adhesion molecule N-CAM (169). In addition, in pancreata of rats, N-CAM shows a local distribution within the islet that is similar to the one I see for mSpry4, *i.e*. high expression in the α cells whereas in β cells the levels of N-CAM are very low (177). It has been shown that differences in cell adhesion properties in two motile cell types can serve as a driving force for segregation and sorting of the cells and less cohesive cells surround a core of more cohesive cells (173). Cirulli and co-workers suggest that the differential expression of N-CAM might make α cells less cohesive than β cells, resulting in their peripheral distribution (177). Here, I demonstrate that mSpry4 inhibits adhesion of cells (**Figure 6-5**). If mSpry4 can affect adhesion properties of cells, the expression of endogenous mSpry4 in α cells might establish a difference in cohesiveness of α versus β cells and thereby has a crucial role in cell segregation and maintenance of islet organization.

However, recently our group reported that N-CAM associates with FGFR to induce intracellular pathways that result in β_1-integrin-mediated cell-matrix adhesion (91). Considering the fact that mice lacking either N-CAM or β_1-integrin and mice ectopically expressing mSpry4 in pancreatic β cells all show a very similar phenotype in endocrine cell sorting I propose the following model: N-CAM modulates adhesion properties of β cells by inducing FGFR signaling important for correct segregation of endocrine cells during pancreas development. Ectopic expression of mSpry4 in β cells attenuates this N-CAM/FGFR signaling pathway and thereby interferes with adhesion and segregation properties of the cells.

Homologous cell contact of β cells influences insulin production (178,179). This suggests that the organization of β cells in the core of the islet is crucial for correct islet function. The perturbed α and β cell localization together with the reduced islet size I describe for mice expressing mSpry4 in β cells could therefore influence blood glucose homeostasis. However, glucose tolerance tests showed that the phenotype on islet architecture caused by mSpry4 expression has no measurable effect on the glucose metabolism of the mice (data not shown), suggesting a compensatory mechanism.

Taken together our results show that the specific expression pattern of mSpry4 in the endocrine cells of the pancreas is necessary for correct pancreas organogenesis. Thus, overexpressing mSpry4 in β cells results in a cell sorting phenotype. Considering our results

that mSpry4 inhibits migration of pancreatic precursor cells *in vitro*, I suggest that the phenotype in Rip1rtTA;$(tetO)_7$mSpry4 mice is due to impaired movement of endocrine cells during pancreas development.

7 mSpry4 in Rip1Tag2 tumorigenesis

7.1 Introduction

7.1.1 Sprouty and cancer

Deregulated, autonomous cell growth, the capacity to invade normal tissues and the ability to metastasize to distant body sites represent defining features of cancer (180). Deregulated RTK signaling has the potential to induce these processes (for review see (181)). Thus, core components of the pathways have been shown to be products of oncogenes. In particular, members of the EGFR family, FGFRs and other tyrosine kinases are commonly over-expressed in many cancers. Similar, downstream targets of the pathway like Ras and Raf are mutated in a broad spectrum of human tumors (for review see (181)).

As the function of Spry proteins has a key role in regulating oncogene-derived proteins, its own expression levels may critically influence tumorigenesis and downregulation could contribute to carcinogenesis due to hyper-stimulation of the p42/44 ERK pathway. Indeed, there is growing evidence demonstrating that Spry is as a tumor suppressor (for review see (182)); (i) over-expression of Spry2 in an osteosarcoma cell line was found to inhibit tumor growth and metastasis when injecting the cells into nude mice, (ii) Spry2 inhibits anchorage-independent cell growth, migration and cell invasion in *in vitro* invasion assays (39), and (iii) knocking down Spry2 in NIH3T3 cells caused mild transformation (183).

Investigating Spry expression in human cancers revealed that Spry1 and Spry2 are consistently downregulated in breast cancer and in liver cancer (87,183). In prostate cancer it was observed that Spry2 expression was reduced in invasive prostate cancer cell lines and high grade clinical prostate cancer compared to bening prostatic hyperplasia (88). These data implicate that loss of Spry might be a late event in tumorigenesis. However, the mechanism of Spry downregulation differs among various cancers. For example epigenetic inactivation of Spry2 represents the main mechanism for the downregulation of the Spry2 gene in prostate cancer (88), whereas promoter methylation has been ruled out in breast and liver cancer (87,183). Interestingly, there is no evidence of any Spry mutation in any of the cancers investigated (182).

In contrast, Spry2 was found to be upregulated in melanoma. In particular, melanoma with B-Raf or N-Ras mutations, the constitutive activation of p42/44 ERK results in an upregulation of Spry2 compared to melanocytes with wt B-Raf and N-Ras (184,185).

Spry2 and Spry4 are able to bind to wt B-Raf and thereby inhibit p42/44 ERK phosphorylation. The fact that both isoforms are not able to bind and inhibit the mutated form of B-Raf suggests that melanomas with this mutation show deregulated p42/44 ERK activation because Spry is rendered functionally ineffective (185). Also, Spry4 expression is upregulated due to aberrant c-Kit activation, in gastrointestinal stromal tumors (GISTs) (186). The drug Imantinib (Gleevec) blocks c-Kit phosphorylation, inhibits proliferation and induces apoptosis in a human GIST cell line (187). It is interesting to note that Spry4 was identified as one of the most significant Imantinib-responsive genes that was consistently downregulated upon treatment of GIST patients with Imantinib (188). Therefore, Spry4 is a potential marker for uncontrolled p42/44 ERK signaling in this disease.

7.1.2 Rip1Tag2 mouse model of β cell cacinogenesis

In the rat insulin promoter 1 (Rip1)T-antigen 2 (Tag2)- transgenic mouse model, expression of the simian virus 40 large T antigen is targeted to the insulin-producing β cells of the endocrine pancreas (97). As a consequence, hyperplastic islets begin to appear at around 4 weeks of age and at 10 weeks, about 50% of the islets are hyperplastic (189). The onset of an angiogenic switch in a subset of hyperplastic islets triggers the formation of new blood vessels, resulting in progression to angiogenic islets at 6 weeks and to solid tumors at 9 to 10 weeks of age (190). At 12 to 14 weeks most of these islets have progressed into well encapsulated, non-invasive benign tumors or adenomas and about 0.5% of islets into malignant, invasive carcinoma (191). Thus, Rip1Tag2 mice provide a suitable model for multistage tumorigenesis in which tumors of all different stages can be reproducibly found and investigated.

