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Syndecan-1 and heparanase as novel regulators of colon cancer stem cell function

Münster 2015

Biologie

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Inaugural-Dissertation
zur Erlangung des Doktorgrades
der Naturwissenschaften im Fachbereich Biologie
der Mathematisch-Naturwissenschaftlichen Fakultät
der Westfälischen Wilhelms-Universität Münster

Vorgelegt von
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aus Indien
-2015-

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Tag der mündlichen Prüfung: _ _19.8.15 _ _ _ _ _ _ _ _

Tag der Promotion: _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _

Summary:

Proteoglycans and glycosaminoglycans and their degradative enzymes are involved in normal tissue homeostasis and cancer. Abnormal changes in their expression cause an imbalance between tissue homeostasis and cancer, resulting in changes in cell plasticity with an increase invasion, metastasis and dedifferentiation. These three processes are regulated by embryonic stem cell like gene signatures which contribute to the formation and maintenance of (cancer) stem cells (CSCs), or tumor initiating cells. The transmembrane heparan sulfate proteoglycan Syndecan-1, provides a structural framework for proper tissue organization in the intestinal tract, and acts as an important coreceptor for multiple signalling pathways. It is a substrate for heparanase (HPSE) the only mammalian enzyme capable of cleaving HS. Increased HPSE expression and reduced Sdc-1 expression during progression of colon cancer is associated with increased invasion and metastasis, and poor patient survival. The aim of this thesis was to investigate whether expression of Sdc-1 and HPSE influences cancer stem cell properties and invasiveness in colon cancer cell lines (Caco2 and HT29). Using an siRNA approach, we demonstrate that depletion of Sdc-1 increased the stem cell phenotype based on *in vitro* sphere-forming and flow cytometry-based assays (side population (SP), ALDH and CD133). Moreover, we observed an increase in matrigel invasiveness linked to EMT-induced conditions. Mechanistically, we observed an increase in integrin-induced actin remodeling via β1- integrin, focal adhesion kinase and Wnt signaling activities. The increase in SP, CD133 and colonospheres could be blocked using a FAK specific inhibitor. The impact of Sdc-1 depletion on the CSC phenotype was substantially enhanced by SP sorting and enrichment, where we also saw a relative increase in the number of colonies following irradiation, indicating that Sdc-1 depletion renders radioresistance to SP-enriched cells. Furthermore, our *in vitro* results correlated with xenograft experiments where we saw an increase in the tumor size in Sdc-1-depleted HT29 cells. To elucidate the interplay between Sdc-1 and HPSE, and the role of HPSE in colon cancer stemness, we applied both stable overexpression and transient knockdown methods (Caco2 and HT29 cells). Sdc-1 depletion regulated HPSE at the transcriptional level through an EGR1-FAK feedback signaling loop. Pharmacological HPSE inhibition caused a decrease in the invasion and SP levels in Sdc-1 depleted. Stable HPSE overexpression increased the SP, but, decreased colonosphere formation through the FAK and Wnt signaling axis. Taken together, our results report for the first time the dynamic interplay between Sdc-1 and HPSE in stemness-associated colon cancer properties via signaling axis involving EGR1, pFAK and Wnt. These findings could form a conceptual framework for establishing novel therapeutic approaches to treat colon cancer.

Contents

1. Introduction

1.1 Cancer

The word cancer broadly describes a collection of diverse diseases. Cancer is accountable for a great number of morbidities and mortality around the world; hence it symbolizes a major health worry concern across the continents (http://globocan.iarc.fr). Several years of cancer research have led to advancement in cancer treatment. However, it is a challenging approach to prevent the tumor recurrences. At the cellular level, cancer initiation and progression is mainly characterized by uncontrolled cellular proliferation and self-renewal of cancer cells ("hallmarks of cancer"), which are under the control of a wide range of signaling networks (Hanahan D and Weinberg RA, 2000). According to the tumor growth properties two main types of cancer can be distinguished: Solid tumors (e.g. colon cancer - abnormal growths) and hematological cancers (leukaemia - abnormal increase of white blood cells). In January 2000, Douglas Hanahan and Robert Weinberg wrote a review on the "The Hallmarks of Cancer" which theorized six common characteristics to all types of cancer (Hanahan D and Weinberg RA, 2000). It includes the properties of sustaining proliferative signaling, evading growth suppressors, resisting cell death inducing angiogenesis, enabling replicative immortality and activating invasion and metastasis. In 2011 they revised their concepts and introduced two additional concepts to understand the dimensions of cancer complexity - inexhaustible self-renewal (replicative) capacity- the notion of cancer stem cells and the reprogramming of energy metabolism. (Hanahan D and Weinberg RA, 2011). During normal physiological conditions the human body maintains tissue homeostasis: cells proliferate, differentiate, migrate and react to signals for apoptosis. Under pathological conditions, cells are not responsive to development controls, induce oncogenic loads and finally lead to cancer initiation and progression. For the initial signals for tumourigenesis, a combination of several pathogenic events is required. For example, successful colon cells require a minimum of mutations in five genes in order to be come malignant (Cho and Vogelstein 1992). In recent years, a mountain of research by several scientists identified the low but unique, population of cells called 'Cancer stem cells' or 'Tumor initiating cells', which are the main reasons for tumor recurrences and relapse (Blanpain, C et al., 2013). It is this subpopulation of cells which are in the focus of this thesis.

1.1.1 Colon cancer:

Colon cancer is the third most commonly diagnosed cancer and one of the leading causes of cancer deaths in both men and women globally (Velde CJ et al., 2005; Kemeny N et al., 2010).

Also known as colorectal cancer (rectal cancer or bowel cancer), colon cancer is the development of cancer by uncontrolled cell proliferation in the colon or rectum (large intestine). Two inflammation- driven diseases, crohn's disease and ulcerative colitis (inflammatory bowel disease) can enhance the risk of colorectal cancer (Lerner I et al., 2011).

During the last decades, due to technological advancement several drugs have been successfully targeting colon cancer, including Adrucil, Fluorouracil, Avastin (Bevacizumab), CAPOX (American Cancer Society: cancer Facts and Figures 2015). In addition, patients benefit from earlier diagnosis (American Cancer Society: cancer Facts and Figures 2015) But therapeutic targeting at the metastatic stage is often not completely successful due to the drug-associated toxicity and ultimately poor patient survival (Helling TS et al., 2014). Even though patients get better with therapies, after a certain time they succumb to the disease due to tumor relapse, which is hypothesized to be linked to the existence of cancer stem cells, as will be outlined below.

At the molecular level, accumulation of complex genetic and epigenetic landscapes in colon epithelial cells transforms into adenocarcinomas and then to invasive colorectal cancer (Baker AM et al., 2014). Most colorectal cancer cases acquire mutations in the *adenomatous polyposis coli* (*APC-tumor suppressor gene*). In fact, it is observed that *APC* inactivation is an early event for initiation of colon cancer in ~70–80% of colorectal adenomas and carcinomas (Kinzler & Vogelstein, 1996). This mutation regulates the multistep signaling pathways involved in tumor progression. Oncogenic loads- i.e. mutational inactivation of tumor-suppressor genes- *APC, TP53,* TGF-β and activation of oncogenes- *RAS* and *BRAF, and* Phosphatidylinositol 3-Kinase drives colorectal cancer. Signaling pathways like several growth factor pathways- epidermal growth factor receptor, vascular endothelial growth factor and activation of prostaglandin signaling and stem cell pathways- Wnt, BMP, Shh further regulate colon cancer malignancy. Mutations in cell cycle regulators- *Cyclin E, FBXW7, CMYC* and *CDK8* control colon cancer tumour cell proliferation (Fearon ER, et al., 2010; D. Markowitz et al., 2011).

1.2 Cancer Stem cells (CSCs)

Previously, cancer was considered as a homogenous mass of fast proliferating cells, which were therapeutically targeted by chemotherapy. Recent studies suggeste that tumor cells are heterogeneous (not all the cells in a tumor behave the same) due to the presence of stem cells, progenitors, and differentiated cells. This heterogeneity is present within the tumor at different regions (intra-tumor) and also among different tumors of same type (inter-tumor) (Morrison SJ et al., 2013).

Several groups independently reported that cancer cells express ES (embryonic stem cells) cell-like signatures in many human cancers (Chen L et al., 2012). The current concept of the cancer stem cell model implies the theory of the famous surgeon Stephen Paget - the 'Seed and Soil' theory. (Fidler, I. J. et al., 2003). In the 19th century, the hypothesis of cancer including stem cells was first identified observing the histological closeness between fetal developing cells and teratocarcinoma (Récamier, J.C.A.et al., 1829). Again in 1971, it was reported that only a small set of myeloma cells was forming tumor colonies (Park, C.H. et al.,1971). Later in 1977, a paper came out describing the isolation of a stemcell-like population from epithelial tumors (Hamburger A. W et al., 1977).

Several independent cell tracing and lineage studies strongly support that malignancies associated with cancer originate from a small population of stem cell-like cells with increased tumor-seeding ability and chemo-radio resistance causing subsequent relapse after treatment (Blanpain, C et al., 2013; Chen J et al., 2012). Such cells are referred to as tumor initiating cells, cancer stem cells or tumor stem cells (Al-Hajj M et al., 2003).

1.2.1 CSCs share the three important hallmarks of stem cells:

Self- renewal (long term renewal capacity) - the potential for long-term stable proliferation and differentiation to maintain a constant stem cell pool.

Differentiation (no renewal capacity) - the capability to develop into fully mature cells in a multi-lineage differentiation process.

Homeostasis Homeostatic control-the capacity to balance between self-renewal and differentiation (Dalerba P et al., 2007; Clevers H et al., 2011)

According to the current cancer stem cell hypothesis, several steps can trigger the tumor formation like mutation in stem cell, progenitor or even the differentiated cells has the capacity to de-differentiate into cancer stem cell like population.

1.2.2 Five Key Characteristics of CSCs:

1. Only a minor portion of cells in a given tumor has tumorigenic capacity when xenografted into mice.
2. Cancer stem cells can be separated and characterized from tumor cells by sorting with specific cell surface markers.
3. Tumors derived from CSCs contain both tumorigenic and non-tumorigenic cells.
4. The cancer stem cell population can be constantly transplanted in several generations,

indicating that these cells have self-renewing strength.

5. These CSCs are resistant to traditional therapies such as chemotherapy, radiotherapy, by virtue of several signaling networks and gene signatures.

Scientists claim that not all cancers follow the CSC concept because all cells are uniformly tumorigenic (Kelly PN et al., 2007; Sugihara E et al., 2013). Based on the concept of tumour heterogeneity in cancer cells, currently two models describe the tumour growth - stochastic clonal evolution models (Reya T et al., 2011) and the cancer stem cell (CSC) hypothesis. Recently, two more models have been developed to understand the concept of heterogeneity of tumor cells in the context of given genetic or epigenetic changes (Blanpain C et al., 2013) (Figure 1).

1) ***"Stochastic" model:*** All tumor cells are equal and they form progenitor and differentiated cells.
2) ***Cancer stem cell (CSC) model:*** Tumor growth is due to the presence of cells having long-term self-renewal giving rise to progenitors (limited proliferation ability) and followed by differentiated cells.
3) ***Clonal evolution in the "Stochastic" model:*** Due to accumulation of mutations in progenitor or differentiated cells.
4) ***Clonal evolution in CSC model:*** Mutations in stem cell, progenitor cell or even the differentiated cells.

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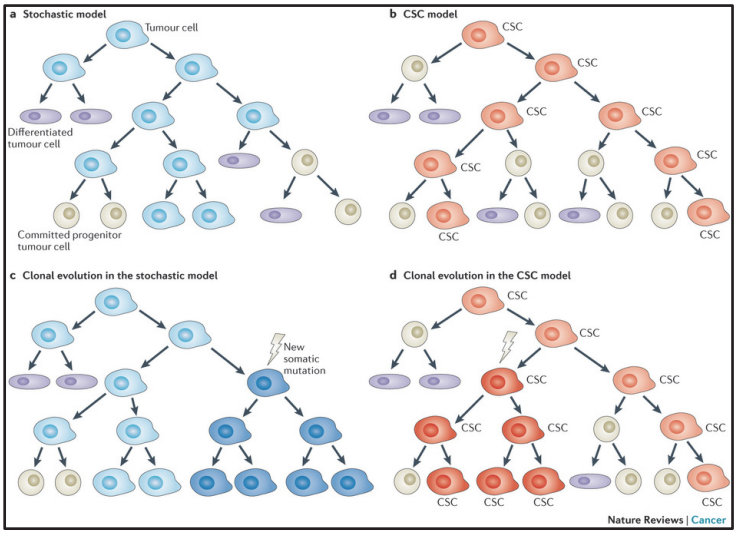


Figure 1. A schematic representation of current cancer stem cell models.

a) In the stochastic model of tumour growth, all tumour cells are equal and every cancer cell has a probability to self-renew or differentiate, defining tumor heterogenesity. b) In the cancer stem cell (CSC) model, only a minor proportion of cancer cells has the ability to form long term- self renewal and give rise to progenitors and eventually differentiate. c,d) In both models, distinct new somatic mutations can induce clonal diversity, which further enhances tumour heterogenesity. Figure adapted from Blanpain C et al., 2013.

1.2.3 Current approaches for isolating Cancer Stem Cells:

a) Cell Markers (surface antigens)

Based on the high expression of surface antigens several markers have been reported in both hematopoietic and solid tumors such as leukemia, (Singh, S. K et al., 2003), liver cancer (Terasaki, M et al., 2003), and colon cancer (Gallinger, S et al., 2007; Pilozzi, E et al., 2007). Subsequently, in breast cancer, CD44 high and CD24 low cells are considered as a cancer stem cell population (Benito-Hernandez *et al.,* 2003). In colon cancer, the combination of $CD44^{+}/CD166^{+}/ESA^{hi}$ assert as the tumorigenic population with tumor initiating properties (Pilozzi, E et al., 2007). Furthermore, CD133 is considered as one of the prominent markers for colon cancer (Ren F et al., 2013).

b) "Side-Population" Cells

The cell population capable of ejecting dye from a specific batch of cells, detectable on a flow cytometer, is called the "side-population" (SP) cells. SP cells were first identified in hematopoietic stem cells by Goodell et al., (1996). Under normal conditions, these pumps (ABC transporter proteins) eliminate metabolic products, drugs and toxic compounds. Likewise in cancer, due to the high expression of these pumps, they non-specifically efflux anticancer drugs. In this way these transporters have a higher protective function for drugs, making the tumor cells more chemoresistant. Based on the property of chemoresistance, these ABCG2-expressing cells are considered to be enriched in cancer stem cells (Dean M., et al 2005; Greve B et al., 2012). Technically, Hoechst 33342 is used a vital DNA dye to stain these side population cells (Y. W et al., 2006; Inoue, H. et al., 2006).

c) Aldehyde Dehydrogenase

One more approach to study the cancer stem cells is based on the high aldehyde dehydrogenase

(ALDH) activity, particularly ALDH1. ALDH oxidizes intracellular aldehydes to carboxylic acids, which are involved in retinoid metabolism (Labrecque, J, et al., 1993). These retinoids are of importance for the differentiation of pre-progenitor cells. Based on the ALDH1 activity in stem cells and progenitor cells they are considered as cancer stem cells (Labrecque J., et al. (2006). These ALDH positive cells are observed in various human cancers like breast, colorectal, liver, leukemia (Charafe-Jauffret, I. K. et al., 2007). The major disadvantage of the Side population and ALDH techniques is that they can't be applied in tissue sections.

d) Sphere Formation- direct functional assay

Sphere formation assay is a simple assay to measure the strength of self-renewing capacity of cancer stem cells. It was first identified to check the sphere formation ability of neural stem cells (Weiss, S. et al 1992). This assay is mainly based on the peculiar property of stem cells to self-renew to form spheres, followed by progenitor cell formation. This method is widely accepted for the characterization of CSC in various cancers- including colorectal and breast (Todaro, M., et al 2005; Ponti, D., et al 2008).

1.2.4 CSCs as targets for therapeutic applications

Currently, cancer is targeted by surgery, chemotherapy, radiation and immunotherapy. The vast majority of anticancer drugs available in the market target the bulk population of the tumor cells, mainly the differentiated cells (Pattabiraman DR et al., 2014). These drugs kill the tumor cells or mass but will not completely act on the cancer stem cells, resulting in tumor recurrence. Due to the advancement in the technology, presently chemotherapy treatments are successful in eradicating the leukemia and lymphoma, which could be because of the killing hematopoietic stem cell population ie., killing again CSCs. For instance, a stem-cell-related inhibitor that targets the Notch pathway is γ-secretase inhibitor. (Tsiftsoglou AS et al., 2009). The important and challenging question to target CSCs is to understand the 'stem cell niche'. The balance between self-renewal, progenitor and differentiated cells tightly controls the homeostatic process. This process is under the control of several intrinsic and extrinsic regulators (matrix proteins and growth factors), called the tumor microenvironment also know as the 'niche' (Sneddon JB et al., 2007). This niche keeps CSC in an undifferentiated state by activating specific signaling pathways. In brain tumors it was observed that the "vascular niche" harbours CSCs. (Yang ZJ et al., 2007). New combinational drugs/therapies may build a platform to kill the CSCs specifically without affecting the normal stem cells and not disrupting the regeneration of tissues.

1.2.5 Cancer stem cells and their link to induced pluripotent stem cells (iPS cells)

Understanding early developmental processes has become easier with the advancement in the iPSC technology. In 2006, the generation of induced pluripotent stem cells from mouse fibroblasts by retroviral transduction of Oct4, Sox2, Klf4 and c-myc has been reported (Yamanaka et al. 2006) - a revolutionary breakthrough that has sparked immense interest. Embryonic stem (ES) and induced pluripotent stem cells (iPSCs) have generated new interested in regenerative medicine applications. Understanding the regulatory mechanisms involved in the maintenance of self-renewal and pluripotency became also a very important area to understand the several diseases including cancer. Several human cancers express ES cell-like signatures (Ittai Ben-Porath et al., 2010) As mentioned above, stem cells and cancer stem cells share similar properties like asymmetric division, self-renewal, unlimited fast proliferation, slow replication and more importantly common regulatory signaling mechanisms. Based on these parallel routes shared between stem cells and cancer stem cells it became a very promising approach to understand the normal stem cells in development and its link to cancer stem cells suggesting new possibilities for cancer therapy. For example, the transcription factor Myc, which is a well-studied oncogene, also facilitates reprogramming into induced pluripotent stem cells (Utikal J et al., 2009). These observations lead further hopes and theories to understand the bilateral connection between pluripotency and tumorigenesis.