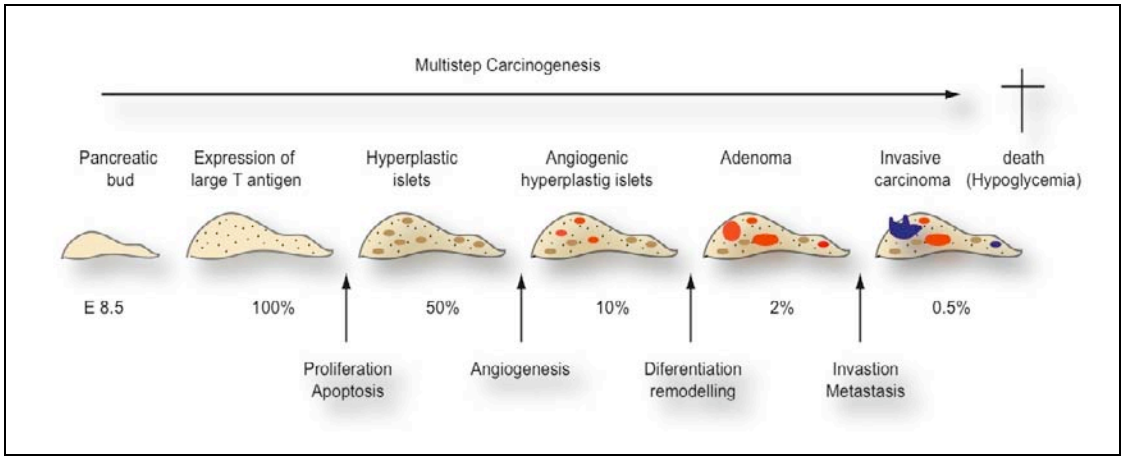

Figure 7-1. Multistep β cell carcinogenesis in Rip1Tag2 transgenic mice.
As indicated, islets (black dots) sequentially progress into hyperplastic islets (large brown ellipses), angiogenic islets (red ellipses), benign adenoma (large red shapes), and malignant carcinoma (blue shape). Percentages indicate 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 (from (192)).

7.2 Results

7.2.1 mSpry4 expression during tumorigenesis in the Rip1Tag2 model

Rip1Tag2 is a well characterized transgenic mouse model of pancreatic β-cell carcinogenesis. To determine if ectopic expression of mSpry4 in β cells affects tumor development in Rip1Tag2 mice, triple transgenic animals (Rip1Tag2;Rip1rtTA;$(tetO)_7$mSpry4) were generated. The expression of the transgene was induced by doxycycline at three timepoints to interfere with different steps of tumorigenesis: i) from week 5 to 8 (initiation of tumorigenesis); ii) from week 7 to 10 (tumor growth); and iii) from week 10 to 12 (progression from benign to malignant tumors). Littermates negative for either of the transgenes (Rip1rtTA or $(tetO)_7$mSpry4 or both) were used as control mice.

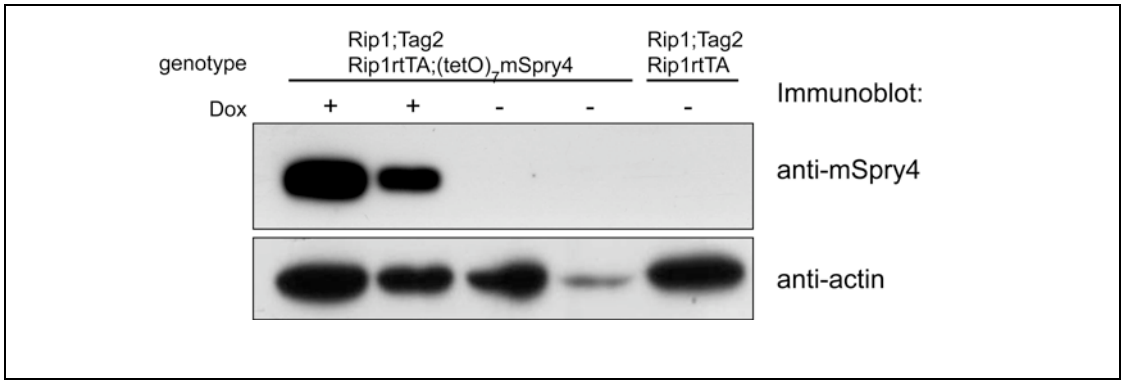

Figure 7-2. Induced expression of mSpry4 in Rip1Tag2 mice.
Rip1Tag2 and Rip1Tag2;Rip1rtTA;$(tetO)_7$mSpry4 mice were treated with doxycycline for 2 weeks or left untreated. Tumor lysates of 12 week old animals were resolved by SDS-PAGE and the resulting immunoblot was sequentially probed with anti-mSpry4 sera and antibodies against α-actin as a loading control.

First, inducible mSpry4 expression in islets and tumors was analyzed by immunoblot of total tumor lysates (**Figure 7-2**) and then by immunohistochemical staining of pancreatic sections using anti-mSpry4 sera (**Figure 7-3**). In pancreata of 8 week old Rip1Tag2 tumor mice, most islets were still comparable in size and architecture to islets of C57Bl/6 mice, with α cells expressing endogenous mSpry4 (**Figure 7-3 A**). In Rip1Tag2;Rip1rtTA;$(tetO)_7$mSpry4 mice, transgenic mSpry4 expression was induced by doxycycline in β cells (**Figure 7-3 B**). Comparable expression was found in small islets of 12 week old triple transgenic mice (**Figure 7-3 C**). However, the number of cells expressing the mSpry4 are markedly declined in most large carcinomas where large areas of the tumors

were negative for the transgene (**Figure 7-3 D**). Immunofluorescence co-staining for mSpry4 and insulin revealed that the expression of the two proteins was not lost concomitantly, indicating that the reduced mSpry4 expression is not due to a loss of insulin promoter activity (data not shown). I therefore conclude that either tumor cells lower the levels of mSpry4 protein or that there is a selection against cells expressing mSpry4 during tumor progression.

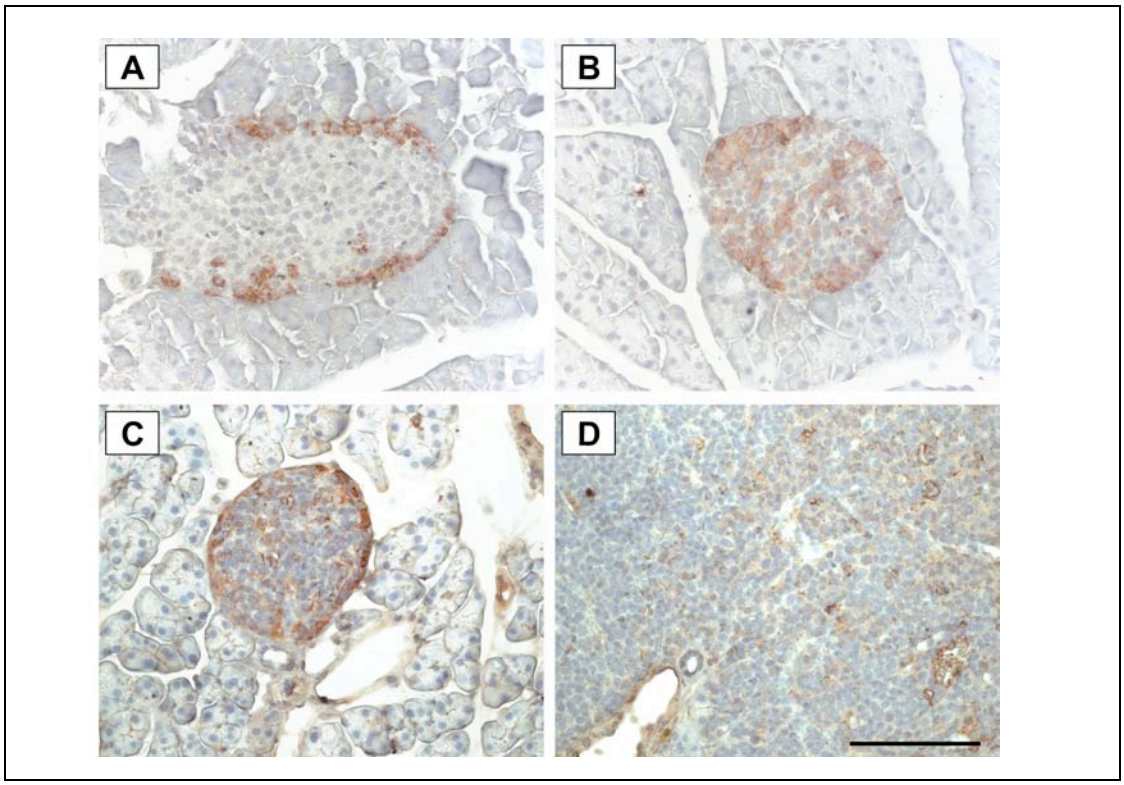

Figure 7-3. Expression of induced mSpry4 in islets and tumors of Rip1Tag2 mice.
Rip1Tag2 (A) and Rip1Tag2;Rip1rtTA;(tetO)$_7$mSpry4 (B-D) mice were treated with doxycycline for 3 weeks. Pancreatic islets of 7 week old mice (A-B) and islets (C) and tumors (D) of 12 week old mice were analyzed for the expression of mSpry4 by immunohistochemical staining using anti-mSpry4 sera. Scale bar: 100μm

To assess whether mSpry4 expression in β cells has any effect on tumor incidence or tumor size, isolated pancreata from 10 and 12 week old Rip1Tag2; Rip1rtTA;(tetO)$_7$mSpry4 mice and from control littermates were analyzed macroscopically (**Table 7-1**). Total number of tumors per mouse was not affected by ectopic mSpry4 expression. In contrast, a moderate, but not significant reduction in tumor volume was observed in triple transgenic mice versus control littermates. These results suggest that while mSpry4 is not affecting the onset of tumorigenesis it has a slight impact on tumor growth.

Next, we investigated tumor cell proliferation by counting tumor cells which stained positive for incorporated BrdU (**Table 7-1**). In all three cohorts the proliferation rate of cells was slightly decreased in tumors expressing transgenic mSpry4 compared to tumors of control mice. Quantification of apoptotic cells by TUNEL assay revealed a significant decrease in cell apoptosis in tumors of 8 week old Rip1Tag2;Rip1rtTA;tetOPmSpry4 mice compared to tumors of control littermates (**Table 7-1**). Surprisingly, the opposite was observed in the cohort of 12 week old animals. Here, the expression of mSpry4 had a marked yet not significant pro-apoptotic effect on tumor cells. A selection against cells expressing high levels of mSpry4 could be responsible for the increase in cell apoptosis. However, staining the TUNEL assay with anti-mSpry4 antibodies did not reveal any correlation between mSpry4 expression and apoptosis (data not shown), suggesting that mSpry4 expressing cells are not more prone to cell death than control cells.