Compelling evidence showed the iPSCs and cancer cells share common characteristics like high telomerase activity, fast progression through the cell cycle, signaling pathways like Wnt, Hedgehog, Notch, and functionally they are involved in self-renewal and unlimited proliferation (Ruiz i Altaba et al., 2011). Therefore, it is a very important approach to model carcinogenesis using non-malignant cells. From these iPSCs cell lines, directed differentiation may provide the platform to understand the early developmental processes or related phenotypes that contribute to cancer development. These studies also provide the biological parallels between induced pluripotency and oncogenesis. More recently, several groups have reported the reprogramming of cancer cells into iPSCs (cancer iPSCs). These approaches will give very valuable information on reprogramming and oncogenic transformation. Further studies may pay an attention to understand the early developmental signals, experimental access to early stages of the disease and more importantly potential cancer inducing and repressing genes. (Chen L et al,, 2012; Kim J et al., 2015).

1.2.6 Contribution of CSCs to invasive cell behaviour

Apart from unlimited proliferation, cancer invasion is one of the processes among the 10 hallmarks of cancer which is most closely related to cancer related death (Hanahan D *et al.,* 2011). The molecular engine involved in the invasion of colon tumours is a hetergenous process. Tissues are composed of epithelial cells and mescenhymal cells. Epithelial cells adhere and form cell-to-cell contacts. In contrast, mesenchymal cells are capable of migrating (Chanmee T et al., 2014). A large proportion of tissues are filled with extracellular space, which is made up with complex of sugars and proteins of the extracellular matrix (ECM) (Yip et al., 2006; Ibrahim SA et al., 2014).

During the process of tumor invasion several complex molecular events are involved. Tumor cells mainly gain two important properties-cell proliferation and ability to migrate. In cancer, cell growth and proliferation are activated by altered regulation of signaling pathways, which are under the control of oncogenic load- tumor suppressor and proto-oncogenes. Tumor cells gain cell migration ability during the process of invasion. These two processes are under the control of ECM space which are filled with several components of adhesion molecules like heparan sulfate proteoglycans, laminin, collagen, fibronectin etc. (Frantz C et al., 2010) Adhesion molecules such as integrins and cadherins play a major role with their inside to outside signaling to sense the cancer cell response to tumor microenvironment (Frantz C et al., 2010). Furthermore, proteolytic enzymes and their inhibitors such as serine urokinase-type plasminogen activator and matrix metalloproteinases (MMPs), cysteine proteases (cathepsin), heparanase and TIMPs recede the extracellular layers. Strikingly, they are also involved in presenting growth factors, cytokinesis, chemokines to the other receptors through the process of paracrine signaling (Lamouille S et al., 2014). These molecules are therefore directly or indirectly involved in several signaling pathways which in turn regulate cancer cell invasion. Deregulated expression of integrins and the release of several proteases remodel the ECM, favoring conditions for cancer cell invasion. Cells form protrusions, cell –matrix interactions form focal contacts, and localization of proteases to the ECM promotes invasion and detachment of the cell from the ECM (Friedl P et al., 2011). In the following section, we will highlight some mechanisms by which CSCs contribute to the invasiveness and metastatic behavior ot tumor cells.

1.2.6.1 Epithelial to mesenchymal transition (EMT)

The process of invasion is also promoted by the adaptive trans-differentiation program called epithelial to mesenchymal transition (EMT), with a change in plasticity of cell- cell junctions. This process ultimately involves the invasion of cancer cells into the basement membrane in the neighboring microenvironment along the lymph and blood vascular systems, an

event that further contributes to the intra and extra-vasation of the tumor cells (Chambers AF *et al.,* 2002; Mani SA *et al.,* 2008). This process is mainly initiated by the increase in the expression of Rac-dependent genes and promotes the cells to gain mobile mesenchymal cells. During the process of EMT, several upstream regulators like TGFβ, *WNT, FGF,* and *EGF* control the activation of transcriptional repressors- *SNAIL1* and *2*, *TWIST* and *ZEB1*, which inhibits the transcription of E-cadherin (Kang Y *et al*., 2004: Burk U *et al*., 2008).

For example, in colon cancer cells a serine protease, TMPRSS4, activates EMT associated with *ZEB* transcription and a decrease in E-cadherin expression (Jung H *et al.,* 2008). Several upstream signals control this process. For example, the TGF-β targets integrin β4 and α6 (Jonathan M *et al.,* 2006) promote EMT conditions in colon cancer progression (Jonathan M *et al.,*2006). Furthermore, in colon cancer, activation of the PI3K/AKT pathway regulates the expression of Snail 1 (Wang et al., 2007a). Deregulation of the Wnt pathway leads to loss of E-cadherin expression further inducing the EMT process in colon cancer (Brabletz et al., 2001). Finally, aregulatory cascade of microRNAs is also involved in EMT process by promoting transcription of genes promoting this process (Burk et al., 2008). Interestingly, colon cancer cells undergoing the EMT process are resistant to immune and chemotherapy (Yang et al., 2006b).

Recent evidence suggests that EMT confers to stem cell properties (Mani et al., 2008). This process of attaining stem cell properties was understood from initial studies using embryonic stem (ES) cells. When human ES cell clusters were grown on matrigel they gained the properties of EMT with a change in molecular switch from E- to N-cadherin along with a gain in the expression of vimentin, metalloproteases, and Snail (Ullmann et al., 2007; Eastham et al., 2007). Interestingly, these cells also maintain the expression of Oct-4 and Nanog without losing their pluripotency. These expression profile and phenotypical studies revealed that ES cells could support a mesenchymal phenotype and also stem cell traits. The other interesting observation of mesenchymal cells controlling stem cells is made from reprogramming adult cells to gain the property of induced pluripotent stem cells. Skin fibroblasts acquired an undifferentiated mesenchymal phenotype when they were cultured with the removal of growth/differentiation factors. So, these cells remained in an EMT state with co-expressing mesodermal and endodermal markers (Lysy et al., 2007). These observations suggest that mesenchymal like stem cells, through the acquisition of a transient EMT conditions can regain pluripotency. So based on these studies, it is possible to consider normal stem cells and cancer stem cells to share a mesenchymal like cell state that may further regulate or preserve stemness. Upon transfection of untransformed immortalized human mammary epithelial cells with Snail1 and stimulation with TGFβ1, cells meet with EMT phenotype. When the cells are transformed by Ras, it regulated the population of

CD44high/CD24low stem cell-like cells with the induction of EMT phenotype (Morel et al., 2008; Mani et al., 2008), resulting in the formation of stem cells. So EMT contributes in the formation of high-grade invasive cells with stem cell-like signatures (Visvader JE et al., 2008). Cells that have undergone the EMT process are able to form spheroids and are high tumorigenic when they are xenografted at low dilution. Whether cancer stem cell in primary tumors drives EMT, or if it is during the process of EMT that these cells are originated is not yet completely understood (Brabletz T et al., 2005).

1.2.6.2 Migrating CSCs

Several recent studies observed that tumor recurrence and remission is due to the presence of metastatic precursors - cancer stem cells (Shiozawa Y et al., 2013; Brabletz T et al., 2012; Dieter et al., 2011). Metastasis is a process that spreads cancer from one part of the body (primary site) to other parts of the body (secondary site). Cancer cells exit from the primary tumor site, attains an EMT phenotype, travels to the blood stream or the lymph system and forms metastatic colonies in the secondary site of the tumor. This process is facilitated by CSCs. These cells having migratory properties and stem-cell-like features are called “mobile cancer stem cells” accordin to the `Migrating Cancer Stem Cell`(MCS)- concept. This process is strongly enhanced by EMT (Mani et al., 2008). It is also observed that circulating tumor cells (CTCs) link to CSC activity. During the process of metastasis, cells infiltrate the circulation and turn into circulating CSCs. These CTCs express stem cell markers (Aktas et al., 2009; Kasimir-Bauer et al., 2012). For example, CTCs obtained from colon cancer patients showed high CD133 positive cells (Hou et al., 2012). It is also observed that tumor- or cancer-associated fibroblasts enhance the metastases through an EMT process associated with CSC-like state (Aktas et al., 2009; Gregory et al., 2008). These cancer stem signatures or mesenchymal stem cell-like phenotype further differentiate to epithelial-like cells at the secondary tumor mass. Consequently, this process occurs through the transient de- differentiation (EMT)- to re-differentiation (MET). Mesenchymal to epithelial transition (MET) is an important driving force for the development of macrometastasis (Brabletz et al., 2001; Brabletz et al., 2005) (Figure 2).

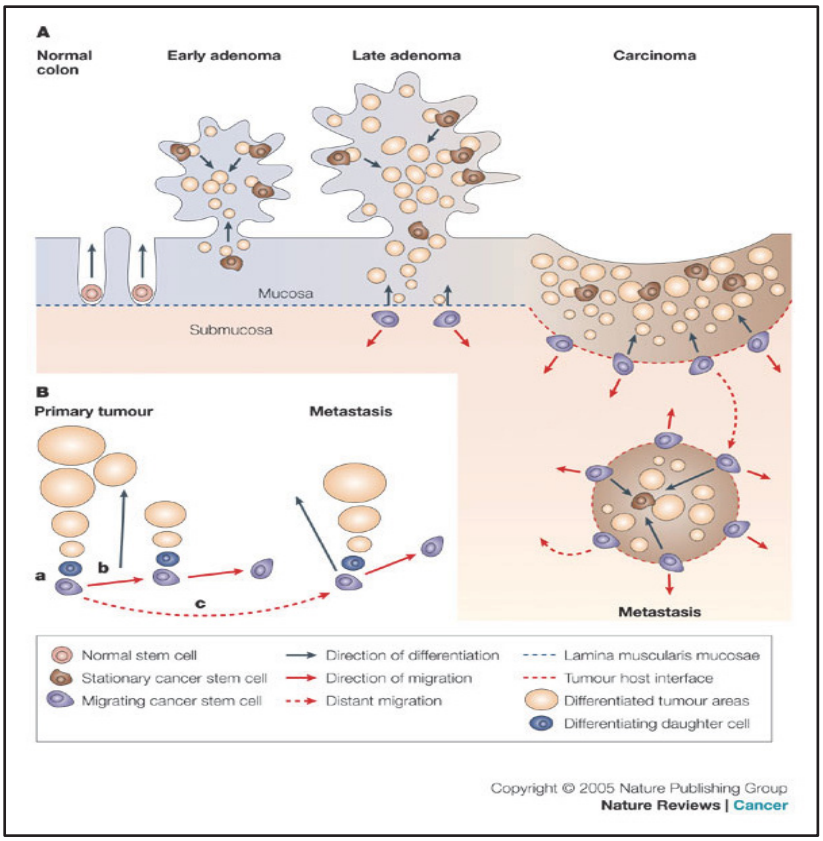


Figure 2. A schematic diagram of colon cancer malignancy – the concept of migrating cancer stem cells.

Normal stem cells are placed at a basal crypt region of the normal colon mucosa. Stationary cancer stem (SCS) cells are located in benign adenomas and can also be observable in carcinomas and metastases. A crucial step en route to malignancy is the promotion of epithelial to mesenchymal transition (EMT) of cancer cells, along with unique CSC-cells, that act as mobile, migrating cancer stem (MCS) cell. B) Precise perspective of the unique function of MCS cells in carcinomas and metastases. MCS undergoes asymmetric division in a primary tumour to form one daughter cell (non stem cell) and stem cell. Daugther cells programmed to proliferate and differentiae (a). This MCS or the new stem cells (cancer stem cells) increase the primary tumour mass (b), or ultimately diffuse through blood or lymphatic vessels and starts metastasis at the secondary location (c). Accordingly, the same mechanism involved in primary carcinomas and metastases. Picture adapted from Thomas Brabletz *et al.*, 2005.

1.2.6.3 De-Differentiation:

Cancer is associated with multiple levels of heterogeneity (one of the hallmarks of cancers). The concept of progression of differentiated cells to "dedifferentiated" cells with stem cell signatures in cancer was controversial for long period of time. However, recent studies show that this transition of differentiated cells to dedifferentiated cells has an important role in cancer stem

cell formation and tumorigenesis (Catherine A 2014; D Friedmann et al.,2014; Tata PR et al., 2013; A Jilkine et al., 2014). The exact mechanism that provides a detailed understanding of the process is not understood. It is stated that the capacity of differentiated cells/transit-amplifying cells to convert into CSCs occurs through the process of dedifferentiation by endogenously expressing pluripotent stem cell factors and their reprogramming networks. These processes are dynamically regulated by several factors like tumor microenvironment, epigenetic changes. It was reported that the expression of reprogramming transcription factors in cancer cells induced the stem cell like features with an unknown dedifferentiation mechanism (Y Li et al., 2012). So if upon inducing pluripotency to cancer cells, transforms them into a stem-cell state, it is possible that in cancer cells pluripotency genes also have the capacity to transit from cancer cell/progenitor to cancer stem cells with the associated process of dedifferentiation. This process, as stated above, will facilitate metastatic spread (Figure 2). Moreover, this dynamic, bidirectional network between differentiated cancer cells and tumor-propagating CSCs has appreciable implications in developing cancer therapeutics (Scheel C et al., 2010) (Figure 3).

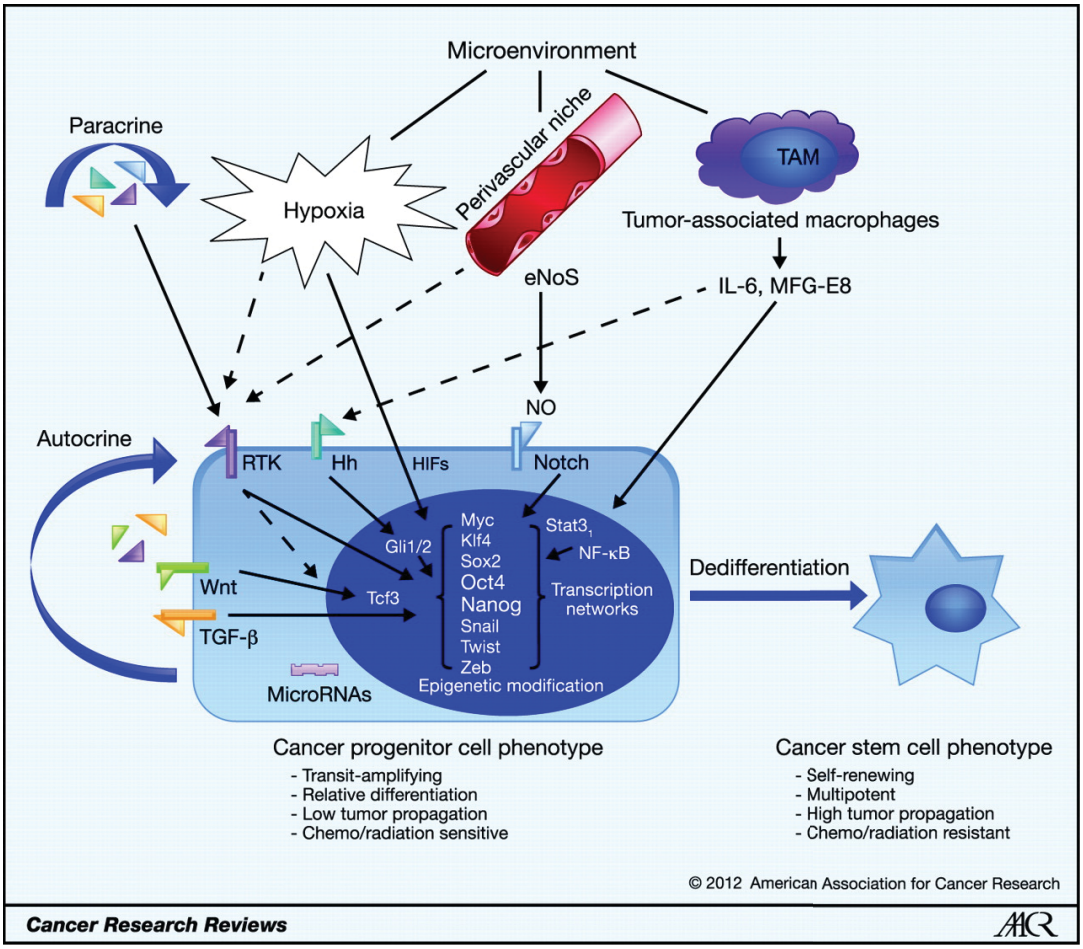


Figure 3. A schematic representation of the dedifferentiation mechanism in cancer.

Cancer progenitor cells gain stem cell like population by a dedifferentiation process. Figure 3 emphasizes the possible functional networks of signal transduction pathways, transcriptional networks, microenvironmental signals, and the epigenetic landscape, that can promote the dedifferentiation of cancer progenitor cells into a CSC phenotype. The embryonic transcriptional factor network acts as a central activator of the dedifferentiation process. Picture adapted from Yunqing Li *et al*., 2012.

1.2.7 Colon cancer stem cells

Colorectal cancer is one of the best-studied models to understand the characteristics of cancer stem cells among all solid tumors due to the presence of active stem cells at the crypt of intestine. The intestine is most widely studied organ in both development and also in malignancy (Barker N *et al.,* 2008). The inner lining of the intestine is one of the most widely dividing tissues in the body. Intestinal stem cells (ISCs) develop greater than 10^{10} new cells every day in the human gut and it has the capacity to renew new cells every 5 days (Nick Barker et al., 2008). In the colon, stem cells and transit amplifying cells (TA) are located at the base of the epithelial crypts (Figure 4). Cells exit from the crypts, enter villi and differentiate into enterocytes, goblet cells, or enteroendocrine cells, whereas the paneth cells escape this process and move to the crypt at the bottom.

Many researchers identified that intestinal stem cells (ISCs) regulate the normal intestine development, maintain tissue homeostasis, avoid tissue damage and controls the tumor growth. In the colorectum, deregulation of the AKT/PKB, Wnt and/or bone morphogenic protein (BMP) signaling pathways disturbs intestinal stem cell self-renewal (Elsa N Garza-Treviño et al., 2015)**.** This crosstalk is regulated by key signaling pathways including the Wnt, Hedgehog (HH) and bone morphogenic protein (BMP) pathways). These pathways directly or indirectly gauge the self-renewal of intestinal stem cells. Disruption of these tumors by oncogenic load leads to tumor promotion and progression. With lineage tracing experiments (Barker N et al., 2007) it was shown that two important genes are involved in the homeostatic control between tissue development and cancer progression. One is the Wnt target gene *Lgr5/GPR49*, which is highly expressed in the crypt base columnar cells (CBCs) (Clevers H et al., 2014).

A single $Lgr5^{+}$ stem cell can form a long-lived, self-renewing “short minimal- gut” (Toshiro Sato et al., 2009). The other interesting factor, Bmi1, which is known to be involved in the self-renewal of neural stem cells and hematopoietic cells, is also highly expressed in the intestinal crypts (Sangiorgi E et al., 2008). Using lineage tracing technology in mouse models, it was clearly shown that homeostasis and malignancy of small intestine epithelium is controlled by Lgr5 and Bmi1, which are involved in the self-renewal, proliferation and give rise to all differentiated

lineages. Among both the genes the concepts and theories of Lgr5 apears most convincing and this set up that Lgr5 is the powerful stem cell marker for tracing the development of intestine. However, the exact link between the Bmi1 and Lgr5 needs to be answered.