Table 7-1 mSpry4 has no effect on tumor incidence, volume, cell proliferation or apoptosis

age Dox treatment	**8 weeks** week 5-8		**10 weeks** week 7-10		**12 weeks** week 10-12	
	ctrl [a]	**mSpry4** [b]	**ctrl** [a]	**mSpry4** [b]	**ctrl** [a]	**mSpry4** [b]
tumor incidence / mouse	N/A		5.1±1.1 *(N=12)*	4.8±1.4 *(N=10)*	7.1±0.8 *(N=11)*	8.7±1.6 *(N=7)*
p value[c]			0.8767		0.3321	
total tumor volume / mouse (mm^3)	N/A		13.4±4.8 *(N=12)*	10.2±4.9 *(N=10)*	78.3±17.8 *(N=7)*	45.3±18.0 *(N=11)*
p value[c]			0.6464		0.2344	
Proliferation rate of tumor cells [c]	85.0±4.7 *n=70;* *N=9*	73.6±5.5 *n=53* *N=7*	93.2±5.0 *n=74* *N=9*	88.2±4.6 *n=72* *N=8*	83.7±8.4 *n=41* *N=5*	73.1±3.9 *n=99* *N=12*
p value[c]	0.1168		0.4593		0.1958	
Apoptotic tumor cells	13.4±0.8 *n=81* *N=11*	9.9±0.8 *n=57* *N=7*	18.8±1.0 *n=89* *N=11*	17.2±1.6 *n=43* *N=6*	13.9±0.7 *n=50* *N=8*	16.0±0.9 *n=75* *N=9*
p value[c]	0.0035		0.3813		0.0698	

a: Rip1Tag2 / Rip1Tag2;Rip1rtTA / Rip1Tag2;tetOPmSpry4
b: Rip1Tag2;Rip1rtTA;mSpry4
c: p value of mSpry4 vs ctrl, analyzed by a two-tailed Student's t test
Values represent mean±SD
n=number of 40x magnification fields, N=number of mice
N/A=not applicable

7.2.2 Expression of mSpry4 in β cells does not inhibit tumor progression

To check for the possibility that transgenic mSpry4 expression could repress tumor progression, tumors were staged according to their morphology into normal/hyperplastic islets, adenomas, and carcinomas of three grades with increasing malignancy (193). In the three age cohorts analyzed, no differences in tumor progression between Rip1Tag2;Rip1rtTA;$(tetO)_7$mSpry4 mice versus control littermates was observed (**Table 7-2**). Thus, we conclude that expression of mSpry4 in β tumor cells leads to moderate inhibition of tumor growth by reducing the proliferation rate of tumor cells and later in tumor development by enhancing apoptosis. However, the presence of mSpry4 does not affect the progression to higher malignancy.

Table 7-2 Expression of mSpry4 in β cells does not inhibit tumor progression

age Dox treatment	**8 weeks** week 5-8		**10 weeks** week 7-10		**12 weeks** week 10-12	
	ctrl [a]	**mSpry4** [b]	**ctrl** [a]	**mSpry4** [b]	**ctrl** [a]	**mSpry4** [b]
normal / hyperplastic islets	62	62	34	38	32	35
Adenoma	19	18	21	21	20	20
	81	**80**	**55**	**59**	**52**	**55**
carcinoma						
G1	15	16	16	16	15	14
G2	3	3	27	22	31	30
G3	1	1	2	3	2	1
	19	**20**	**45**	**41**	**48**	**45**
	n=213 *N=11*	*n=217* *N=9*	*n=219* *N=11*	*n=224* *N=10*	*n=161* *N=12*	*n=218* *N=12*

a: Rip1Tag2 / Rip1Tag2;Rip1rtTA / Rip1Tag2;tetOPmSpry4
b: Rip1Tag2;Rip1rtTA;mSpry4
n=number of islets, N=number of mice
values represent % of total islets

7.2.3 Ectopic mSpry4 has no effect on proliferation, adhesion and stimulation of β tumor cells

To investigate the effect of mSpry4 on tumor growth at a cellular level, we established several independent β-tumor cell lines from Rip1Tag2;Rip1rtTA;$(tetO)_7$mSpry4 mice and from control littermates. The inducible expression of mSpry4 in β-tumor cell lines isolated from triple transgenic animals was confirmed by immunoblot (**Figure 7-4**).

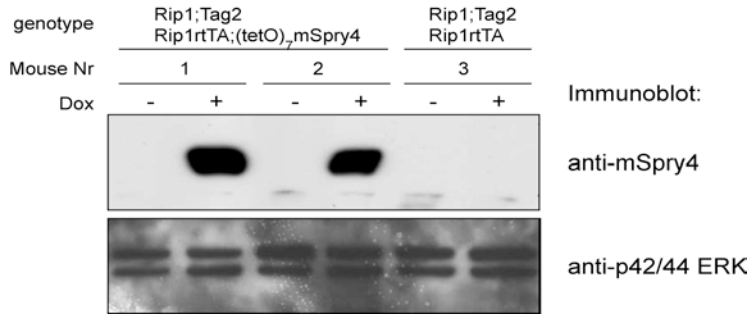

Figure 7-4. Inducible expression of mSpry4 in β tumor cells.
Tumor cell lines were isolated from Rip1Tag2; Rip1rtTA and Rip1Tag2;Rip1rtTA;$(tetO)_7$mSpry4 mice. To induce the expression of the transgene mSpry4, cells were treated with doxycycline for 2 days. Lysates were resolved by SDS-PAGE and resulting immunoblots were sequentially probed with anti-mSpry4 sera and antibodies against anti p42/44 ERK as a loading control. The immunoblot shown is representative of 3 independent experiments.

Next, the proliferation rate of four different cell lines was analyzed. In contrast to the *in vivo* data where we find a moderate decrease in BrDU incorporation (**Table 5-1**), the expression of mSpry4 has no significant effect on β tumor cell proliferation (**Figure 7-5**).