Among all the available markers for colorectal cancer, $CD133^+$ was the first marker to isolate and characterize the colon cancer stem cell properties (Ricci-Vitiani et al., 2007). Mushasi-1, a marker for neuronal stem cells is also proposed as a candidate marker for colon epithelial crypt cells (Okano H et al., 2002). A study also reported that a cell population characterized by the combined expression of $CD133^+$ and $Msi1^+$ has the highest metastatic ability compared to other marker combinations (Todaro, M et al., 2008). In addition to cell surface antigens/markers, high expression of ABCG2 and ALDH1 in colon cancer cells is considered as a well-established marker for detecting colon cancer stem cell population (Garza-Treviño EN et al., 2015). Colon cancer stem cell can generate *in vitro* undifferentiated colonospheres under serum-free conditions. In addition, several studies confirm the formation of tumors in NOD/SCID mice with implantation of a low percentage of colon cancer stem cells (Todaro, M et al., 2008). All these strategies suggest that colon cancer is a prime model to understand the concepts and theories of cancer stem cells.

1.2.8 Colon cancer stem cells - Role of Wnt signaling

It has been shown that few colon cancer cells in the primary tumor can acquire the properties of cancer stem cells through the process of EMT, and more importantly this process promotes secondary tumor formation in the process of metastasis (Brabletz T, et al., 2005). Even though the environmental factors that trigger the re-differentiation of cancer stem cells to metastases at the secondary site is not completely understood, it was clearly observed that Wnt signaling activities are coordinately involved in EMT-driven metastases (Barker N et al., 2007). This key switch of the Wnt signaling pathway is regulated depending on the tumor stage, primary growth and metastasis. In colon cancer, hyperactive canonical WNT signaling is mainly involved in promoting tumor-initiating cells (tumor stemness) with complex oncogenic events (Arnaud Duquet et al, 2014). A previous study showed that Wnt signaling acts as an antagonist in driving metastasis of colon cancer by coordinating the feedback regulation between Wnt and HH-GLI signaling, which further controls the expression levels of stemness associated genes *SOX2, OCT4* and *KLF4* in colon cancer (Ruiz i Altaba A. 2011). So rather than Wnt signaling, HH-Gli regulated stemness-associated genes which could induce a reprogramming event in cancer stem cells that promotes

high invasion and metastasis. These observations also gave a platform to understand the activities of Wnt signaling and associated genes in regulation of the normal and malignant ISCs indicating the link between intestinal stem cell biology and cancer These findings showcase the mechanisms by which the tumor cells are restricted from metastasizing, which is very useful in light of developing therapeutic targets.

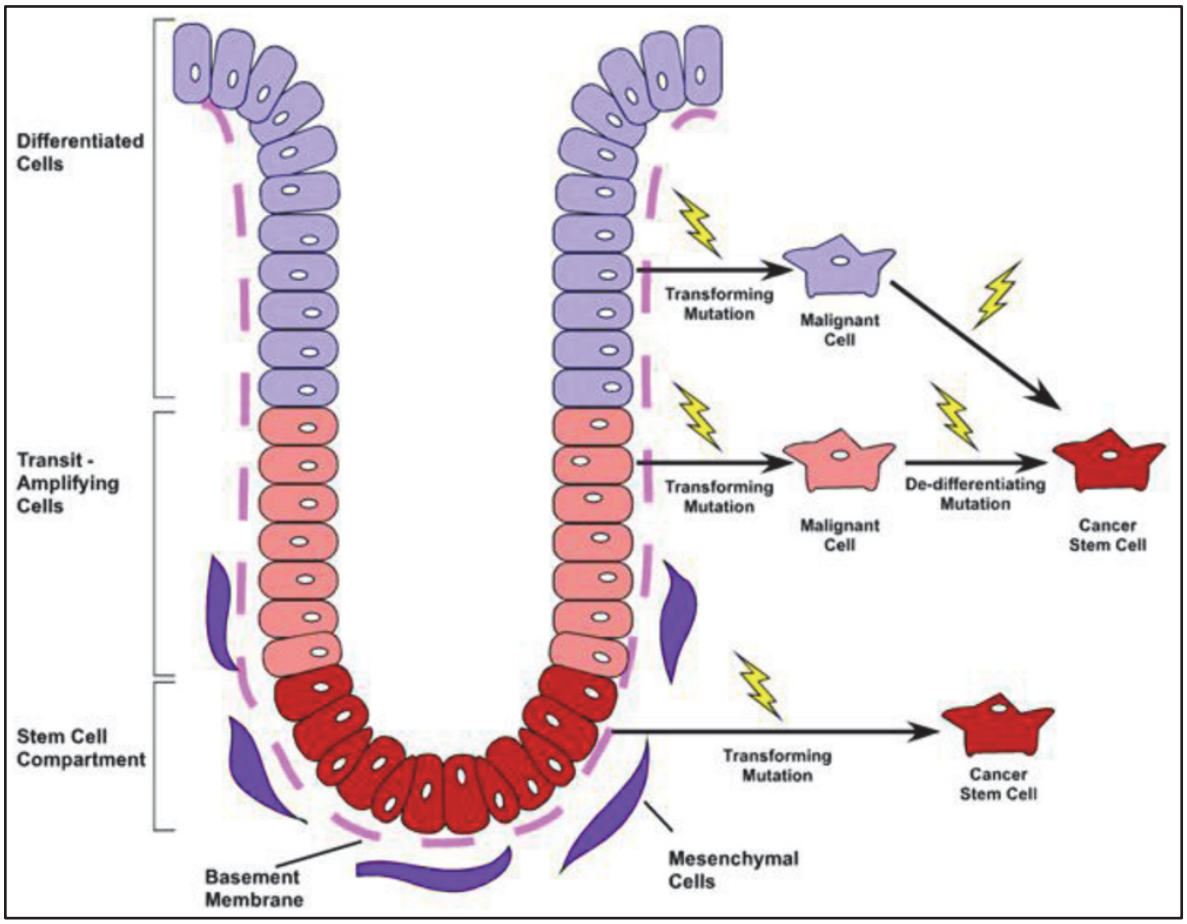


Figure 4. A schematic diagram of the human colonic crypt. (Left) The stem cell compartment is located at the base of the crypt. Transit-amplifying (TA) cells arise from this population and further differentiate into the enterocytes, goblet cells, or enteroendocrine cells (functional cells) of the colon. (Right) The common source of the colon CSC is not clearly understood. One particular transforming mutation (mutation that can transform completely) in a somatic intestinal stem cell could give rise to a CSC, whereas two mutations (one transforming and one de-differentiating) act as a positive selection force to drive the TA or differentiated colonic cell into a CSC. Figure adapted from Anderson E.C. et al., 2011.

1.3 Extracellular matrix dynamics

The extracellular matrix (ECM) and its associated components are involved in various functions in the normal cellular and tumor microenvironment. ECM remodeling is an important trigger for several cellular functions like branching, angiogenesis, morphogenesis and importantly ECM dynamics also involves in the regulation of stem cell niches. These dynamic changes involve changes in cell fate behavior through cell-cell contact and cell-ECM interactions (Daley et al., 2008).

Metalloproteinases (MMP) and disintegrins (ADAMTS) are the two important enzymes involved in the ECM remodeling targeting the glycoproteins and proteoglycans (Barter MJ et al., 2010). More importantly, the proteoglycan-associated enzymes like heparanase, involved in glycosaminoglycan (GAG) degradation, and GAG structural modification enzymes (SULF1 and SULF2) regulate the ECM dynamics that are under the tight homeostatic control of several signaling pathways (Ibrahim SA et al., 2014; Gomes AM et al., 2015). It is well appreciated that biophysical dialogue between ECM components plays multiple roles in the stem cell niches (Gattazzo F et al., 2014). This functional link is one of the reasons for the use of ECM receptors as surface markers for characterization of stem cells (Raymond et al., 2009). ECM receptors are in fact involved in several downstream signaling loops in turn regulating stem cell properties (Gattazzo F et al., 2014). Moreover, deletion of ECM receptor integrins inhibits the MYC levels decreasing the numbers of neural stem cells (Frye et al., 2003). One mode of how ECM molecules regulate stem cell niches is by the enhancement of signaling of FGF2, BMP4, and Wnt by heparan sulftate proteoglycans (Lanner F et al., 2010). Furthermore, in neural stem cells, FGF2 and BMP4 signaling is modulated by the expression levels of tenascin C (Garcion et al., 2004).

1.3.1 ECM dynamics in cancer progression

Mounting evidence suggests that ECM components and their degradative enzymes are associated with normal tissue homeostasis, while their dysregulation is associated with cancer (Lu P et al., 2011). For example, enhanced or decreased proteoglycan levels have a prognostic value for breast cancer and colon cancer (Fujiya M et al., 2001; Yip GW et al. 2006). Expression of various ECM remodeling enzymes is misregulated in many human cancers. For example, Heparanase, MMPs, sulfatases, are highly expressed in many cancers, whereas some HS sulfotransferases are silenced (Ibrahim SA et al., 2014; Vijaya Kumar A et al., 2014; Gotte and Yip Cancer Res 2006; Yip GW et al. 2006). Interestingly, recent reports showed the regulation of

the “metastatic niche,” by the “cancer stem cell niche”, with abnormal changes in the ECM dynamics (Lu P et al., 2012; Filatova A et al., 2013).

1.3.2 Integrin signaling

During 1980s, several interesting findings identified integrins as highly expressed receptor proteins in the ECM, attracting many scientists in the field of biology. Integrins are involved in cell-cell adhesion and cell-matrix contacts, in turn regulating several downstream signaling pathways through cytoskeleton interactions (Hynes RO et al., 2202). Admitting the importance of integrins as one of the major components in the ECM region, several non-integrin receptors also occupy this region. Interestingly, symbiotic association between integrins and surface receptors act cooperatively in t integrin-mediated cell adhesions (Xian X et al., 2011).

Integrins are dimeric (heterodimers) transmembrane proteins consisting of α (alpha) and β (beta) subunits. In mammals, there are eighteen α and eight β subunits. Integrins structurally have three conformations in major - inactive, active and ligand bound states (Alberts B et al., 2002) It is stated that inactive integrins have a bent – V shaped confirmation whereas, if the ligand is binding to the integrin it attains the straight conformation, ie., the plasma membrane is rearranged towards the integrin ligands (Gahmberg CG et al., 2009). Integrins are involved in both “outside-in” and “inside-out” signaling. During “inside-out signaling” cytoskeleton-associated proteins such as vinculin and talin binds to the β-subunit of integrins, increase the integrin affinity to bind to extracellular ligands, in turn regulating cell migration, invasion and also the cell adhesion process through ECM binding and assembly. Whereas in “outside-in” signaling, the ligand binds at the extracellular domain of the integrin clusters, and activates changes in the cytoskeleton and gene expression regulating several downstream pathways. This signaling network in turn regulates the cell polarity, survival, migration and proliferation (Mas-Moruno C. et al., 2010). The other interesting function of integrins is their interaction with growth factor receptors, thus mediating crosstalk regulating several signaling cascades and cellular responses (Morgan MR et al., 2007).

1.3.3 Interplay between Integrins and focal adhesion kinase (FAK)

Integrins remodel the ECM and control three important phenomena at the cellular level - focal adhesions, podosomes and invadopodia. Focal contacts are activated by the clustering of integrins which have the capacity to drive the mechanical forces of the cell body through the focal adhesion kinase (FAK)/Src signaling complex (Schwartz MA et al., 2010). FAK is a non-receptor

tyrosine kinase that is activated upon integrin activation and promotes the complex network of signaling pathways important for cell survival, proliferation, migration and invasion (Golubovskaya VM et al., 2014). Recent reports showed the importance of FAK in the EMT process and also in the maintenance of cancer stem cell traits (Williams KE et al., 2015). The N-terminal domain of FAK is called FAK the FERM domain, which has the function of preventing the activation of kinases. FAK is brought to the adhesion site through integrin and paxillin. Upon binding of the ligand to integrins, FAK is autophosphorylated at Tyr397 creating the affinity to bind to the SH2 domain of Src. This FAK/Src complex in turn phosphorylates and promotes the activation of several proteins further regulating the several signaling pathways and gene expression (Seong J et al., 2011).

1.4 Heparan sulfate proteoglycans

Proteoglycans are composed of a core protein and a glycosaminoglycan (GAG) part, which are covalently attached to each other. Based on the composition of GAG, four main classes of GAGs exists: heparan sulfate (HS), chondroitin sulfate (CS), dermatan sulfate (DS) and keratan sulfate (KS). Heparan sulfate proteoglycans (HSPG) are principally decorated with heparan sulfate (HS) GAG chains, but may contain additional modifications, e.g. CS (Bernfield *et al.,* 1999). HSPG are mainly found at the cell surface or in the extracellular matrix Heparan sulfate is structurally closely related to heparin. They mainly differ in their disaccharide composition. Both have alternating units of glucosamine (GLcN) with glucuronic acid (GLcA) or iduronic acid (IdoA). HS contains high proportion of glucurnonic acid, wheras heparin contains more iduronic acid, and a higher degree of overall sulfation (Esko *et al.,* 2002). HSPGs are major ECM components that interact with a plethora of ligands (Esko et al., 2009). Using several genetic models, it was shown that the composition of GAG and the expression of HSPGs control the cellular fate due to changes in the ECM dynamics (Kramer et al., 2003). An important feature of HSPGs is that they are involved in both embryonic development and in cancer, showing the possibilities to apply the principles and theories of development to understand the cancer progression.

According to the location, HPGSs are mainly three types: syndecans, (transmembrane proteoglycan), glypicans (glycosylphosphatidylinositol-anchored proteoglycans), agrin, perlecan, type XVIII collagen (extracellular matrix secreted HSPGs) and serglycin (secretory vesicle proteoglycan) (Bernfield et al. 1999). Membrane proteoglycans mainly act as a coreceptors for

growth factor receptors (e.g., fibroblast growth factors, FGFs) (Rapraeger et al. 1991), chemokines, cytokines, morphogens and several ECM ligands. Transmembrane proteoglycans coordinate with integrins and several ECM proteins (e.g., fibronectin) acting as cell adhesion receptors (Bernfield et al. 1999). HSPG binds to chemokines and thereby regulate the leukocyte recruitment during inflammation (Wang et al. 2005, Vijaya Kumar et al. 2015). Glypicans are involved in morphogen gradient formation, which is an important signal for development (Jackson et al. 1997). The HS chain of HSPGs is cleaved by the enzyme-heparanase, whereas core proteins of some transmembrane HSPGs can be cleaved by matrix metalloproteinases (MMPs). For example, the extracellular domains of Syndecans can be shed by MMPs, and get transported along the ECM space and present HS bound growth factors to other receptors (Bernfield et al. 1999). With these functions they are involved in several cellular fates: cell proliferation, adhesion, migration, invasion, survival, matrix assembly, differentiation, endocytosis, inflammation, defense mechanism, angiogenesis and also in stem cell niche maintenance (Symes et al., 2010).

Depending on the tumor type and stage, HSPGs are deregulated in various cancers and indeed correlates with poor prognosis in several malignancies (Cattaruzza et al., 2008; Frankel et al., 2008). Sonic Hedgehog (Shh) signaling - an extremely complex signaling pathway involved in embryonic development and in cancer is well established (Gupta S *et al*., 2010).. Recent evidence indicates that this pathway involves in a variety of cancer stem cell properties. For example, a few reports highlighted the importance of the proteoglycan interaction with Sonic Hedgehog in promoting human malignancies (Rosalind A Segal et al., 2009: Glise et al., 2005). In this context, the Cardin-Weintraub motif present in the N terminus of SHH ligand binds to heparan sulfate, triggers a specific transduction cascade, and regulates the transcriptional factor, Gli, thus driving continuous proliferation (Denis M et al., 2009).

1.4.1 Heparan sulfate proteoglycans and the stem cell niche

Stem cell niches are very complex locations, which still need to be completely understood (Nurcombe and Cool 2007). A few reports showed the contribution of HSPGs in stem cell maintenance and differentiation: Using embryonic stem cells (ESCs), it was shown that HSPGs expression is involve in the self-renewal i.e., pluripotent state and also in the differentiation of committed cells to specific lineages through a promotion of Wnt, FGF and BMP signaling (Sato et al. 2004). One important observation was that the GAG composition and the HSPG expression levels were very crucial for the maintenance of self-renewal of pluripotent cells and also for differentiation to a specific cell fate (Johnson et al. 2007). By biochemistry-based studies, it was

shown that normal ESCs produce majorly around 80% of HS and a lesser percentage of chondroitin sulfate (CS). Strikingly, the level of CS was increased along with HS during ESC differentiation (Nairn et al., 2007). During the transition of ESCs from an undifferentiated to a committed cell type the level of HS sulfation was increased (Hirano et al., 2012). The level of ECM components is very important during embryoid body (EB) formation. For example, during the differentiation of mesoderm lineage, particularly cardiac lineage high expression of fibronectin is important. GAG expressed during the EB formation binds to ECM molecules and regulates lineage specificity (Shukla et al., 2010). For example, there was a strong increase in the expression of the HS biosynthetic enzymes Ndst-4, 3-HSst-3a, and 3-HSst-5 (highly expressed in brain tissues) during the transition of undifferentiated ESCs into a $Sox1^{+}$ neural differentiated cells (Johnson et al., 2007). From ex vivo studies, it was observed that mouse embryonic stem cells carrying mutations in HS biosynthetic genes (Ext1) were not capable to differentiate upon withdrawal of leukemia inhibitory factor (Kraushaar et al. 2010). Supporting this observation, deletion of Ndst1/2 in mouse embryonic stem cells reduced FGF binding due to reduced sulfation and cells were able to differentiate into specific lineage fate (Lanner et al. 2010). The important observation was lack of Ext1 and Ndst1/2 failed to differentiate into ectoderm, mesoderm and endoderm but static to maintain the expression of ESC markers (Lanner et al., 2010; Forsberg et al., 2012). So it is suggested that HS- binding ligands Wnt, BMP, FGFs may have anti-differentiation effects (Ying et al., 2003). Upon closer examination it is observed that HS binds to FGFs in ESC cells. For instance, presence of HS in ESC cells inhibits expression of the pluripotency gene *Nanog*. Additionally, in Ext deleted ESCs a high level of Nanog expression was observed (Kraushaar et al., 2010). This indicates that lack of HS and associated enzymes may not be important for pluripotent status. These findings paid important contributions of HSPGs in understanding the stem cell based disease models and also improving the iPSC technology protocols.

1.4.2 Syndecans

Syndecans are family of conserved transmembrane heparan sulfate proteoglycans classified into Syndecan 1, 2, 3 and 4 depending on their molecular weight, location and function (Götte M et al., 2003). Invertebrates have only one type of syndecan (Bernfield et al. 1999). They play major role in the process of embryonic development, angiogenesis and in tumorigenesis. Each syndecan has three main structural domains: the conserved cytoplasmic, and transmembrane domains and an extracellular domain (Figure 5). The extracellular domain part is finely decorated with the GAGs heparan sulfate (HS) and less of chondroitin sulfate (CS) at least for some syndecan

members (Couchman JR et al., 2003). The exact role of CS is not clear, but the HS of syndecan binds to a variety of ligands- FGFs, transforming growth factor-β (TGF-β), vascular endothelial growth factors (VEGFs), and platelet-derived growth factors. It also binds to several extracellular matrix proteins, such as fibronectin, collagen, laminin and the plasma protein antithrombin-1 (Tkachenko E et al., 2005).

Depending on the cell type and location, the expression of syndecans has different functions. Each syndecan has spatial expression pattern during development. Syndecan-1 is highly expressed in epithelial and mesenchymal cells, syndecan-2 in mesenchymal, epithelial, endothelial cells and fibroblasts and neuronal cells, syndecan-3 in musculo-skeletal tissues and neuronal cells, syndecan-4 is almost expressed is all cell type particularly high expression in fibroblasts (Götte M et al., 2003; Couchman JR et al., 2003). In a signaling and functional context, it is generally considered that syndecan-1 and syndecan-3 form one group and syndecan-2 and syndecan-4 form another subfamily. For example, in general syndecan-1 and syndecan-3 frequently inhibit cell growth whereas syndecan-2 and syndecan-4 promotes cell growth. One possible structural reason could be the higher amino acid sequence homology of Syndecan-1 and Syndecan-3 compared to Syndecan-2 and Syndcan-4 (Tkachenko E et al., 2005).

1.4.2.1 Syndecan-1

Among the HS proteoglycans, Syndecan-1 (Sdc-1) represents the predominant epithelial HSPG preserving the structural integrity of the intestine (Bode, L et al., 2008). It binds to an array of proteins: FGF2, VEGF, HGF, along with several ECM components fibronectin, collagen I, III, V and tenascin. Functionally, within the tumor microenvironment, Sdc-1 acts as a co-receptor for growth factor signaling and mediate cell proliferation, survival, invasion, cell matrix assembly (Bernfield M et al., 1992). Sdc-1 participates in integrin-mediated signaling events by cooperating with integrins and other cell adhesion receptors to facilitate cell-ECM attachment, cell–cell interactions, and cell motility thus providing a physical and functional link to the cytoskeleton. Sdc-1 KO mice showed viable offsprings, but abnormality in the re-epithelialization after injury was observed with increased leukocytic infiltration (Kumar AV et al., 2015). Syndecan-1 decreases Wnt-1 signaling activities in Sdc-1 knockout mice compared to the wild type in mammary gland specific tumors (Liu BY et al., 2003). It was observed that Sdc-1 is essentially involved in controlling intestinal inflammation and DSS treatment to Sdc-1 KO mice worsened the phenotype with high lethality (Floer, M et al., 2010). The exact signaling mechanism of syndecans is an area of effective research. Even though the mechanisms are understood with many studies it

is still a matter of controversy. Syndecan-1 is involved in inflammation (Götte M et al., 2003) with an increase in the leukocyte–endothelial interactions in Sdc-1 KO mice (Götte M et al., 2002). One possible mechanism is the enhancement of leukocyte integrin-ICAM-1 interactions in the absence of Sdc-1 (CPVijaya Kumar et al. 2015). Increased expression or shedded syndecan-1 has an inhibitor effect on cell proliferation due to lack of FGF2 induced proliferation (Mali M et al., 2003). Downregulation of syndecan-1 expression in epithelial cells by siRNA results in a change in cell plasticity with loss of cell polarity and decreased the expression of E-cadherin levels, which suggest an important transition switch during development and in wound healing through epithelial–mesenchymal transition (Couchman JR et al., 2001). Syndecan-1 overexpressing mice had delayed dermal wound repair due to the inhibitory role of soluble syndecan-1 ectodomain, which enhanced proteolysis in the granulation tissue. (Elenius, V et al., 2004).

1.4.2.1.1 Role of syndecan-1 in malignancy

It has been reported that during tumor development, complex changes occur in the expression pattern of Sdc1 and loss of syndecan-l is associated with local tumor stage and metastasis (Gotte, M et al., 2007; Hashimoto, Y et al., 2008). Sdc-1 functions as a double-edged sword depending on the tumor context it either promotes or inhibits tumorigenesis. However, it is poorly understood how Sdc-1 integrates these two routes of signaling and what the consequences for malignant cell behavior are. It is observed that Sdc-1overexpression or shed form of Sdc-1 inhibits the cell growth, cell migration and invasion. However, in certain conditions heparanase can promote degradation of the HS within the syndecan-1 ectodomain to become an activator. For instance, if HS is bound to FGF2, it promotes mitogenicity (Kato M et a1., 1998). So, in a way functions of syndecan-1 may depend on the availability of heparanase, which can generate HS fragments capable of enhancing signaling. In breast cancer, highly expressed syndecan-1 is able to bind to the FGF-1 and FGF-2 and increases mitogenicity (Fernig et al., 2000). Notably, in MCF-7 cells siRNA knockdown of Sdc-1 reduces FGF-2-dependent activation of MAP kinase activity (Floer et al., 2010). In myeloma cells, high expression of syndecan-1 is prone to poor survival like breast cancer. It was reported that syndecan-1 acts as a coreceptor that binds to HGF and mediates mitogenic signaling (Derksen et al., 2002). Furthermore, the soluble form of syndecan-1 inhibits the proliferation compared to the transmembrane form in breast cancer cells, which may be because of competitive binding of ligands (Nikolova et al., 2009). In contrast, in endometrial cancer cells, overexpression of Sdc-1 increased cancer cell proliferation through the activation of nuclear factor kappaB (Oh et al., 2009). Moreover, addition of purified Sdc-1 ectodomain induced apoptosis with the downregulation of cyclin D1 in myeloma cells (Dhodapkar et al., 1998). The

expression of cell adhesion molecule E-cadherin (E-cad) is proportional to the expression of syndecan-1 in many tumor cells like colon carcinoma, prostate cancer, and in breast cancer cell lines (Bernfield et al., 1999; Nikolova et al., 2009). For example, loss of cell surface syndecan-1 decreases the E-cadherin expression levels in cell culture models. In colon cancer, decreased expression of syndecan-1 indicates an aggressive tumor type with poor patient survival and prognosis (Day RM et al., 1999; Fujiya M et al., 2001; Conejo JR et al., 2000; Mennerich, D et al., 2004). So, as syndecan-1 expression maintains epithelial integrity, loss of expression results in a change in epithelial morphology, polarity promoting epithelial-mesenchymal transition (Lind et al., 2004) but interestingly, syndecan-1 expression restored the epithelial phenotype (Leppä S et al., 1992, 1996).

Consistent with these findings, the malignant transformation of Caco2 cells resulted in a decrease in the syndecan-1 expression (Levy P et al., 1996). This loss of syndecan-1 expression changes the epithelial morphology, forms filopodia and exhibits the anchorage-independent growth with changes in focal adhesions (Bernfield et al., 1999). So, these syndecan-1 low cells acquire a migratory phenotype altering the ECM dynamics through the activation of integrins and FAK signaling pathways, which further regulates the Rho-GTPase-dependent actvities in tumor cells (Ishikawa T et al., 2010).

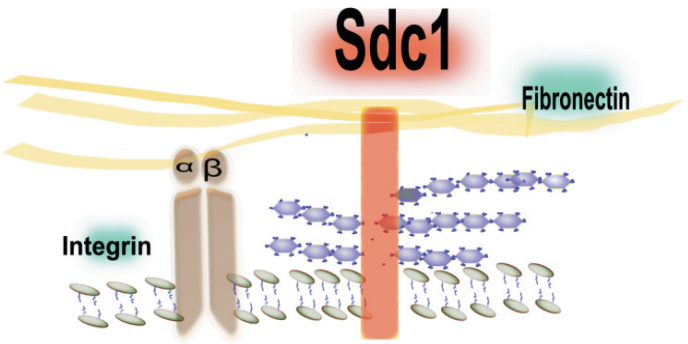


Figure 5. Schematic diagram of the structure of Syndecan-1: It acts as a transducer between the ECM and the cytoskeleton. Syndecan-1 coordintae with integrins and regulate cell adhesion and

cytoskeltol rearrangement. It possesses conserved transmembrane domains, a cytoplasmic domain and a unique ectodomain. The ectodomain is exposed to the ECM and it is which is decorated with heparan sulfate (HS) glycosaminoglycan chains. Several growth factors, chemokines and ECM components like firbonectin bind to the extracellular HS. The cytoplasmic domain is involved in the activation of several cytoplasmic kinases, and is capable of binding scaffolding proteins which can interact with cytoskeletal elements (not shown).

1.4.3 Heparanase

Mounting evidence suggests that ECM components and their degradative enzymes are associated with the normal tissue homeostasis and cancer. Several reports showed the influence of heparanase in tissue remodeling, cancer progression with invasion, metastasis and angiogenesis (Vlodavsky I et al., 2001) properties. With its endo-β glucuronidase activity heparanase cleaves the HS chains into large fragments of about 2-10kD or around 10-20 sugar units, which are sequestered with protein ligands promoting their biological functions (Vreys V et al., 2007; Zcharia E et al., 2009). The pH optimum for heparanase catalysis peaks at 5 and the enzyme possesses little activity above 7 (Freeman C et al., 1998). Heparanase is expressed as a latent 65-kDa pro-enzyme that is enzymatically inactive and requires cellular processing to form an active dimer of 8 and 50kDa subunits (Vlodavsky I et al., 2001). Recently, a close homologue, heparanase 2 has been described, which appears to show enzymatic inhibitory functions (Levy-Adam F et al., 2010). Heparin, which is endogenously released by activated mast cells and eosinophils, controls the enzymatic activity of heparanase (Temkin V et al., 2004).

a) Expression: The transcriptional control of heparanase is not well understood. The expression of heparanase differs between normal and pathological conditions. In normal/noncancerous cells, expression is low due to heparanase promoter methylation, and also wild type p53 suppresses the heparanase transcription by directly binding to its promoter (Ogishima T et al., 2005; Baraz L et al., 2006). In cancer, mutational inactivation of p53 promotes heparanase transcription. It is observed that the transcription factor early growth response 1 (EGR1) acts as an activator or a repressor of heparanase promoter depending on the cell type and tissue (de Mestre AM et al., 2005).

b) Role in inflammation. Heparanase controls the sequestering and release of chemokines/cytokines in the ECM region and is involved in the leukocyte recruitment and migration at the inflamed sites. It was furthermore reported that heparanase involves in the activation of leukocytes with endothelial cells and initiates the innate immune response through

toll-like receptor (TLR) 4 (Akbarshahi H et al., 2011).

c) Role in malignancy. The role of heparanase in cancer progression is known for almost three decades. It is very clearly observed that heparanase is overexpressed in highly invasive and metastatic tumors (Vlodavsky I et al., 1983). Later on, many reports demonstrated that heparanase promotes and enhances tumor growth in many human cancers (including colon, prostate carcinoma, myeloma, and breast) (Edovitsky E et al 2004 ;Lerner I, et al., 2008). Further studies with heparanase gene silencing clearly showed the inhibitory activities of malignancy showing the direct evident of heparanase in pro-tumorigenic functions (Roy M et al., 2005). Interestingly, it was reported that heparanase acts as a pivotal gene in inflammation-driven colon cancer (Lerner I et al., 2011).

Heparanase cleavage of HS generates fragments bound to cytokines such as FGF2, VEGF etc., which travel across a gradient along the ECM space and present these ligands to several receptors, in turn facilitating various signaling networks (Figure 6). With this function, heparanase acts as a pro-tumorigenic agent, and at cellular level it promotes cell proliferation invasion, metastasis and angiogenesis (Myler HA et al., 2002). Strikingly, enzymatically inactive mutant heparanase also regulates adhesion dependent signaling. It was shown that the inactive latent enzyme triggers the PI3K/Akt signaling pathway without the involvement of heparan sulfate. Further studies in several cancer cell lines showed that latent or enzymatically inactive form is involved in several signaling networks (Gingis-Velitski S et al., 2004; Cohen E et al., 2008). These observations lead to further investigations of the role of heparanase in signaling. As a result of these studies, it was confirmed that heparanase regulates several downstream targets including Akt, Src, p38 MAPK, EGFR, and STAT3, even though the exact functions and receptors are not clear (Zetser A et al., 2006; Cohen-Kaplan V et al.,2008; 2012). Strikingly, heparanase exposure increased the levels of β1 integrin in a glioma cell line (Zetser A et al., 2003), adding another level of non-cytokine-dependent signaling. Several drugs are currently developed to target the expression of heparanase in several human cancers, these include small-molecule inhibitors, phosphomannopentaose sulfate (PI-88), polyanionic molecules like laminarin sulfate, modified heparin, the fully sulfated HS mimetic PG545 and a novel inhibitor SST0001, another modified heparin (Ritchie JP et al. 2011).

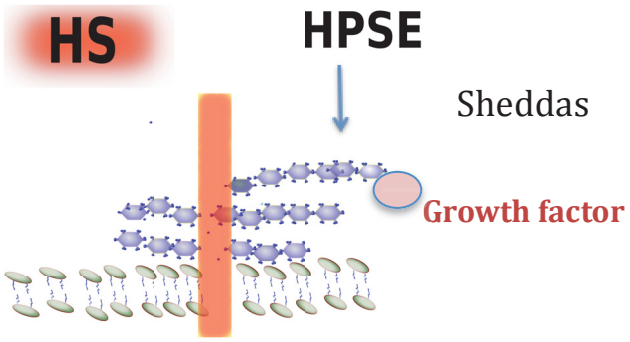


Figure 6. Schematic diagram of some of heparanase functions. The side chains of HSPGs, bind to several ligands (eg: growth factors, cytokines, morphogens). Heparanase cleaves HS at specific sites, generates small fragments and presents them to several unknown receptors through the process of paracrine signaling.. Cleavage of HS also, remodels the ECM membrane, promotes several adhesion dependent signals, which further controls the several cytoskeletol-signaling events. These functions of heparanase regulate tumor invasion and metastasis.

1.4.4 The Syndecan-1- heparanase axis

Recent studies indicate that the interplay between Syndecan-1 and heparanase show important functional connections in the progression of colorectal cancer and myeloma. For example, in colon cancer progression there is a gradual increase in the expression of heparanase (*HPSE*) (Friedmann Y et al., 2000) and decrease in the Syndecan-1 (Sdc-1) (Hashimoto, Y et al., 2008) expression from the well differentiated to poorly differentiated colon carcinoma. Deeply invading colon carcinoma cells showed decreased expression of Sdc-1 (Fujiya M et al., 2001) and increased expression of HPSE (Takaoka Met al., 2003;
Levy P et al.,1996). Overall loss of Sdc-1 and increase in *HPSE* levels showed a change in cell plasticity with an increase in invasiveness but the underlying mechanism is not understood.

Even though it is not fully understood, it gives an impression that heparanase regulates the structure of syndecan-1 by degrading its HS chains, and augments growth factor signals (Purushothaman A et al., 2012; Ma P et al., 2006). Interestingly, this heparanase/syndecan-1 axis is involved in tumour and host cell signaling events including immune cells, endothelial cells, and fibroblasts in the tumor stroma. It was observed that heparanase enhances the HGF expression and in myeloma cells syndecan-1 binds to the hepatocyte growth factor HGF. Apparently, the cross talk between heparanase, syndecan-1 and HGF promotes myeloma malignancy (Derksen PW et al., 2002). Up-regulation or addition of heparanase enhances syndecan-1 shedding in myeloma cells. It

is reported that heparanase activates ERK signaling, and regulates matrix metalloproteinase-9 (MMP-9) to cleave syndecan-1 (Purushothaman A et al., 2008). Notably, heparanase clusters syndecan-1 and promotes cell adhesion through a cascade of signaling networks, which include the Rac1, Src and PKC pathways in glioma cells (Levy-Adam F et al., 2008). Strikingly, activated heparanase modifies heparan sulfate, increases the affinity for efficient binding of FGF2 and regulates focal adhesion kinase phosphorylation (Reiland J et al,, 2006). It could also be possible that enhanced heparanase may not directly cleave heparan sulfate chains but may increase the expression of MMP, which in turn mediates the sheddase activity of syndecan-1 (Purushothaman A et al., 2008)

Interestingly, syndecan-1 and heparanase are also expressed in the nucleus. The exact mechanism is not understood but it was reported that heparanase in the nucleus decreases the nuclear syndecan-1 and favours the histone acetyl transferase enzyme (HAT) activity (Purushothaman A et al., 2011). Recent reports also showed the expression of heparanase and syndecan-1 in exosomes (Thompson CA et al., 2013), which could be the transport mechanism to the nucleus.

1.5 Aims of the thesis

Dysregulation of heparan sulfate proteoglycans (HSPGs) and associated enzymes involved in their processing and biosynthesis has been known for decades (Iozzo RV et al., 1982; Götte et al., 2007). Their use as diagnostic and prognostic markers in several cancers underscores their clinicopathological relevance. The HSPG Syndecan-1 (Sdc-1) touches upon modern aspects of biology serving multiple biological roles. Even though Sdc-1 functions such as its role as a coreceptor for multiple signaling pathways and as a matrix receptor are well-studied, current strategies to target Sdc-1 are not yet successful due to its diverse functions of Sdc-1 in the tumor microenvironment. In colon cancer, e.g., a decrease in the expression of Sdc-1 correlates with poor patient survival in spite of its role as a coreceptor for FGF signaling. Restoring the functions of cell surface Sdc-1 may enhance the FGF signaling leading to more aggressive phenotype, and drugs targeting the shed Sdc-1 (soluble Sdc-1) may enhance the efficiency of fibronectin binding to integrins thereby enhancing their downstream signaling. Moreover, targeting of Sdc-1 at the expression level can cause off target effectss by increasing the expression of other syndecans (Gharbaran R et al., 2015), thus hindering the success of current colon cancer therapeutics. To overcome these problems, we aim to determine novel Sdc-1dependent molecular mechanisms in order to target the Sdc-1 influenced downstream signals with combinational therapies and drugs which may provide more efficient anti-tumor effects.

With reference to the clinical data, loss of Sdc-1 expression and increase in HPSE expression are observed during progression from well-differentiated to poorly differentiated colon carcinoma, ultimately correlating with poor patient survival (Vlodavsky I et al., 1983; Day RM etal., 1999; Fujiya M etal.,2001; Edovitsky E et al 2004; H Wang et al., 2010). This expression is gauged by the degree of cancer cell plasticity, which acts as a positive selective force in controlling the cancer stem cell population thus speeding carcinogenesis (Scheel, C et al., 2012). Owing to the role of Sdc-1 and HPSE in tumor growth, invasion and metastasis we aim to investigate the contribution of Sdc-1 in cancer stem cell properties in two well-established colon cancer cell lines (Caco2 and HT29).