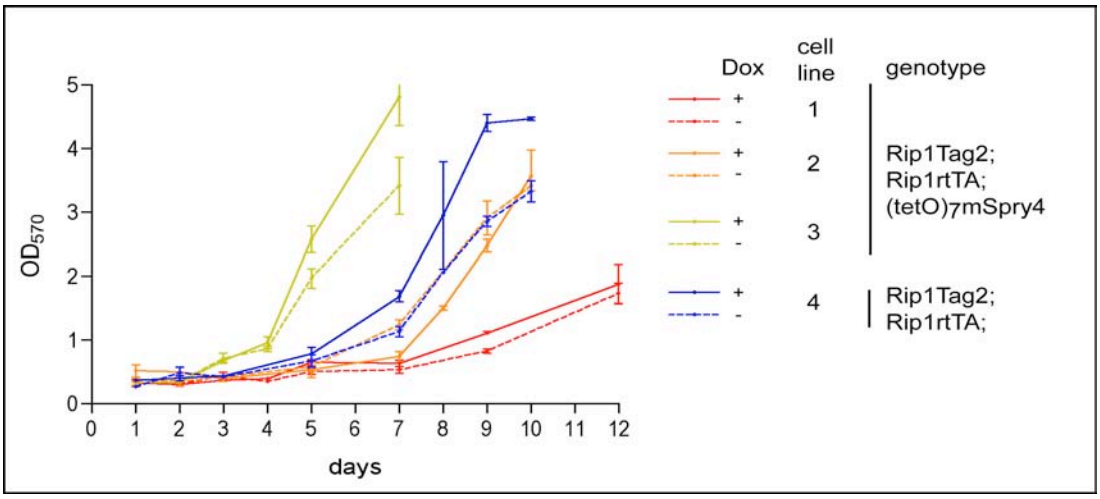

Figure 7-5 mSpry4 expression has no effect on proliferation of Rip1Tag2 tumor cells. *Tumor cell lines isolated from 3 different Rip1Tag2;Rip1rtTA;(tetO)$_7$mSpry4 mice and one Rip1Tag2; Rip1rtTA mouse were grown with (continuous line) or without (dashed line) doxycycline and the cellular proliferation rate was measured by MTT assay.*

Sprouty proteins are inhibitors of the Ras/ERK pathway. To test, if mSpry4 functions as such an inhibitor in β tumor cells, we performed stimulation experiments with various growth factors. β tumor cell lines from Rip1Tag2;Rip1rtTA;(tetO)$_7$mSpry4 mice were cultured with or without doxycycline for 2 days to induce mSpry4 expression and then stimulated with different growth factors following overnight starvation. When cell lysates were analysed for pp42/44 ERK by immunoblot, constitutive ERK signaling was observed in these β tumor cells (**Figure 7-6**). Also there was no further stimulation of the ERK pathway by any of the growth factors tested. Independent of starvation or stimulation with different growth factors, induced mSpry4 expression could not attenuate the levels of activated p42/44 ERK.

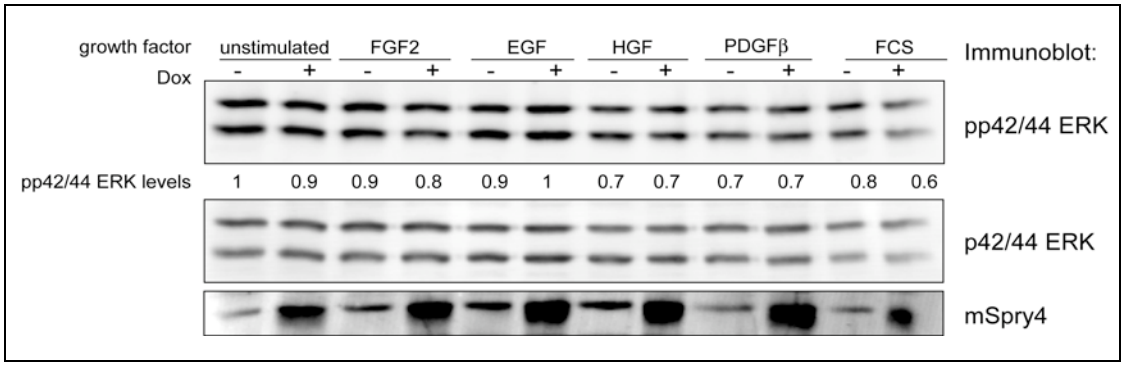

Figure 7-6. mSpry4 does not affect RTK signaling pathways in Rip1Tag2 tumor cells. *Tumor cells isolated from a Rip1Tag2;Rip1rtTA;(tetO)$_7$mSpry4 mouse were treated with doxycycline for 24h, starved overnight and then stimulated for 30 min with the growth factors indicated. Lysates were resolved by SDS-PAGE and the resulting immunoblots were sequentially probed with the indicated antibodies. The immunoblot shown is representative of 3 independent experiments.*

7.2.4 Tumor cell transplantation

Next, we investigated if mSpry4 could influence the tumorigenic potential of β tumor cells *in vivo*. Cells lines established from Rip1Tag2 tumors have the capacity to grow and form tumors when xenografted into immune-deficient mice (89). Therefore, a β-tumor cell line of a Rip1Tag2;Rip1rtTA;(tetO)$_7$mSpry4 mouse was injected subcutaneously into the two flanks of nude mice. Then, mice were treated with doxycycline to induce the expression of mSpry4 in the grafted cells, or left untreated. After six weeks, mice were sacrificed and tumor incidence and tumor volume were determined (**Table 7-3**). Results revealed that doxycycline induced expression of mSpry4 was not able to interfere with tumor formation and growth. Taken together, experiments with β tumor cells demonstrate that these cells are refractory to the inhibitory activity of mSpry4, which is consistent with the rather moderate effects seen in the Rip1Tag2 tumorigenesis model.

Table 7-3 Injection of Rip1Tag2;Rip1rtTA;(tetO)$_7$mSpry4 tumor cells into nude mice

	- Dox	+Dox
tumors / flank injected	5 / 8	4 / 8
volume / tumor (mm^3)	220.9 ± 117.4 *(N=4)*	240.4 ± 185.7 *(N=4)*
p value	0.9324	

N=number of tumors
Data were analyzed by a two-tailed Student's t test. Values represent mean±SD

7.2.5 Future work involving mSpry4 and angiogenesis in the Rip1Tag2 mouse model

The formation of new blood vessels, *i.e.* sprouting of new capillaries from existing vessels (angiogenesis) is fundamental for expansion of tumor mass (194). The process of angiogenesis is complex (for review see (195)). The basement membrane surrounding the endothelial cell of existing vessels is locally degraded and endothelial cells change shape and invade into surrounding stroma. This invasion is accompanied by proliferation of the endothelial cells at the leading edge of the newly forming vessel. Angiogenesis is induced by factors including VEGFs, PlGF, FGF2, Interleukin-8, tumor growth factor β, platelet-derived growth factor, which are secreted during carcinogenesis by tumor cells, and cells from the tumor stroma. By binding to their corresponding receptor on endothelial cells of existing blood vessels, these factors have the potential to induce the formation of new blood vessels, the so-called angiogenic switch (196,197). Indeed, preclinical studies demonstrate that FGF2 antagonists inhibit tumor vascularization and thereby attenuate tumor growth (196,198). Also, the VEGF-specific antibody bevacizumab (Avastin) has been shown to exert antivascular effects in cancer patients (199).