Specifically, we want to address the following key questions:

1) Contribution of Sdc-1 to colon cancer stem cell properties, its possible link to the process of epithelial-to-mesenchymal transition (EMT), and the underlying molecular signaling mechanisms (Figure 7).
2) To investigate the molecular interplay between Sdc-1 and HPSE and their influence on colon cancer stemness-related molecular signatures and functions. (Figure 8).

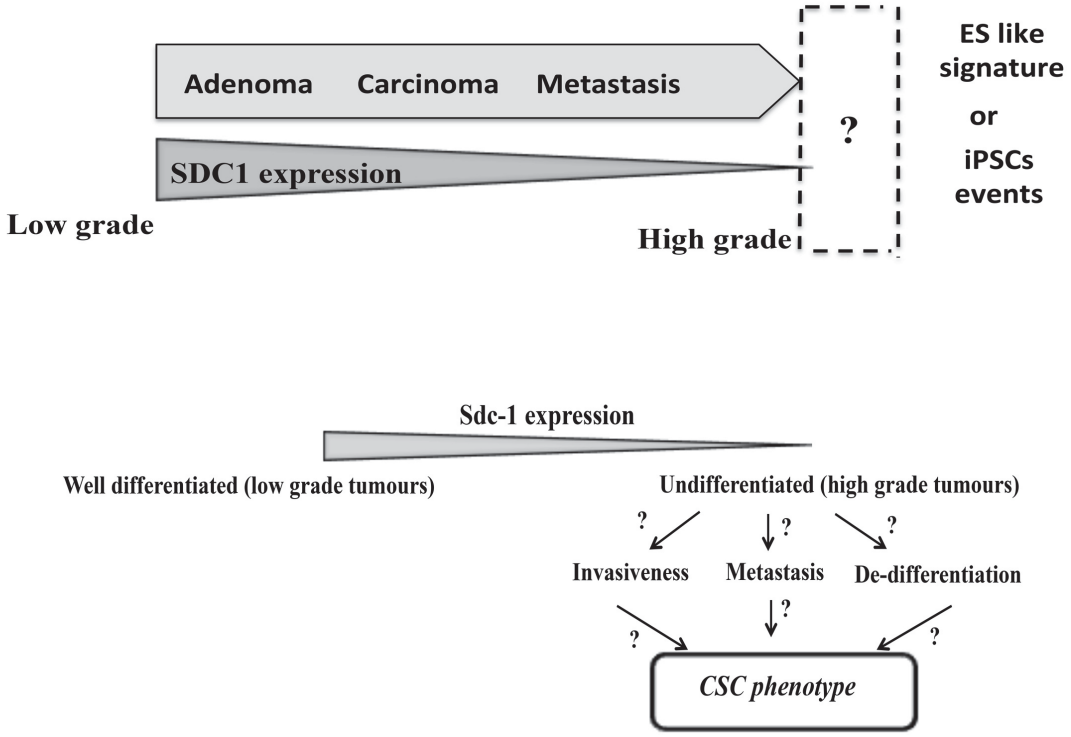


Figure 7. Potential roles of Sdc-1 in colon CSC function.

During colon cancer development, complex changes occur in the expression pattern of Sdc1 from well differentiated to undifferentiated tumours. This loss of Syndecan-1 is associated with a change in phenotypic plasticity with an increase in invasiveness, metastasis and dedifferentiated cells. Empirical evidence showed that this change in phenotypic plasticity allow cancer cells to dynamically enter into embryonic stem-cell or induced pluripotent stem cell like state. So, we are interested to investigate if the decrease in Sdc-1 expression has any influence on stem cell like properties in cancer cells.

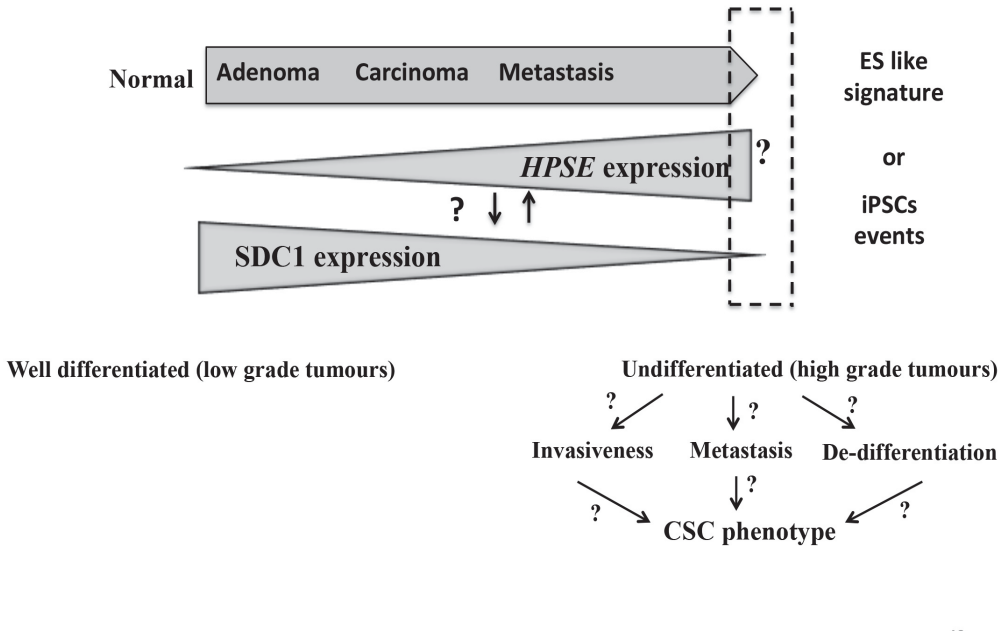


Figure 8. Possible functions of heparanase and its interplay with syndecan-1 in CSC function.

During colon cancer progression, a decrease of Sdc-1 and increase of HPSE expression are observed from well differentiated to undifferentiated tumours. This complementary change in expression is associated with change in phenotypic plasticity with an increase in invasiveness, metastasis and dedifferentiated cells. So, we are interested to investigate the interplay of Sdc-1 and HPSE and the role of this axis in invasion, and its influence o stem cell like properties in colon cancer cells.

2. Materials and methods:

Caco-2

ATCC® Number: HTB-37™

Type: Colorectal adenocarcinoma

Species: human

Culture medium: RPMI 1640 supplemented with 1% Glutamine, 1% Penicillin/Streptomycin and 10% FCS

Culture conditions: 37 °C, 5% CO_2, 100% relative humidity

HT-29

ATCC® Number: HTB-38™

Type: Colorectal adenocarcinoma

Species: human

Culture medium: DMEM high glucose supplemented with 1% Glutamine, 1% Penicillin/Streptomycin and 10% FCS

Culture conditions: 37 °C, 8% CO_2, 100% relative humidity

COLO 205

ATCC® Number: CCL-222™

Type: Colorectal adenocarcinoma, derived from metastatic site

Species: human

Culture medium: DMEM high glucose supplemented with 1% Glutamine, 1% Penicillin/Streptomycin and 10% FCS

Culture conditions: 37 °C, 8% CO_2, 100% relative humidity

2.1.2 Cell culture media und supplements

BMCyclin®	Hoffmann-La Roche, Basel, CH
DMEM High Glucose (4,5 g/l)	Gibco Invitrogen, Paisley, GB
DMEM High Glucose (4,5 g/l) without phenol	PAA Laboratories GmbH, Pasching, A

red	
DPBS 10X	Gibco Invitrogen, Paisley, GB
Fetal calf serum (FCS)	PAA Laboratories GmbH, Pasching, A
Freezing medium	70% culture medium, 20% FCS, 10% DMSO
HEPES-reagent (1 M)	Gibco Invitrogen, Karlsruhe
Insulin 10 mg/ml	Sigma-Aldrich, Steinheim
L-Glutamine (200 mM)	PAA Laboratories GmbH, Pasching, A
OPTI-MEM I	Gibco Invitrogen, Paisley, GB
Penicillin/Streptomycin (100X)	PAA Laboratories GmbH, Pasching, A
RPMI 1640	PAA Laboratories GmbH, Pasching, A
Trypsin/EDTA (0.05%, 1X)	Gibco Invitrogen, Paisley, GB
Kanamycin	Biochrom AG, Berlin

2.1.3 Bacterial media

Luria-Bertani (LB) medium	1% (w/v)Tryptone 0.5% (w/v) Yeast extract 1% (w/v) NaCl
	0.5% (w/v) Yeast extract 1% (w/v) NaCl 1.5% (w/v) Agar

2.1.4 Chemicals and Cytokines:

1,4-Dithiothreitol (DTT)	Merck KGaA, Darmstadt
3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromid (MTT)	Sigma-Aldrich, Steinheim
Acetic acid 100% (Glacial)	Merck KGaA, Darmstadt
Agar	AppliChem, Darmstadt

Agarose	Sigma-Aldrich, Steinheim
Ammonium persulfate (APS)	Bio-Rad Laboratories Inc., Hercules, USA
Bidistilled water	Clinic Pharmacy of the University Hospital Münster, Münster
Boric acid (Crystalline)	Merck KGaA, Darmstadt
Bovine serum abumin (BSA)	Sigma-Aldrich, Steinheim
Bromophenol blue	Merck KGaA, Darmstadt
Dimethylsulfoxide (DMSO)	Carl Roth GmbH & Co. KG, Karlsruhe
Ethanol absolute	Merck KGaA, Darmstadt
Ethylendiamintetracetic acid (EDTA)	AppliChem, Darmstadt
FAK inhibitor (PF-573228)	Sigma-Aldrich (Deisenhofen, Germany)
Gelatin Type A: porcine skin, G-2625	Sigma-Aldrich, Steinheim
Glycerol	Merck KGaA, Darmstadt
Glycine	Merck KGaA, Darmstadt
GRGDSP-peptide	R&D Systems GmbH, Wiesbaden
HGF	Calbiochem (EMB Biosciences Inc. La Jolla, CA)
Human fibronectin	BD, Becton & Dickinson Biosciences, Heidelberg
Hydrochloric acid (HCl) fumed 37%	Merck KGaA, Darmstadt

Laminin	Sigma-Aldrich, Steinheim
Methanol	Merck KGaA, Darmstadt
Methylene blue	Sigma-Aldrich, Steinheim
N,N-Dimethylformamide 99%	Sigma-Aldrich, Steinheim
PMA	Fluka, Buchs, Switzerland
Potassium chloride (KCl)	Merck KGaA, Darmstadt
ProMMP-2, ProMMP-9	Merck KGaA, Darmstadt
Skimmed milk powder	Sigma-Aldrich, Steinheim
Sodium bicarbonate ($NaHCO_3$)	Merck KGaA, Darmstadt
Sodium carbonate (Na_2CO_3)	Merck KGaA, Darmstadt
Sodium Chloride (NaCl)	J.T. Baker, Phillipsburg, NJ, USA
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich, Steinheim
Sodium Fluoride (NaF)	Sigma-Aldrich, Steinheim
Sodium hydroxide (NaOH)	Merck KGaA, Darmstadt
Sodium orthovandate ($NaVO_3$)	Sigma-Aldrich, Steinheim
Tris (Tris-(hydroxymethyl)-aminomethane)	Carl Roth GmbH & Co. KG
Tris-Hcl	Merck KGaA, Darmstadt
Triton X-100	Sigma-Aldrich, Steinheim
Triton X-114	Sigma-Aldrich, Steinheim
Tryptone	AppliChem, Darmstadt
Tween 20	Fluka, Buchs, Switzerland
WNT inhibitor IWP-2	Sigma-Aldrich (Deisenhofen, Germany)
WNT1 ligand	Peprotech (Hamburg, Germany)

Xylol/Xylene	Merck KGaA, Darmstadt
Y-27632 dihydrochloride monohydrate	Sigma-Aldrich, Steinheim
Yeast extract	AppliChem, Darmstadt
β-Glycerophosphate	Sigma-Aldrich, Steinheim

2.1.5 Buffers and solutions:

2.1.5.1 Buffers:

Unless stated, all buffers were diluted with bidistilled water. Stock solutions (10X or 5X) of the buffers were prepared and immediately diluted before using. The required pH was adjusted, if necessary, by adding appropriate volume of HCl or NaOH.

2.1.5.1.1 Protein chemistry and Western blot:

Blocking buffer (diluting buffer of the secondary antibody)	5% (w/v) Skimmed milk powder 0.1% (v/v) Tween 20 in TBS 1X
Blotting buffer, 10X	0.25 M Tris 1.92 M Glycine
Blotting buffer, 1X	10% (v/v) Blotting buffer, 10X 20% (v/v) Methanol
Diluting buffer of the primary antibody	5% (w/v) BSA in PBS
Fractionation lysis buffer	50 mM HEPES pH 7.5 50 mM NaCl 1 mM $MgCl_2$

	2 mM EDTA
	10 mM NaF
	1 mM DTT
	1% (v/v) Protease Inhibitor Cocktail
	10 mM β-Glycerophosphate
	1 mM $NaVO_3$
Resolving buffer, 2.5X, pH 6.7	1.875 M Tris
	0.5% (w/v) SDS
RIPA buffer	20 mM Tris-HCl pH 7.4
	137 mM NaCl
	1% (v/v) Triton X-100
	2 mM EDTA
	1% (v/v) Protease Inhibitor Cocktail
	10 mM β-Glycerophosphate
	10 mM NaF
	1 mM $NaVO_3$
Running buffer, 5X	0.5 M Tris
	1.92 M Glycine
	0.5% (w/v) SDS
Sample buffer, 5X	1 M Tris-HCl pH 6.8
	25% (v/v) Glycerol
	5% (w/v) SDS

	0.1% (w/v) Bromophenol blue
Stacking buffer, 5X, pH 8.9	0.3 M Tris 0.25% (w/v) SDS
Stopping solution (film development)	1% (v/v) Acetic acid
Stripping buffer, pH 2.5	0.1 M Glycine 0.15 M NaCl
TBS, 10X, pH 7.6	0.15 M Tris-HCl 1.37 M NaCl
Washing buffer	0.1% (v/v)Tween 20 in TBS, 1X

2.1.5.1.2 Cell viability assay (MTT):

MTT ((3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) solution	0.5% (w/v) MTT in PBS sterile filtered (0.22 µm)
Stopping buffer, pH 4.7	10% (w/v) SDS 50% (v/v) N,N-Dimethyl formamide

2.1.5.1.3 Cell adhesion assay:

Blocking buffer	0.5% BSA in DMEM without FCS

Borate buffer, pH 8.5	0.01 M Boric acid
Fixative	3.7% (v/v) PBS-buffered formaldehyde
Washing buffer	0.1% BSA in DMEM without FCS

2.1.5.1.4 Immunofluorescence microscopy:

Blocking buffer	10% (v/v) Aurion BSA in PBS
Diluting solution of primary and secondary antibodies	1% (w/v) BSA in PBS
Fixative	Ice cold methanol or 3.7% (v/v) PBS-buffered formaldehyde
Permeabilization solution	0.1% (v/v) Triton-X100 in PBS

2.1.5.1.5 Zymography:

Destaining solution	Acetic acid/Isopropanol/Bidistilled water, 1:3:6
Resolving bufffer, 2.5X, pH 6,7	1.875 M Tris 0.5% (w/v) SDS
Running buffer, 5X	0.5 M Tris 1.92 M Glycine 0.5% (w/v) SDS

SDS-Loading buffer	20 mM Tris-HCl 20% (v/v) Glycerin 2% (w/v) SDS 0.01% (w/v) Bromophenol blue
Stacking buffer, 5X, pH 8.9	0.3 M Tris 0.25% (w/v) SDS
Staining solution	0.5% (w/v) Coomassie-Brilliant-Blue R250 in destaining solution
Substrate buffer, pH 8.5	50 mM Tris-HCl 5 mM $CaCl_2$

2.1.5.1.6 Molecular biology:

TBE, 10X	0.9 M Tris 25 mM EDTA 0.9 M Boric acid
Buffer E1	50 mM Tris-HCl (pH 8.0) 10 mM EDTA 100 µg/ml RNase
Buffer E2	200 mM NaOH 1% (w/v) SDS
Buffer E3	3.1 M Potassium acetate (pH 5.5 with acetic acid)
Buffer E4	100 mM Sodium acetate (pH 5.0 with acetic

	acid) 600 mM NaCl 0.15% (v/v) Triton X-100
Buffer E5	100 mM Sodium acetate (pH 5.0 with acetic acid) 800 mM NaCl
Buffer E6	100 mM Sodium acetate (pH 5.0 with acetic acid) 1.5 M NaCl

2.1.5.2 Solutions ready to use:

4', 6'-diamidino-2-phenylindole (DAPI)	Molecular Probes, Karlsruhe
Acrylamide/Bis Solution 30%	Bio-Rad Laboratories Inc., Hercules, USA
Aurion BSA-c® (10%)	AURION Immuno Gold Reagents & Accessories, Wageningen, NL
DNA Ladder 100bp 500µg/mL	New England Biolabs, Inc., Ipswich, USA
DPBS 10x	Gibco Invitrogen, Paisley, GB
Ethidium bromide solution 1%	AppliChem, Darmstadt
Formaldehyde solution 37%	Merck, Darmstadt
G150 (Developer)	AGFA, Dübendorf, CH
G354 (Fixative)	AGFA, Dübendorf, CH
Goat serum (Normal)	DakoCytomation, Glostrup, Denmark
PageRuler™ Prestained Protein Ladder	Fermentas GmbH, St. Leon-Rot
Ponceau S solution	Sigma-Aldrich, Steinheim
Prestained SDS-PAGE Standard High Range	Bio-Rad Laboratories Inc., Hercules, USA
Prestained SDS-PAGE Standard Low Range	Bio-Rad Laboratories Inc., Hercules, USA

Protease Inhibitor Cocktail 100x	Sigma-Aldrich, Steinheim
Protein A/G PLUS-Agarose Immunoprecipitation Reagent	Santa Cruz Biotechnology Inc., Santa Cruz, USA
TaqMan Universal PCR Master Mix, No AmpErase®UNG	Applied Biosystems, Foster City, USA
N,N,N',N'-tetramethylenediamine (TEMED)	Bio-Rad Laboratories Inc., Hercules, USA
Transfection reagent DharmaFECT® 1	Dharmacon, Lafayette, USA
Trypan blue solution 0.4%	Sigma-Aldrich, Steinheim
VECTASHIELD® Mounting Medium	Vector Laboratories, Burlingame, CA, USA