In the development of the placenta, kidney and the lung, Spry was shown to play a profound role in regulating tubular morphogenesis indicating that Spry may also be implicated in angiogenesis (38,84,154-156). Indeed, Spry negatively regulates proliferation and migration of endothelial cells and expressing mSpry4 in endothelial cells of cultured mouse embryos inhibits branching and sprouting of small vessels resulting in abnormal embryonic development (18,46).

Therefore, the next goal of the project is to investigate mSpry4 function on tumor-driven angiogenesis in the Rip1Tag2 mouse model. To this end, we analyzed mSpry4 expression in vessels of pancreata of C57/Bl6 mice and in tumors of Rip1Tag2 mice. Using CD31 as a marker for blood endothelial cells we found in the exocrine pancreas that mSpry is present in larger vessels but absent in smaller capillaries (

Figure 7-7 A-C). Also, we could not detect any expression of mSpry4 in newly formed blood vessels in tumors of Rip1Tag2 mice (

Figure 7-7 D-F).

Thus, we are planning to ectopically express mSpry4 in endothelial cells. Therefore, we will cross the $(tetO)_7$mSpry4 transgenic mice to two different transgenic lines in which an endothelial cell specific promoter, the vascular endothlial-cadherin promoter (provided by L. Benjamin, BBS, Harvard University) or the Tie2 promoter (provided by U. Deutsch, Theodor Kocher Institut, University of Berne) respectively, controls synthesis of the doxycycline transactivator (tTA). Double transgenic animals are expected to express the transgene mSpry4 specifically in endothelial cells unless they are fed with doxycycline in their drinking water ((200) and unpublished data).

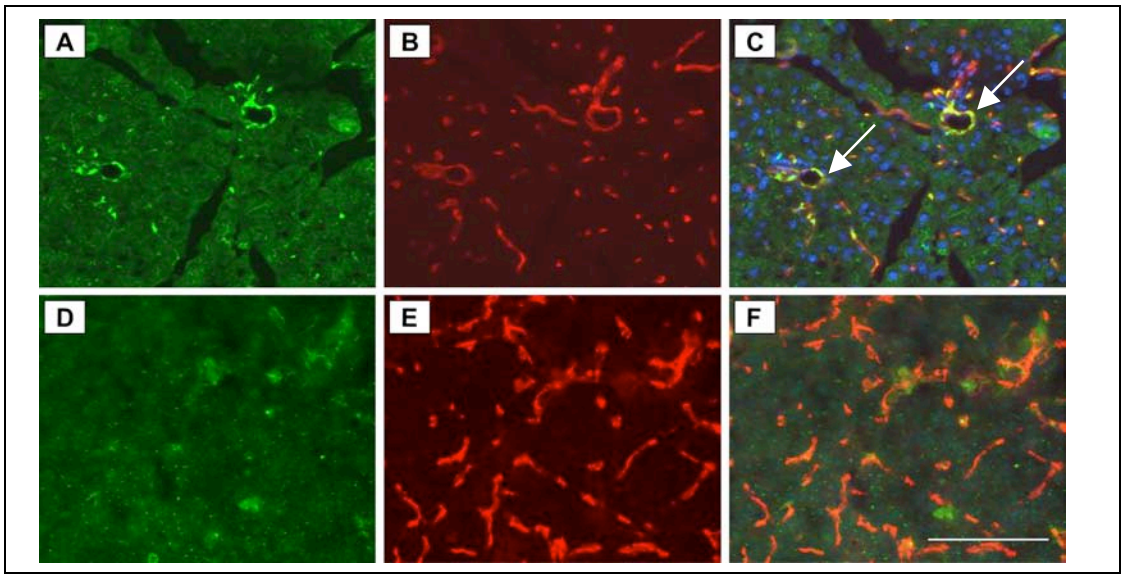

Figure 7-7 mSpry4 expression in blood vessels.
Paraffin sections of the exocrine pancreas of C57Bl/6 mice (A-C) and tumors of Rip1Tag2 mice (D-F) were analyzed by immunfluorescent co-staining using anti-mSpry4 sera (A, D, green) and CD31 (B, E, red) as a marker for endothelial cells in blood vessels. Merge of pictures (C, F). C, arrow: blood vessels co-expressing mSpry4 and CD31. Scale bar: 100µm

7.3 Discussion

Here I investigate a possible role of mSpry4 expression in β cells on tumorigenesis in the Rip1Tag2 mouse model. I observe that the effect of transgenic mSpry4 on β cell carcinogenesis is not significant. However, the presence of mSpry4 leads to a modest decrease in tumor size (**Table 7-1**), which is most probably due to reduced cell proliferation. This observation together with the fact that larger tumors negatively select against mSpry4 expression (**Figure 7-3**) suggests that Rip1Tag2 tumors are not completely mSpry4-refractory.