2.1.5.2.1 Antibodies:

Antibody	**Source**
rabbit polyclonal anti-phospho FAK Y925	Cell Signaling, Beverly, MA, USA
rabbit polyclonal anti FAK	Cell Signaling, Beverly, MA, USA
mouse anti-human E-cadherin	Becton Dickinson Biosciences, Heidelberg,Germany
rabbit monoclonal anti-human TCF4	Cell Signaling, Beverly, MA, USA
rabbit monoclonal anti-human EGR1	Cell Signaling, Beverly, MA, USA
mouse anti-human anti-Integrin β1	Millipore, Darmstadt, Germany
rabbit polyclonal anti-Vimentin	Santa Cruz Biotechnology, USA
Rabbit polyclonal anti-Vinculin	Santa Cruz Biotechnology, USA
mouse anti-human α-Tubulin	Sigma-Aldrich, Munich, Germany
Horse radish peroxidase-conjugated goat-anti-mouse IgG	Merck-Millipore, Darmstadt, Germany
Horse radish peroxidase-conjugated goat-anti-rabbit IgG	Merck-Millipore, Darmstadt, Germany

Horse radish peroxidase-conjugated anti-rabbit IgG	CellSignaling, Beverly, MA, USA
Horse radish peroxidase-conjugated anti-goat IgG	Merck KGaA, Darmstadt, Germany
Mouse anti-human IgG-PE	MiltenyiBiotec GmbH, Germany
Mouse anti-human IgG-PE	MiltenyiBiotec GmbH, Germany
Alexa-Fluor 488 anti-mouse IgG, Goat	Invitrogen Corporation, Carlsbad, USA
Alexa-Fluor 555 anti-mouse IgG, Rabbit	Invitrogen Corporation, Carlsbad, USA

2.1.5.2.2 Oligonucleotide primers, TaqMan®-probes, SYBR green primers:

Taqman probes:

Gene	**Primer code**
18S rRNA	Hs99999901_s1
CDH1(E-cadherin)	Hs00170423_m1
KLF4	Hs00358836_m1
MMP-2	Hs00234422_m1
MMP-9	Hs00234579_m1
SDC1	Hs00174579_m1
HPSE	Hs00180737_m1
COX-2	Hs00153133_m1
miR10b	Tm2218
miR 200b	Tm002251

SYBR green primers:

Gene	Forward Primer	Reverse Primer
ITGA2	GGGAATCAGTATTACACAACGGG	CCACAACATCTATGAGGGAAGG
FN1	CGGTGGCTGTCAGTCAAAG	AAACCTCGGCTTCCTCCATAA
ARHGAP28	CAGAAATGGTTACGGAGGCTC	TGCTTCAGTTAAGCCAAATCTG
ACTB1	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT
ZEB 2	TGGGCTAGTAGGCTGTGTCCA	TCATCTTCAACCCTGAAACAGA
SNAI1	CCTGTTTCCCGGGCAATTTA	TTCTGGGAGACACATCGGTCA
TCF7L2	AAACCAGCTGCCGCTTTTATG	GCAACATCAACATGCCTAGGTT
CD133	TCAAAGATTGGCCATGTTCCAC	TGTCAGATGGAGTTACGCAGGT
EPCAM	GATGGGTGAGATGCATAGGGAAC	CGTCCCACGCACACACATT
LGR5	TGTTCTCTCTGGATAACCCACTTGA	CAGCCATTTGGTTTGGATGTAT
SOX2	TGGCGAACCATCTCTGTGGT	CCAACGGTGTCAACCTGCAT
VIM	TCAGCATCACGATGACCTTGAA	CTGCAGAAAGGCACTTGAAAGC
NANOG	GGTCTCGTATTTGCTGCATCGT	TTGAAACACTCGGTGAAATCAG
KLF2	AGACCACGATCCTCCTTGACG	ACCCGGTGGGAGAGAAGATG

2.1.5.2.3 Reaction system (Kits):

Affymetrix One Cycle cDNA synthesis	Affymetrix, Santa Clara, CA
Diff-Quik® staining system for invasion filters	Dade Behring, Düdingen, Switzerland
JETSTAR 2.0 MAXI columns with lysate filtration unit (LFU)	GENOMED GmbH, Löhne
LUC-Pair™ miR Luciferase Assay	GeneCopoeia, Inc., Rockville, MD, USA

Micro BCA™ Protein Assay	Thermo Fisher Scientific Inc., Rockford, USA
RNA-Preparation system basic*rna-OLS*	OMNI Life Science GmbH, Hamburg
RNA-Transcription system ”First strand cDNA Synthesis Kit”	Fermentas GmbH, St. Leon-Rot
Western Blot Detection system „Super Signal West Pico Chemiluminescent Substrate”	Thermo Fisher Scientific Inc., Rockford, USA

2.1.6 Equipment and Materials

Agarose gel chamber SUB-CELL® GT	Bio-Rad Laboratories Inc., Hercules, USA
Autoclave Hiclav® HV-50	HMC Europa GmbH, Engelsberg
BD Falcon® High-Clarity Polypropylene Conical Tube (15 mL, 50 mL)	Becton Dickinson Biosciences, Heidelberg
BioDoc Analyze system	Biometra GmbH, Göttingen
Cell culture flasks BD Falcon® (25 cm², 75 cm², 150 cm²)	Becton Dickinson Biosciences, Heidelberg
Cell culture plates Multiwell® Falcon®, (6-Well, 24-Well, 96-Well)	Becton Dickinson Biosciences, Heidelberg
Cell scraper Costar®	Vitaris AG, Baar
Centrifuge Biofuge fresco (Molecular biology)	Kendro-Heraeus, Berlin
Centrifuge Multifuge 3 S-R(cell culture)	Kendro-Heraeus, Berlin
Cover slips (different sizes)	Diagonal GmbH & Co. KG, Münster
Cryo-freezing device (NU200)	Nunc GmbH & Co. KG, Wiesbaden
Cryotubes	Brand GmbH & Co. KG, Wertheim
ELISA-Reader VersaMax® Microplate Reader	Molecular Devices, Sunnyvale, CA, USA

Gel electrophoresis-voltage source PowerPac® 300	Bio-Rad Laboratories Inc., Hercules, USA
GeneChip Fluidics 450 station	AFFYMETRIX, INC.Santa Clara, CA, USA
GeneChip Hybridization Oven	Manual Tissue Arrayer MTA-1, Beecher Instruments, Silverspring, USA
Incubators BB6060	Kendro-Heraeus, Berlin
Invasion Chamber BD BioCoat® Matrigel®	Becton Dickinson Biosciences, Heidelberg
Inverted microscope Axiovert25	Carl Zeiss, Inc. Göttingen
Labmixer Vortex Genie 2	Scientific Industries, Bohemia, NY, USA
Laminar flow hood HS15	Kendro-Heraeus, Berlin
Light microscope with camera Axiovert-100	Carl Zeiss AG, Jena
Magnetic stirrer Ikamag® Ret	Ika-Werke GmbH & Co. KG, Staufen
Microlumat plus LB 96V	Berthold technologies, Bad Wildbad
Micropipets Eppendorf Research® variabel	Eppendorf, Hamburg
Microwave Micromat	AEG-Electrolux, Nürnberg
Migration assays, Costar® Transwell-Clear inserts, 8 µm pore size	Corning Life Sciences, Schiphol-Rijk, NL
Neubauer Haemocytometer	Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen
Nitrocellulose membrane Hybond™ ECL	GE Healthcare, General Electric Company, Chalfont St. Giles, GB
PCR-Reaction plate 96 Well Multiply® (real time PCR)	Sarstedt AG & Co., Nümbrecht
PCR-Reaction tube Multiply®-µStrip 8-fold (conventional PCR)	Sarstedt AG & Co., Nümbrecht
PCR-Thermocycler Applied Biosystems 7300 real-time PCR system (real time PCR)	Applied Biosystems Foster City, CA, USA
PCR-thermocycler Biometra T1 (conventional	Biometra GmbH, Göttingen

PCR)	
Pipette tips epT.I.P.S.	Eppendorf, Hamburg
Pippettes, serological (1 mL, 5 mL, 10 mL, 25 mL, 50 mL)	Becton & Dickinson Biosciences, Heidelberg
Savant SpeedVac concentrator	Thermo Fisher Scientific Inc., Rockford, USA
SDS-PAGE chambers Minigel-Twin	Biometra GmbH, Göttingen
Sensitive balance TE 214S-oCE	Sartorius, Göttingen
Slides for IHC, SuperFrost® plus, 75 x 25mm	R. Langenbrinck Labor und Medizintechnik, Emmendingen
Slides, normal 76 x 26 mm	Diagonal GmbH & Co. KG, Münster
Standard eppendorf tubes 1,5 mL, 2mL	Eppendorf, Hamburg
Sterile filters Millex® GP	Millipore, Bedford, MA, USA
Thermomixer	Eppendorf, Hamburg
Ultracentrifuge avanti 30 compact	Beckman Instruments, München
UV-Spectrophotometer BioDoc Analyze	Biometra GmbH, Göttingen
Video cameras (Models XC-ST70CE and XC-77CE)	Hamamatsu/Sony, Japan
Water bath GFL-1004	GFL, Burgwedel
X-ray films CL-XPosure™	Thermo Fisher Scientific Inc., Rockford, USA

2.1.7 Softwares

Adobe Photoshop 7	Adobe Systems Inc., San Jose, USA
AMIRA software	TGS Inc., San Diego, CA, USA
AxioVision 4.3 (evaluation of Migration and Invasion assays)	Carl Zeiss AG, Jena
HiPic and WASABI softwares	Hamamatsu/Sony, Japan
JAVA programs and the NIH ImageJ software	http://rsb.info.nih.gov/ij/

MS Excel	Microsoft Corporation, Redmond, USA
MS Power Point	Microsoft Corporation, Redmond, USA
MS Word	Microsoft Corporation, Redmond, USA
Sequence Detections Software (SDS) Version 1.4 (evaluation of real-time PCR-Analysis)	Applied Biosystems Foster City, CA, USA

2.2 Methods:

2.2.1 *Adherent cell culture*

The human colon cancer cell lines Caco2, HT29 and Colo205 were provided by the German Collection of Microorganisms and Cell Cultures, Department of Human and Animal Cell Cultures, Braunschweig, Germany. Cells were maintained with RPMI-1640 (HT29) or DMEM (Caco2) supplemented with 10% (v/v) FCS, 1% (v/v) glutamine and 1% (v/v) penicillin/streptomycin in a humidified atmosphere of 5% and 7% CO_2 at 37°C. Cells were cultured to 80% confluence.

2.2.2 *siRNA knockdown*

siRNA knockdown was performed using siRNA #12634, #17258, # 4537 (Ambion, Cambridgeshire, UK) targeting the coding region of Syndecan-1, GPCR5A, and EGR1,and a negative control siRNA (negative control #1, Ambion). In pilot experiments, we optimized conditions for the efficient transfection of Caco2 and HT29 cells. We found that optimal conditions were achieved with serum starvation for 5h before transfection at 50–70% confluence using 40nM siRNA and Dharmafect reagent (Dharmacon, Lafayette, CO, USA) according to the manufacturer's instructions. Fresh medium was added 16 h after transfection, and experiments were conducted 48 h after transfection. Target downregulation was confirmed by qPCR.

2.2.3 *Stable overexpression of Heparanase*

Caco2 cells were stably transfected with a pcDNA3.1 control plasmid (Invitrogen, Karlsruhe, Germany), a HPSE overexpression plasmid or a plasmid for overexpression of HPSE double mutated in Glu225 and Glu343 (Hulett MD et al., 2000) (plasmids were donated by Israel Vlodavsky, Hadassah-Hebrew University Medical Center, Jerusalem, Israel). Stable clones were selected using 800ug/ml G418. Caco2 cells were maintained in RPMI media containing 10% fetal calf serum (FCS), 1% glutamine, 1% penicillin/streptomycin and 800 μg/ml G418 in a humidified atmosphere of 5% CO_2 at 37°C.

2.2.4 *Promoter reporter assay*

For HPSE promoter assays, we used a plasmid where the 1.9-kb region of the human heparanase promoter [*HPSE* (-1791/+109)-LUC] had been subcloned upstream of the *LUC* gene in a pGL2 basic reporter plasmid (Promega, Madison, WI, USA) (Elkin *et al.*, 2003; Zcharia *et al.*, 2005). 24h after siRNA transfection cells were replaced with serum media for 6h and procced for co-transfection of the reporter construct of 1ug/ well (6 well) using FuGENE 6 Transfection Reagent (Promega) according to the manufacturers protocol. Control cells were transfected with basic pGL2 plasmid containing the *LUC* gene alone (without promoter). 46h after transfection, luciferase assay was done using the Luciferase Reporter Assay system **(**Promega-E1500**).** The relative light units were determined in each sample with a luminometer and results were normalized against beta-galactosidase activity measured by a colorimetric assay. Data are presented as the means of quadruplicates±s.d., and all experiments were repeated at least three times with similar results.

2.2.4 *Enrichment of Caco2 and HT-29 cells with sphere formation capacity*

a) Enrichment of spheres

Sphere cultures of Caco2 cells were generated by plating single cells obtained from adherent culture in low attachment plates (Corning) at the concentration of 1000 cells/ml into RPMI-1640 supplemented with B-27 (Life Technologies), 25 ng/ml basic fibroblast growth factor (bFGF, Sigma-Aldrich), 20 ng/ml mouse recombinant epidermal growth factor (EGF, Sigma-Aldrich), and 4 ng/ml Heparin (Sigma-Aldrich) ("Sphere media"). Unless indicated otherwise, sphere cultures of HT29 cells were generated by plating single cells obtained from adherent culture in low attachment plates at the concentration of 1000 cells/ml into DMEM supplemented with 5% FBS B-27, 25 ng/ml bFGF, 20 ng/ml mouse recombinant EGF, and 4 ng/ml Heparin.

b) Sdc-1 silencing in sphere cultures

For siRNA treatment 9,500 Caco2 and HT29 cells were plated in 24 well-plates under adherent conditions 48 hours before the treatment. The transfection was performed either with 50 nM Sdc1 siRNA (#12634, Ambion) or negative control siRNA (Ambion), using the INTERFERin® reagent (Polyplus Transfection TM) according to the manufacturer's instructions. After 24 hours, the cells were washed, trypsinized (0.25% Trypsin-EDTA, Gibco, Life Technologies, Germany), counted and seeded in suspension culture. Fresh sphere medium was added each three days. Caco2 and HT29 spheres were counted after 7 days.

c) ***FAK inhibitor (PF-562271) treatment***

siRNA-treated Caco2 cells were seeded at a density of 20,000 cells/cm^2. After 24 h, the growth medium was changed and cells were either treated with the inhibitor PF-562271 (Sigma-Aldrich) (10µg/ml), DMSO (0.05%) or with vehicle only (controls). After 48 h, cells were washed, trypsinized, counted and plated as single cells in sphere medium containing the same inhibitor or DMSO, respectively. Media were replaced after 3 days, and spheres generated from each cell type and treatment were counted after 7 days from suspension plating.

c) ***HPSE inhibitor (SST0001) treatment***

siRNA-treated Caco2 cells were seeded at a density of 20,000 cells/cm^2. After 24 h, the growth medium was changed and cells were either treated with the inhibitor SST0001 (10µg/ml), DMSO (0.05%) or with vehicle only (controls). After 48 h, cells were washed, trypsinized, counted and plated as single cells in sphere medium containing the same inhibitor or DMSO, respectively. Media were replaced after 3 days, and spheres generated from each cell type and treatment was counted after 7 days from suspension plating.

2.2.5 Cell Viability assay: (MTT assay)

48 h after control and Sdc-1 siRNA transfection, 5×10^3 cells/well were seeded in complete growth medium into a 96-well plate and incubated at 37°C overnight. Subsequently, cells were incubated with 10 µl/ well of MTT dye solution for 4 h at $37C^o$. The number of viable cells was directly proportional to the production of formazan, which was dissolved in 100 µl/well stopping buffer for 20 h, and measured spectrophotometrically at 570/650 nm in a Softmax Microplate reader.

2.2.6 ***Cell adhesion assay***

Briefly, ninety-six-well plates were coated with 50µg x mL^{-1}, fibronectin, 100µg x mL^{-1} *GRGDSP peptide* and 10 µg x mL^{-1} BSA as a negative control overnight at 4 °C, and washed twice with washing solution (0.1% BSA in DMEM). The plate was then incubated for 45 min with 0.5% BSA in DMEM (blocking solution) to block nonspecific binding at room temperature, and washed once. Seventy-two hours after transfection, control and Sdc-1 siRNA-transfected cells were released from the plates with 2 mM EDTA in NaCl/Pi, washed twice, and resuspended in blocking solution. Cells ($2.5*10^4$ per well) were added to the coated wells, and allowed to attach for 1 h at 37 °C in a cell culture incubator. Nonadherent cells were removed by three gentle washes with NaCl/Pi

buffer, and subsequently fixed with 3.7% NaCl/Pi-buffered formaldehyde for 30 min. Attached cells were stained with 1% methylene blue in 0.01% borate buffer (pH 8.5) for 30 min following four washes with borate buffer, the cells were lysed in ethanol/0.1 M HCl (1 : 1), and the released stain was quantified in a Softmax Microplate reader at 620 nm.

2.2.7 *Invasion assay*

Bio Coat Matrigel Invasion Chamber (BD Biosciences, Heidelberg, Germany) assays were performed exactly as recommended by the manufacturer using an invasion time of four days for Caco2 50,000 cells.

For inhibitor studies, compound SST0001 was added to both compartments 24 h after cell plating (Upper chamber 0.5ug/500ul and lower chamber 0.7ug/700ul).

Relative invasiveness was expressed as percentage of the cell number on compound-treated inserts compared with control inserts.

2.2.8 *Quantitative real-time PCR*

Total cellular RNA was isolated using rna-OLS (OMNI Life Science, Hamburg, Germany) and reverse transcribed (Advantage First strand cDNA synthesis kit; Fermentas, St. Leon-Rot, Germany). qPCR and melting curve analysis were performed using Qiagen QuantiTect SYBR Green PCR kit in a LightCycler (Roche, IN). Expression of additional mRNAs was analyzed using TaqMan probes on an ABI PRISM 7300 Sequence Detection System following the standardized cycling conditions recommended by the manufacturer. The $2^{-\Delta\Delta}$ Ct method was used to determine relative gene transcript levels after normalization to 18S rRNA expression.