However, the *in vivo* results in the Rip1Tag2 model imply that transformed β cells are rather insensitive to mSpry4. This is further confirmed by experiments with isolated β-tumor cell lines from these transgenic mice. Despite the fact that mSpry4 expression can be induced with doxycycline neither proliferation of the cells (**Figure 7-5**) nor the activation of p42/44 ERK by different growth factors (**Figure 7-6**) is affected. Also, mSpry4 expression does not influence the potential of the cells to form tumors when reinjected into mice (**Table 7-3**). β tumor cells are transformed by expression of the large T antigen of the tumor virus SV40. The most characterized interactions of this transforming protein are those that inactivate the tumor suppressor genes Rb and p53, both implicated in cell cycle regulation (201). However, Sachsenmeier and co-workers revealed that T antigen-driven inhibition of Rb and p53 is insufficient for cell transformation (202). Little is known about other effects of large T antigen driven cell transformation but there are probably plenty of other pathways affected. For example, in large T antigen transformed cells PDGF signaling acts independently of Src kinases (203). Also, large T antigen was shown to cooperate with an activated Ras oncogene to transform primary rat embryo fibroblasts (204). In β-tumors we observe a constitutive activation of Ras/ERK pathway (**Figure 7-6**). Thus, the inhibitory effect of mSpry4 on Ras/ERK signaling might be abolished by a constitutive activation of the pathway downstream of mSpry4 function. Similarly, it was shown that Spry2 and Spry4 expression is not effective in melanoma cell lines, that possess a mutated form of Raf resulting in a constitutive activation of the Ras/ERK pathway (185).

Analyzing cell apoptosis I find a significant decrease in TUNEL positive cells in 8 week old Rip1Tag2;Rip1rtTA;tetOPmSpry4 mice when compared to tumors of control littermates. These data suggest that mSpry4 has an anti-apoptotic effect on tumor cells. As discussed previously, in Rip1Tag2 tumors, Rb is inactivated due to large T antigen-mediated

cell transformation. Inactivation of Rb often sensitizes cells to apoptosis, although this effect is strongly cell context-dependent (201). Interestingly, in *Drosophila* it could be shown that a concomitant reduction of EGFR signaling is necessary for Rb-mediated cell apoptosis (205). Thus, as a potentiator of the EGF pathway, Spry might modulate cell apoptosis in β-tumor cells where the expression of large T antigen inactivates Rb.

8 General Discussion

8.1.1 Spry, important regulators of RTK signaling

In this work I investigated (i) the biochemical aspects of Spry function, namely the functional consequences of Spry interacting with Caveolin-1 and (ii) biological consequences of mSpry4 on pancreas development and on tumorigenesis in the Rip1Tag2 mouse model.

Most animal cells develop according to cues from their environment that have been produced by other cells. Among these signals are various growth factors which bind to their specific RTK, inducing downstream pathways in the targeting cell. However, the biological outcome of the signaling event is not only determined by the growth factor itself but strongly depends on the cell receiving it. For example, FGF stimulation of fibroblasts results in cell proliferation whereas stimulation of the same RTK in neuronal cells results in cell differentiation (for review see (1,72)). Moreover, the intracellular pathways that are activated by different growth factors can be very similar; *e.g.* various RTK downstream cascades result in p42/44 ERK activation. It was suggested that cellular responses are determined by variable duration and intensity of p42/44 ERK activation by different growth factors (206).

Spry proteins are key regulators that modulate the strength, duration and range of activation of RTKs allowing for controlled growth and differentiation. The impact of Spry as a modulator of different RTKs is exemplified in PC12 cells. In PC12 cells, EGF treatment results in transient activation of p42/44 ERK thereby inducing increased proliferation of the cells. However, sustained p42/44 ERK phosphorylation mediated by FGF stimulation leads to differentiation into a neuron-like phenotype (207). Ectopic expression of Spry2 in PC12 cells sustains EGF-induced ERK activation and induces neurite outgrowth, thus imitating the effect of FGF stimulation (29).

In addition, Spry2 activity in PC12 cells underlines the broad functional spectrum of Spry proteins; Spry proteins act on various RTK signaling pathway differentially, *i.e.* depending on the growth factor and cellular context they have the capacity to inhibit or potentiate p42/44 ERK activation. This implies, that Spry activity itself must be subject of a complex regulatory system. To some extent, the diversity of Spry function can be explained by the four different isoforms, each of them differentially interfering with RTK downstream signaling cascades (51,53,78,139). However, taking Spry2 as an example demonstrates that single isoforms can have dual functions; acting as a potentiator of EGF

signaling in one instance (23,44,47,53,85), our data presented here are in agreement with the work from others showing that Spry2 inhibits EGF-mediated p42/44 ERK phosphorylation in other cellular contexts (our results and (26,27,40)). The cellular context-dependent function of single isoforms implies that Spry activity is modulated by the presence of other proteins and/or post-transcriptional modifications. The interaction of Spry with Caveolin-1 examined here represents one mechanism of such a modulation. Various possible combinations of the different Spry isoforms in the presence or absence of Caveolin-1 provides a wide range of RTK signaling regulation.

8.1.2 Conserved function of Spry proteins: regulation in branching morphogenesis

Many essential organs the lung, vascular system, kidneys, and most glands-are composed of branching networks of epithelial tubes. Branching morphogenesis involves reiterated cycles of bud initiation, bud outgrowth and bud arrest. Therefore, a fine balance between stimulating and inhibitory signals is critical for the proper pattern of branching morphogenesis (for review see (151)).

As discussed in the introduction, Spry is an important regulator of branching morphogenesis in many different developing organs by controlling bidirectional signaling between different tissues like the mesenchyme and the epithelium. Here I show that ectopic expression of mSpry4 in pancreatic β cells perturbs the organization of endocrine cells in the islets of Langerhans. During pancreas development, epithelial precursor cells invade the surrounding mesenchyme and form aggregates that develop into islets of Langerhans. Thereby, mesenchyme-derived FGFs are thought to act via a paracrine mechanism to stimulate migration of the epithelial precursor cells into the mesenchyme (for review see (161)). The resemblance of pancreatic islet formation to events of branching morphogenesis discussed before made it tempting to speculate that Spry is involved in pancreas development. Support for this hypothesis is provided by the very specific expression pattern of mSpry4 in α cells and defective islet formation in the double transgenic mice ectopically expressing mSpry4 in β cells. Taken together, all these data illustrate that the role of Spry proteins in developing organs is conserved, namely they regulate the responsiveness of a particular group of cells to signals coming from the surrounding tissue. We suggest that the role of Spry in pancreas development is analogous to the proposed role for Spry in other developmental systems.

However, the mechanisms by which this is achieved might be very divergent in different organs. Namely, Spry proteins have been shown to regulate branching

morphogenesis and epithelial-mesenchymal interactions mediated by various growth factors, among them FGF, GDNF, and WNT (156-158). Also, our biochemical data using PANC-1 cells suggest that in pancreas development, Spry might inhibit migration of cells by a mechanism other than attenuation of growth factor-mediated p42/44 ERK activation. Finally, investigating the impact of Spry in organogenesis exemplifies the strong cell context-dependent function of these proteins. Interestingly, in many of these developmental systems discussed before, Spry expression is detected throughout the organ. For example, Spry expression in the Wolffian duct includes the region where the ureteric bud normally forms (156). The molecular mechanisms that enable cells to escape the inhibitory effect of Spry remain to be elucidated. Here, my work demonstrating how Caveolin-1 differentially modulates the activities of Spry proteins, makes Caveolin-1 a suitable candidate to accomplish this task.

8.1.3 Spry as a tumor suppressor

While there is good evidence that Spry isoforms have various functions in directing the formation of branched tubular structures during development, less is known about the function of Spry isoforms in mature animals. However, in the mammary gland, Spry2 is upregulated when these epithelial cells are actively proliferating and branching (87). Also, Spry is upregulated in the actively proliferating neointimal smooth muscle cells after vascular injury (208). This would suggest that similar to its function in developing organs, Spry may have a role in the adult organism opposing the actions of growth factors in modulating growth and migration in physiological and pathological conditions. Therefore, any deregulation, especially downregualtion could have profound implications in terms of uncontrolled growth, possibly contributing to carcinogenesis due to hyper-stimulation of the Ras-p42/44 ERK pathway.

Indeed, there is growing evidence for Spry proteins to be tumor suppressors (for review see (182)). Spry1 and Spry2 are consistently downregulated in breast cancer (87), in liver cancer (183), and in prostate cancer (86). Interestingly, Spry is upregulated in benign human neurofibromas and benign prostatic hyperplasias, but downregulated in malignant tumors (88,209). Thus, loss of Spry expression appears to be a late event in tumor development and might play a role in progression from benign to malignant state. In line with this, it was suggested that Spry is involved in a negative signaling network that is responsible for oncogene-induced senescence (210). In some primary cells, activated Ras leads to an initial burst of proliferation followed by cellular senescence due to the

accumulation of p53, p16 and ARF (211). Courtois-Cox and coworkers provide a model whereby Ras-mediated p42/44 ERK and PI3K activation induces a negative feedback loop that leads to a suppression of Ras signaling below baseline levels (210). The data suggest that this dramatic termination of Ras signaling triggers senescence in primary cells. Further, Spry2 and Spry4, which are highly upregulated in Ras-activated primary cells seem to be involved in this negative regulatory loop. These data provide evidence, that loss of Spry proteins might enable tumor cells to escape oncogenic-induced senescence and would therefore be responsible for an important step in tumor progression. Further support for a possible function of Spry as a tumor suppressor is provided by the fact that over-expression of Spry2 in an osteosarcoma cell line was found to inhibit tumor growth and metastasis (212). In addition, over expression of Spry2 can inhibit anchorage-independent cell growth, and cell invasion in *in vitro* invasion assays (39) and, knocking down endogenous Spry2 in NIH3T3 causes a mild transformation (183).

Here I investigated a possible role for mSpry4 in β cell carcinogenesis in the Rip1Tag2 model. No detectable effect on tumorigenesis by induced expression of mSpry4 in β cells was observed. I assume that mSpry4 is not functional in these cells because of constitutive activation of the p42/44 ERK (probably due to the large T antigen) by a mechanism that is further downstream from the point where mSpry4 interferes with the pathway. Similar observations were made in melanoma cells; in most melanomas the p42/44 ERK pathway is constitutively activated due to oncogenic mutations in B-Raf or N-Ras, rendering Spry2 ineffective as an inhibitor of p24/44 ERK phosphorylation (184,185).

Another aspect of Spry function in tumorigenesis concerns the capacity of Spry to act as an inhibitor of angiogenesis. Ectopic expression of mSpry4 in cultured mouse embryos reveals that the protein inhibits branching and sprouting of the vasculature during angiogenesis in the developing embryo (46). The importance of angiogenesis for the growth of solid tumors is well recognized. In the absence of access to an adequate vasculature, tumor cells become necrotic and/or apoptotic, restraining the increase in tumor volume that should result from continuous cell proliferation (for review see (194)). Therefore, using the Rip1Tag2 mouse model for β cell carcinogenesis and expressing ectopic mSpry4 in endothelial cells we expect a reduction in tumor size due to an inhibition of angiogenesis.

8.1.4 Concluding remarks

RTKs and their signaling pathways are implicated in many cellular processes thereby fulfilling a crucial role in cell homeostasis during development and in the adult

organism. Aberrant signaling through these pathways results in malignant transformation of the cells (for review see (181)). Here, the crucial role of Spry proteins in the development of various organs is exemplified by our results showing that aberrant mSpry4 expression in the pancreas has a severe effect on the formation of islets of Langerhans. Investigating the effect of mSpry4 on tumorigenesis in the Rip1Tag2 model, I find that Spry is ineffective in transformed tumor cells where the p42/44 pathway is constitutively active, thus confirming results found by others (184,185).

Further, I discussed the need for a tight regulation of Spry activity that guarantees their cell-context specificity and functional differences among the four isofoms. An important step towards understanding these regulatory mechanisms is to investigate proteins that differentially interact with Spry proteins depending on their isoforms and on the cellular context. Here, I illustrate that Caveolin-1 represents such an interacting partner. However, more work needs to be done to understand the complexity of the Spry proteins and as G. Christofori wrote in his recent review on Spry function: Whatever future experiments will reveal, it's going to be complicated (6).

9 References

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11 Publications

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Function of mSpry4 in pancreas development and β cell carcinogenesis
(in preparation)

Miguel A. Cabrita, Fabienne Jäggi, Sandra P. Widjaja, Gerhard Christofori
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