2.2.9 *Immunoblot and Immunoprecipitation*

2.2.9.1 *SDS-PAGE and immunoblot*

30-60 µg total protein was mixed with 5X sample buffer (1:4) and boiled at 95^{o}C for 5 min. The boiled samples were briefly centrifuged and loaded onto the gel along with 5 µl pre-stained marker. Electrophoresis was performed at 15 mA/gel for the stacking gel for about 15 min. Then, the current was increased to 20 mA/gel for the separating gel until the marker reached the end of the gel. After gel running, the gels were removed from electrophoresis chamber and the stacking gel was cut with scalpel. The resolving gel was equilibrated in transfer buffer at 4C^{o} to remove SDS. At the meantime, the membrane was pre-wetted for 2 min in distilled water and then in transfer buffer at 4^{o}C for 10 min. The safety cover of the Trans-Blot SD cell was removed and a gel

sandwich was prepared in the following order: anode, soaked filter paper, membrane, gel, soaked filter paper air bubbles between layers were rolled out using glass pipette. The cathode plate was added and 16 volt was applied for 1-2 h. The membrane was subsequently removed and prepared for immunodetection. The membrane bound proteins can be detected using specific antibodies. In the present study, we entirely used the indirect method using horseradish peroxidase (HRP) conjugated secondary antibodies. Before the addition of the primary antibody, the membrane first must be incubated with blocking buffer to prevent interactions between non-specific binding sites of the membrane and the antibodies. Secondly, the membrane can be probed with the primary antibody that will bind the corresponding epitope of the protein of interest. Excess primary antibody is removed by washing. Consequently, secondary antibody directed to the to the Fc portion of the primary antibody will be added. Enhanced chemiluminescent substrates produce light in proportion to the amount of protein, which can be detected with the aid of X-ray films. HRP catalyzes the oxidation of luminol in the presence of hydrogen peroxide. The luminol, which is in an excited state immediately following the reaction, decays via a light-emitting pathway. This emission of light can be enhanced up to a 1000-fold by the addition of compounds, such as phenols. The image of the blot is visualized by exposing the blot to a film or a digital camera. After the transfer, the blot was stained Ponceau S stain for 2 min to determine the efficiency of protein transfer. The blot was destained in water for additional 5 min and directly used for antibody incubation. Blocking of the membrane was performed in blocking buffer for 1 hr at RT followed by incubation with the appropriate dilution (2.1.5.2.1) of primary antibody overnight at 4°C with gentle shaking. The membrane was washed 2X with washing buffer for 5 min each followed by 1X washing for 20 min. Consequently, the membrane was incubated with the corresponding HRP conjugated secondary antibody diluted in blocking buffer for 1 h at RT with gentle shaking followed by 3X washing for 5 min each. The blot was then incubated with appropriate amount of chemiluminescent substrate solution for 1 min. The excess substrate was drained and the membrane was put in a polythene sheet for detection. In a dark room, the signal was detected by exposing the membrane to an X-ray film and the film was developed by briefly swirling in developer, acetic acid 1% and fixer, rinsed briefly in tap water and finally dried.

2.2.9.2 *Immunoprecipitation (IP)*

For IP, lysates of Caco2 cells were prepared 72 hr after transfection with control or Syndecan-1 siRNA as described above. Two hundred microgram protein was incubated with 1:50 dilution of primary antibody rabbit monoclonal anti human EGR1 at 4°C on a rocker platform overnight. Afterwards, the mixture was incubated analogously with 20 µL resuspended protein A/G-PLUS-

Agarose. Immunoprecipitates were pelleted by centrifugation (1,000g, 5 min, 4'C), washed four times with RIPA buffer and boiled in 40-µL SDS sample buffer (5 min). SDS-PAGE, Western blotting, stripping and reprobing were performed as described above using 30–60 µg of protein/lane on 7.5– 12% gels.

2.2.9.3 *Zymography*

Conditioned media from the upper chambers of invasion filters were collected on ice and centrifuged for 10 min at 4°C. The supernatant was diluted with SDS sample buffer for zymography in a ratio of 1:4 and loaded onto a 10% polyacrylamide gel containing 1 % gelatin. Mmp2 and Mmp9 were loaded as positive controls. Electrophoresis was carried out at 4°C at 80V.The gel was rinsed twice with 2.5% TritonX-100, followed by H_2O for 20 min prior to overnight incubation in developing buffer (50 mM Tris/HCl, pH 7.8, 5 mM $CaCl_2$, 0.05% Brij) at 37 °C. Then, the gel was placed in 0.5% Coomassie blue for 30 mins and rinsed in destaining solution (acetic acid/isopropanol/water at a ratio 1:3:6) for 30 mins.

2.2.10 *TOPFLASH/FOPFLASH Reporter Assay*

24h after siRNA transfection, cells were cultured in serum-containing media for 6h and processed for co-transfection of TOPFLASH/FOPFLASH luciferase reporters (addgene, Cambridge, MA, USA, plasmid # 1256 and 12457). 1ug of plasmid/ well (6 well) was tranfected using FuGENE 6 Transfection Reagent (Promega, Mannheim, Germany), according to the manufacturer's instructions. Per well, 0.5 ng *Renilla* control luciferase plasmid was cotransfected to normalize for transfection efficiency. 46h after transfection, cells were lysed with 1× passive lysis buffer (GeneCopoeia, Rockville, MD, USA), and luciferase activity was assayed in a luminometer using the Dual-Luciferase Reporter Assay Kit (GeneCopoeia). TOPFLASH and FOPFLASH values were normalized to *Renilla* activity, and fold induction was calculated as normalized relative light units of TOPFLASH divided by normalized relative light units of FOPFLASH.

2.2.11 *Affymetrix microarray expression analysis*

Affymetrix gene array analysis was performed by our collaboration partners (Prof. Dr. G.W. Yip and colleagues) at the Department of Anatomy, National University of Singapore. The initial sample preparation steps and independent target confirmation were performed by Sampath Kumar Katakam. Total RNA was isolated from three biological replicates of control and Syndecan-1 siRNA transfected cells using the basic RNA-OLS Kit (OLS, Bremen, Germany). Preparation

of biotin-labeled cRNA using the 1-cycle labeling protocol, hybridization, and scanning of the arrays was performed essentially as previously described (Nikolova et al. 2009). 3.6 µg of purified RNA and poly-A controls were used to generate cDNA, which was used to synthesize biotin-labeled cRNA. Fragmented cRNA was hybridized to Human Genome U133 plus 2.0 arrays for 16h at 60°C in a GeneChip Hybridization Oven 640 at 60 rpm. The arrays were washed and stained in a GeneChip Fluidics 450 station (Affymetrix), followed by scanning (Affymetrix GeneChip Scanner 3000). The raw data image was processed with GeneChip Operating Software v1.2 and analyzed using GeneSpring GX 11.0 with Robust Multiarray Average (RMA) normalization. A list of differentially expressed genes was generated using the filtering criteria of $p < 0.05$ after Benjamini Hochberg false discovery rate control and fold change of at least 2. The Database for Annotation, Visualization and Integrated Discovery (DAVID) 2008 was used to verify the annotations of the filtered genes (n = 396) and classify the genes into different ontology groups. The GEO accession number of this screening is GSE58751.

2.2.12 *Flow cytometry*

2.2.13 *Side population analysis*

Side population (SP) cells were first identified in murine HSCs by Goodell et .,1996). These SP cells are resistant to anticancer drugs with their unique effleuxing properties via ATP-binding cassette transporter proteins such as ABCG/Brcp1. It is based on the unique property to efflux lipophilic fluorescent dyes out of the cell. SP cells have been considered as a stem cell population in many cancers like colorectal, breast and liver cancer (Haraguchi, N et al., 2006; Olempska, M et al., 2007). The assay was performed 72 hr after control and Syndecan-1 siRNA transfection using the Hoechst 33342 dye exclusion technique. $1x10^6$ cells were incubated in DMEM containing 2% (v/v) FCS for 90 min at 37°C either with 5 µg/mL Hoechst 33342 (Sigma-Aldrich) or in the presence of 50 µM verapamil (Sigma-Aldrich). Finally, 2 µg/mL propidium iodide was added for cell death discrimination, and cells were stored on ice until analysis. Cells were analysed on a CyFlow Space (Partec, Münster, Germany) using a 16 mW 375 nm UV laser for excitation, emission was measured at 475 nm (BP 455/50) and at 665 nm (LP 665 nm). Signals were slivered by a dichroic mirror of 610 nm to measure Hoechst signal intensity in both channels. All cells with a low Hoechst fluorescence and which were not visible in the verapamil control were gated (R2) as SP cells. SP analysis was further combined with CD133 analysis. In this case the SP staining was done first and followed by the cell surface marker protocol. For stimulation experiments WNT1 ligand was used at a concentration of 50ng/ml and

for WNT signaling inhibition, IWP-2 was used at a concentration of 10uM/ml for 1 hr. SST0001 was used at a concentration of 10mg/ml for 1 hr. SP cells and non-SP cells were sorted and enriched in adherent conditions using MammoCult media (Stem Cell Technologies, Cologne, Germany) and in suspension conditions using sphere media.

2.2.14 *Identification of ALDH-1 positive cells*

The ALDEFLUOR™ fluorescent reagent detects a unique approach to the identification of stem or progenitor population based on their expression of the enzyme aldehyde dehydrogenase (ALDH), especially ALDH1. This enzyme mediates the oxidation of intracellular aldehydes to carboxylic acids in the cytosol and is involved in retinoid metabolism (Labrecque, J et al., 1993). It was first identified in the hematopoietic stem cells (HSC). In mice it has been shown that in HSCs, retinoids mainly contribute for the terminal differentiation of late progenitors and the self-renewal of early precursor cells (Purton, L. E et al., 1999; Chute, J. P et al., 2006). Several studies showed that $ALDH^+$ populations has high tumorogenic properties in many cancers. For example in colorectal and breast cancers (Dalerba, P et al., 2007). ALDH-1 activity was assessed 72 hr after siRNA transfection by using the ALDEFLUORTM kit (StemCell Technologies, Köln, Germany). Briefly, 1x 10^6 cells were resuspended in assay buffer containing ALDH substrate (1 µmol/L). Half of this suspension was used as a negative control and transferred into another tube containing 50 mmol/L of the specific ALDH-1 inhibitor diethylaminobenzaldehyde (DEAB). The cells were incubated for 1 hr at 37°C in a water bath in the dark and agitated every 10 minutes. After a final centrifugation at 400g for 5 min the cells were resuspended in 1 ml assay buffer and stored on ice prior to flow cytometry on a CyFlow Space (Partec) using the 488 nm blue laser for excitation. Fluorescence emission was measured at 545 nm (BP 527/30 nm). Gates were set by comparing the fluorescence of the DEAB control with that of the original sample.

2.2.15 *Cell surface marker detection*

Monoclonal mouse anti-CD133 PE conjugated antibody (clone AC133) was used to quantify stem cells, mouse IgG1-PE was used as isotype control (both from Miltenyi Biotec, Bergisch Gladbach, Germany). 10 µl of each antibody was given to 1 x 10^6 cells suspended in 90 µl PBS and incubated for 20 min in the dark. Flow cytometric analysis was performed on a CyFlow Space (Partec, Münster, Germany) equipped with a 25 mW 638 nm red laser diode. Fluorescence emission was detected at 640 nm (BP675/20 nm), as also measured over the whole population by setting a region gate (RN1) in FL2.

2.2.16 *Activated β1 integrin detection*

Monoclonal mouse anti-human-Integrin β1 antibody clone HUTS-4 (Millipore) was used to analyse the active conformation of human β1 integrins. About 1 x 10^6 cells were incubated with 2.5 µg of β1 integrin antibody for 1hr at room temperature. Cells were then washed once with PBS and resuspended in 100µl of the 1:1000 diluted Alexa 488 conjugated goat anti-mouse secondary antibody (Invitrogen, Oregon, USA). Incubation took place for 30 min at room temperature in the dark. After a final washing step cells were ready for flow cytometric analysis. Flow cytometric analysis was performed on a CyFlow Space (Partec, Münster, Germany) equipped with a 25 mW 638 nm red laser diode. Fluorescence emission was detected at 640 nm (BP675/20 nm), as also measured over the whole population by setting a region-gate (RN1) in FL2.

2.2.13 *Radiation exposure*

Irradiation of transfected colon cancer cells was performed at room temperature using 6 MV photons of a linear accelerator (Varian Medical Systems, Palo Alto, California, USA). The dose rate was 2Gy per minute and a dose of 2Gy was applied.

2.2.14 *Colony forming assay*

To measure the colony forming ability, 2x10^5 knockdown cells were either irradiated or left untreated and subsequently trypsinised and counted. 1 x 10^3 cells were resuspended in 1 ml culture medium, plated into 3.5 cm petri dishes with a 2.5 mm grid (Nunc, Langenselbold, Germany) and incubated for about 6 days in a CO_2 incubator at 37°C. Cell colonies with more than 50 cells were counted using a microscope (Olympus, Hamburg, Germany). The survival fraction was calculated as follows: plating efficiency treated/plating efficiency control.

2.2.15 *NOD/SCID mice xenograft model*

In vivo xenograft experiments were performed by our collaboration partners (Dr. Rolland Reinbold and colleagues) at the ITN, Segrate, Milan, Italy. Generation of siRNA-treated and stably transfected Colon cancer cell lines, as well as post-mortem analysis of tumor tissues were performed by Sampath Kumar Katakam, partially in Milan and in Münster.The specific small interference RNA (siRNAs) used to knockdown Syndecan-1 in the xenografted cells was siRNA #12634 Sdc1 siRNA, (Ambion, Cambridgeshire, UK), and the control siRNA was negative control #1, (Ambion). For siRNA treatment HT29 cells were plated in adherent condition 72 hours before

the treatment, $5x10^4$ cells cells in 6 well-plates. The transfection was performed with both siRNAs, Sdc1 siRNA and Control siRNA, using the INTERFERin® reagent (Polyplus Transfection TM) according to the manufacture's instructions. The siRNA concentration used in the experiments was 50 nM. After 48 hours from transfection the cells were washed, trypsinized (0.25% Trypsin-EDTA, Gibco, Life Technologies), counted and seeded at the density of $1x10^4$ cells/cm^2. After 48 hours from replating the cells were washed, trypsinized, counted and prepared for injection. HT29 cells treated with both siRNAs were injected into NOD/SCID mice. Mice received $1x10^5$ cells or $2x10^5$ cells for both conditions. Injections were subcutaneous in the neck. Tumor development was checked daily until suspect tumors were detected. Tumor size was estimated using calipers by measuring the difference in the suspect tumor width, which includes the width of the animal skin, subtracted from width of the folded animals skin of a control region of the animal.

2.2.16 *HPSE activity assay*

HPSE activity was measured using a commercial HS degrading enzyme assay kit (Takara.Mirus.Bio, Madison, WI) which is based on the measurement of HPSE-induced degradation of biotinylated-HS (b-HS) fragments. The assay carried out according to the manufacturer's protocol. Briefly, $1x10^6$ cells were collected and suspended in 400 mL extraction buffer. After cell debris removal by centrifugation, the supernatant was incubated with biotinylated-HS (b-HS) for 45 minutes at 37°C. The mixture was then incubated on a FGF-coated plate and undegraded b-HS was detected by HRP-streptavidin. This b-HS fragment is specifically designed so that it cannot bind to FGF when being degraded by HPSE, thus resulting in a lower HRP signal. The absorbance was mea sured at 450 nm.

2.2.17 Statistical analysis

Data were expressed as mean ± SEM or SD, as indicated. Statistical analysis was performed using the Sigma Stat 3.1 software (Systat Software, Point Richmond, CA). An unpaired t-test was used when groups passed the normality test, otherwise, the Mann–Whitney U-test was used. A two-sided P-value <0.05 was considered statistically significant.

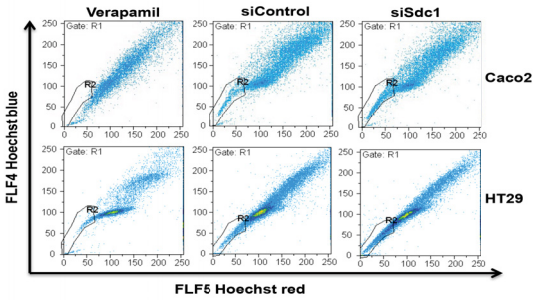
3 Results:

3.1 Part 1

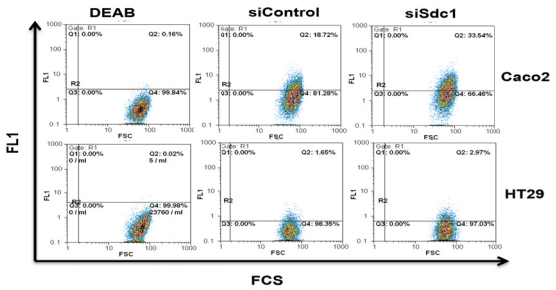
3.1.1 *Silencing of Syndecan-1 enhances the cancer stem cell phenotype in cultured human colon cancer cell lines.*

To characterize a potential role for Sdc-1 in human colon cancer stem cell function, we employed an siRNA knockdown approach in the human colon cancer cell lines Caco2 and HT29, which are known to contain a CSC population (Haraguchi N et a., 2008; Yeung TM et a., 2010). Several approaches have been utilized to identify stem cell like populations from various human malignancies, including the side population (SP) phenotype, analysis of cell surface markers, and aldehyde dehydrogenase (ALDH) activity assays (Tirino V et al., 2013). Analysis of the side population phenotype, which is based on the expression of cell surface transporters (ABCG2) to exclude vital dyes such as Hoechst 33342 preferentially from CSCs, but not from non-CSC differentiated cells, revealed a relative increase in the SP of Sdc-1silenced compared to control Caco2 and HT29 cells (Fig.1.1A,D). As the role of the SP in colon CSCs is controversially discussed (Xie ZY et al., 2013) we next tested the activity of ALDH isoform 1 (ALDH1) as an additional colon cancer stem cell-associated parameter (Huang EH, Hynes MJ, Zhang T, Ginestier C, Dontu G, Appelman H, Fields JZ, Wicha MS, Boman BM. (Huang EH et al., 2009). Similar to the impact on the SP, Sdc-1 silencing significantly increased ALDH1 activity in both Caco2 and HT29 cell lines (Fig.1.1B,D). Colon CSCs have also been shown to be enriched in the CD133+ population (Ricci-Vitiani L et al., 2007). In Sdc-1-silenced Caco2 and HT29 cells, there is an increase in CD133 expression compared to controls (Fig.1C,D). Quantitative analysis of the SP revealed a 60% and 55% increase, ALDH 60% and 150% increase and CD133 measurements showed a 10% and 70% increase in Caco2 and HT29 respectively upon Sdc-1 KD compared to the controls (Fig.1.1C,D). To complement our analysis, we investigated a panel of additional CSC and pluripotency markers. Sdc-1depletion was associated with significantly increased mRNA expression of the intestinal stem cell markers *CD133, EPCAM,* and *LGR5* (Hirsch D et al., 2014,(Xie ZY et al., 2014) and the pluripotency-associated markers *KLF2, SOX2 and NANOG* (Fig. 1.1E). These results suggest that Sdc-1 may be involved in the regulation of the colon CSC compartment.

1.1A)



1.1B)



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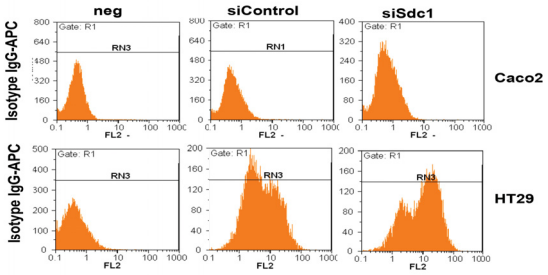

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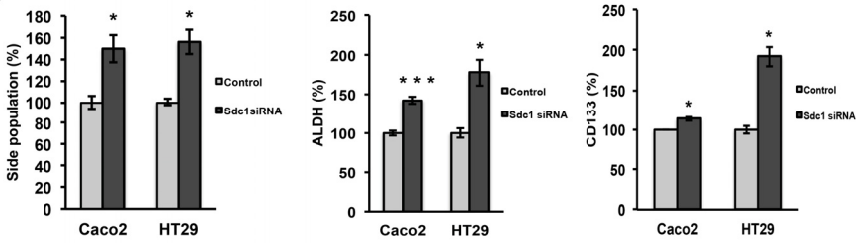


1.1E)

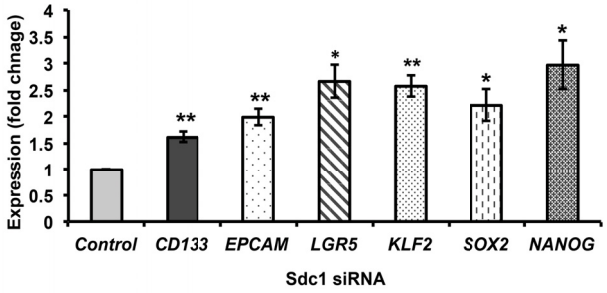


Figure* 1. *Syndecan-1 siRNA knockdown enhances the stem cell phenotype in colon cancer cells. Flow cytometric analysis of putative stem cell pools in the Sdc-1silenced colon cancer cell lines Caco2 and HT29 reveals enhanced SP phenotype, ALDH1 activity and CD133 expression in Sdc-1silenced cells. (Fig.1.1A) Side population analysis. Control and Sdc-1depleted cells were stained with Hoechst 33342 dye in the presence (left) or absence (center, right) of 50 μM verapamil and analyzed by flow cytometry. Gate R1, SP cells appeared as a dim 'tail'. (Fig.1.1B) Representative example displaying aldehyde dehydrogenase (ALDH) activity. Control and Sdc-1 knockdown cells were incubated with fluorescent ALDH substrate in the presence or absence of the inhibitor diethylaminobenzaldehyde (DEAB), followed by flow cytometric analysis. FL1 (green fluorescence) and FSC (forward scatter). (Fig.1.1C) Example of flow cytometric analysis for the cell surface expression of CD133 protein. Control and Sdc-1 knockdown cells were incubated with isotype IgG-APC and *CD133* APC antibodies followed by flow cytometry analysis. (Fig.1.1D) Quantitative analysis revealed a 60% and 55% increase of SP (D), 60% and 150% increase of ALDH1 activity and a 10% and 70% increase of *CD133* expression in Caco2 and HT29, respectively upon syndecan-1 knockdown compared to the controls. Data are expressed as mean percentage +/- SEM relative to the controls (set to 100%) (n=3-5, *=P< 0.05, ***=P< 0.001). (Fig.1.1E) SYBR green qPCR analysis of pluripotency-associated genes in Sdc-1silenced Caco2 cells show a significant increase in the mRNA expression of *CD133, EPCAM, LGR5, KLF2, SOX2,* and *NANOG* compared to the controls. Data are expressed as mean percentage +/- SEM relative to the controls (set to 100%) (n=3-5, *=P< 0.05, **=P< 0.01).

3.1.2 Sdc-1 levels regulate self-renewal and tumorigenicity of colon CSCs. Next we extended our study to investigate the self-renewal capacity of Sdc-1silenced cells. The formation of tumorspheres is one of the key characteristics of cancer stem-like cells. Such cell aggregates are often considered to be a surrogate for tumors as they appear to mirror the cellular architecture and composition and behaviour of tumors in vivo (Zucchi I et al., 2007). To determine whether absence of Sdc-1 is critical for the self-renewal, we generated spheres from the Sdc-1silenced colon cancer cells lines using serum-free suspension cultures (Fig.1.2A). Interestingly, as we saw a highly significant increase of the sphere-forming ability in Sdc-1deficient cells (Fig.1.2B). Next, we performed MTT assays to assess possible changes in viability caused by the depletion of Sdc-1.

We observed a significant increase in the viability of Sdc-1knockdown cells both in the Caco2 and in the HT29 cell line (Fig.1.2C). Overall, these results support the idea that Sdc-1 is implicated in the regulation of self-renewal in colon CSCs.

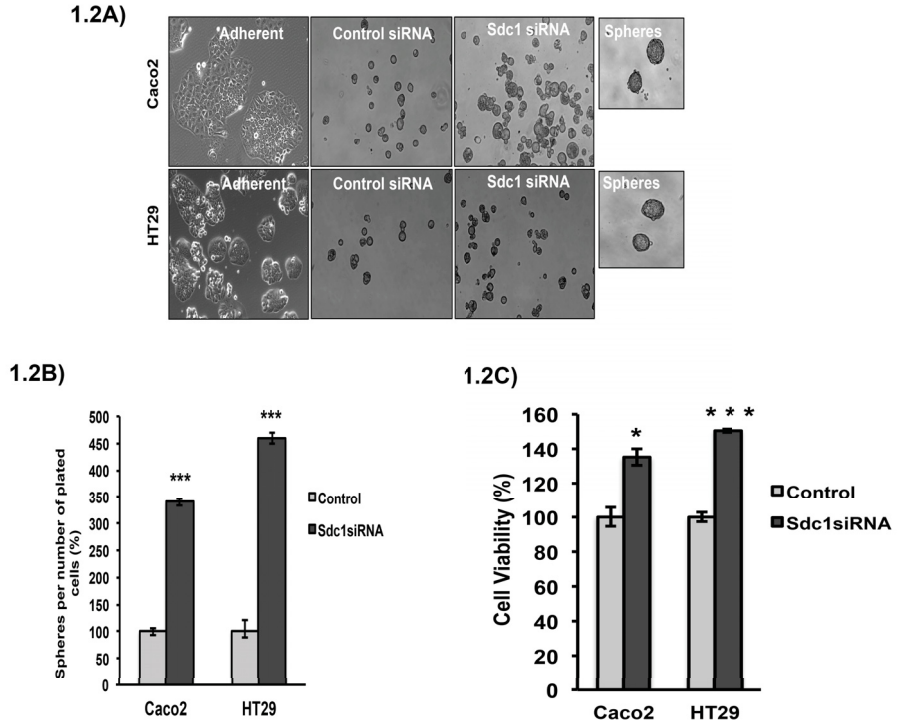


Figure 1.2. Syndecan-1 silencing increases the formation of spheres in Caco2 and HT29 cells. Caco2 and HT29 cells were transfected with a control siRNA or Sdc-1 siRNA, followed by sphere enrichment. After 1 week, the spheres formed by Sdc-1 transfected Caco2 and HT29 cells are larger and there are much more aggregates compared to controls (central panel) (Fig.1.2A). The insert shows enlarged Sdc-1depleted spheres. Each sphere is not much larger than 64 to 100 cells. (Fig.1.2B) Quantitative analysis of the sphere formation efficiency in control and Sdc-1siRNA treated Caco2 and HT29 cells. Syndecan-1 siRNA-treatment results in a significant reduction of sphere formation efficiency ($P<0.001$, $n=6$). (Fig.1.2C) Quantitative analysis of cell viability, as determined by MTT assay in control and Sdc-1 siRNA-treated Caco2 and HT29 cells. Data are expressed as mean percentage +/- SEM relative to the controls (set to 100%) ($n=13$, *=$P< 0.05$, **=$P< 0.01$).

3.1.3 Sdc-1 silencing has an impact on the canonical Wnt signaling pathway.

Canonical Wnt signaling is an important gatekeeping pathway in the regulation of colon cancer stem cell properties (Ragusa S et al., 2014; Fevr T et al., 2007). Work in cell lines and animal models has demonstrated the importance of different syndecan members, and of specific heparan

sulfate structures for Wnt signaling in development and tumorigenesis (Liu BY et al., 2003; Ibrahim SA et al., 2013; Astudillo P et al., 2014; Vijaya Kumar A et al, 2014). However, the function of Sdc-1 in this context has not been elucidated in colon cancer. To test this possibility, we first evaluated the expression of the Wnt effector TCF7L2, a transcription factor required for intestinal epithelial stem cell proliferation (van Es JH et al., 2012). qPCR revealed a significantly increased expression of *TCF7L2* in Sdc-1depleted Caco2 and HT29 cells (Fig.1.3A). Increased expression was confirmed at the protein level by Western blotting (Fig.1.3B). By using a TOPFlASH luciferase reporter plasmid, which contained the β-catenin/TCF binding sites in the promoter region of a luciferase gene, we could furthermore demonstrate an increase in the TCF/LEF-1 transcriptional activity in the Sdc-1silenced cells (Fig.1.3C). To provide additional evidence that the Wnt signaling pathway is modulated by Sdc-1 in colon CSCs, Sdc-1 depleted and control cell lines were stimulated with Wnt1, a relevant ligand in colon CSCs (Mao J et al., 2014) or treated with the small molecule WNT inhibitor IWP-2 (Arensman MD et al., 2014). Wnt-1 stimulation resulted in an increase in the control SP, whereas the SP of Sdc-1 depleted cells showed an even further increase. Importantly, application of the Wnt inhibitor IWP-2 abolished the increase in the proportion of SP cells caused by the knockdown of Sdc-1 (Fig.1.3D). Along with the increased expression of the Wnt target genes *LGR5, CD133,* and *EPCAM* (Hirsch D et al., 2014) in the Sdc-1 silenced cells (Fig.1.1E), these results indicate that the effect of Sdc-1 levels on colon CSC population is at least in part mediated through the Wnt signaling pathway.

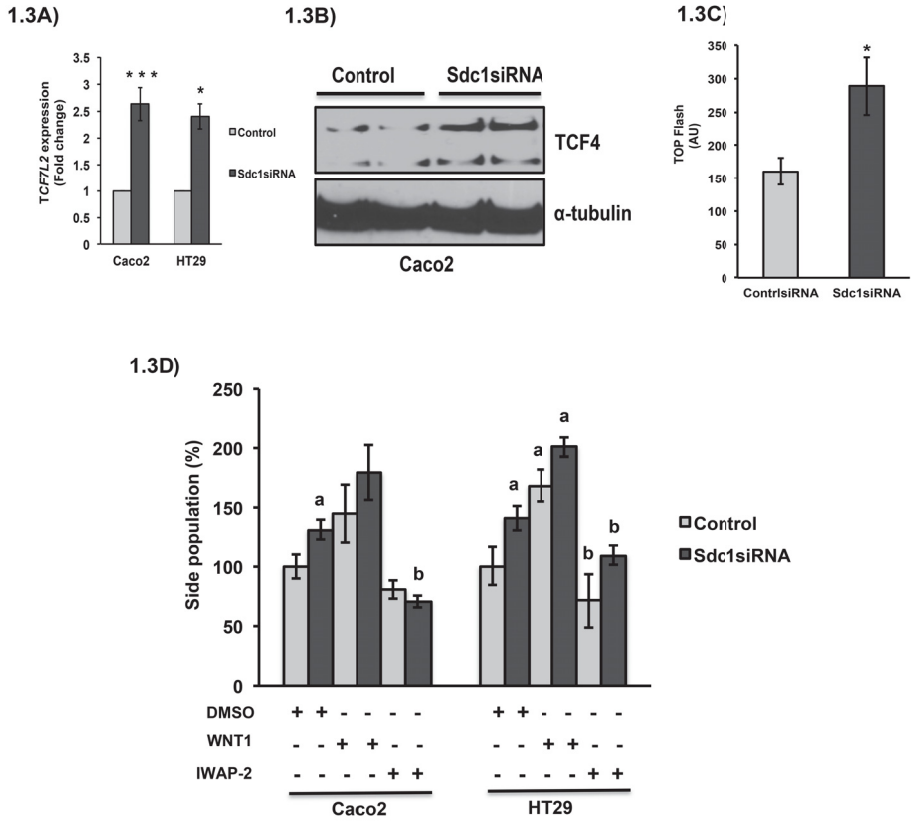


Figure 1.3. Syndecan-1 siRNA depletion enhances the activity of the Wnt-pathway in colon cancer cells. (Fig.1.3A) SYBR green qPCR analysis of TCF7L2 expression in Caco2 and HT29 cells shows a 2.5-fold increase in Sdc-1delpeted cells compared to controls. **=p≤0.01, *=p>0.05, n≥3, error bars=SEM. (Fig.1.3B) Western blot analysis showing the increased expression of *TCF7L2*/TCF4 upon Sdc-1 knockdown compared to the controls. Data shown are triplicates from a single experiment representative of three independent experiments. (Fig.1.3C) Assessment of β-catenin/Tcf– dependent transcriptional activity in Sdc-1 silenced Caco2 cells using Top flash assay indicates 80% increase in its activity. *=p>0.05, n≥4, error bars=SEM. (Fig.1.3D) Side population analysis in cells stimulated with WNT1 ligand for 1 hr shows, a ~40% & ~20% increase in the side population pool in Sdc-1 depleted Caco2 and HT29 cells respectively compared to the untreated cells. Treatment with the WNT inhibitor IWP-2 abolishes the Sdc-1dependent increase in the SP. a=p<0.05 compared to DMSO control, b=p<0.01 compared to DMSO Sdc-1 siRNA, c= p<0.01 compared to WNT1 Sdc-1 siRNA, n≥3, error bars = SEM.

3.1.4 Sdc-1 knockdown induces an EMT-like phenotype in colon cancer cells.

The process of epithelial-to-mesenchymal transition (EMT) is an important contributing factor to the acquisition of an invasive phenotype in cancer. Notably, EMT has been linked to a CSC phenotype, and it has been suggested that EMT may trigger reversion of into a CSC-like phenotype (Singh A et al., 2010; Götte M et al., 2010; Scheel C et al., 2012; Findlay VJ et al., 2014). Histopathological investigations have demonstrated that a reduced expression of Sdc-1 is associated with a more advanced TNM stage and lymph node metastasis in colon cancer (Hashimoto Y et al., 2008; Pap Z et al., 2009; Mennerich D et al., 2004). Owing to the critical role of EMT-related invasiveness and a potentially linked CSC function, we assessed the invasiveness of Sdc-1depleted cells using the Matrigel invasion chamber assay. Sdc-1 knockdown substantially increased invasiveness of Caco2 cells compared to controls (Fig.1.4A). Notably, increased invasiveness of Sdc-1depleted cells was associated with a decreased expression of the epithelial homotypic cell adhesion molecule E-cadherin, a known suppressor of metastasis (Miyaki M et al., 1995). Dedifferentiation and decreased expression of adhesion molecules, E-cadherin and ZO-1, in colorectal cancer are closely related to liver metastasis (Karamitopoulou E et al., 2011) as revealed by Western blotting (Fig.1.4B). Consistent with this finding, the mRNA expression of the EMT-related mesenchymal markers Snail, vimentin, fibronectin and ZEB2 was significantly upregulated, and expression of the EMT-related microRNAs miR-10b and miR-200b (Scheel et al., and Weinberg et al., 2012) was significantly downregulated in Sdc-1-depleted Caco2 cells

compared to controls (Fig.1.4C). These data are in accordance with the previously observed inverse correlation of Snail and Sdc-1 expression in prostate cancer (Poblete CE et al., 2014). This suggest that altered EMT may contribute to the invasion-related phenotype of Sdc-1depleted colon cancer cells.

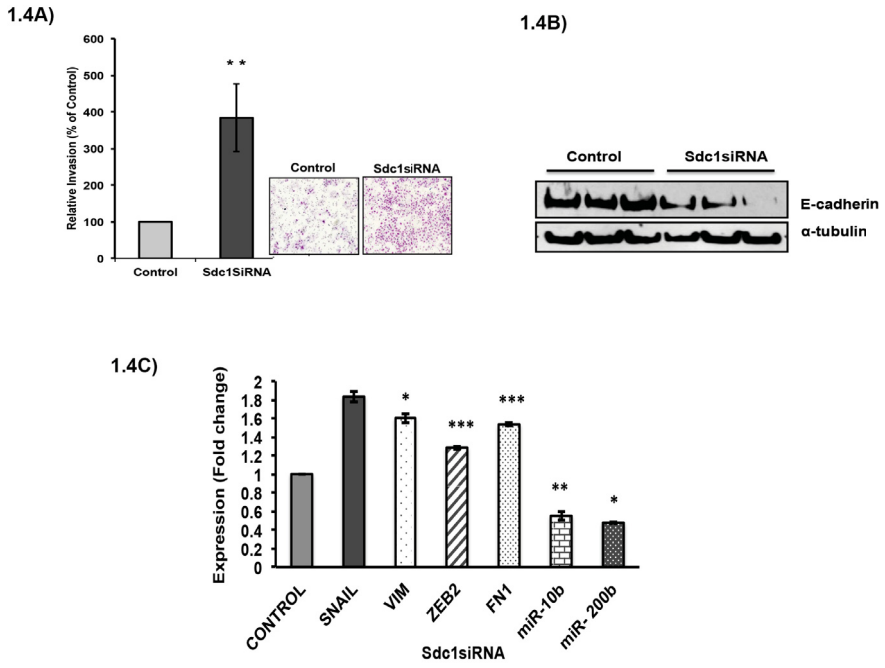


Figure 1.4. Silencing of Sdc-1 increases EMT-associated functional and molecular changes in Caco2 cells. (Fig.1.4A) Sdc-1 knockdown significantly increases matrigel invasion by 3.8 fold in Caco2 cells. (error bars=SEM N=4 ** ** P<0.01 Left panel = representative images of stained matrigel matrix filters. t = 72 h. (Fig.1.4B) Western blot analysis reveals the decreased expression of E-cadherin upon Sdc-1 knockdown compared to the controls. Data shown are triplicates from a single experiment representative of three independent experiments. (Fig.1.4C) SYBR green and TaqMan® qPCR analysis of EMT regulator genes in Sdc-1 silenced Caco2 cells indicates a significant increase in EMT- related genes (like Snail, Vimentin, Zeb-2, fibronectin), and a significant decrease in the expression of the EMT-related microRNAs miR-10b and miR-200b. N=9, error bars=SEM, *=P<0.05, **=P<0.01, ***=P<0.001.

3.1.5 Sdc-1 depletion results in an increased activation of integrin signaling in colon cancer cells.

To understand the possible molecular mechanisms by which Sdc-1 depletion may mediate an invasive CSC phenotype, we performed a transcriptomic Affymetrix microarray analysis for

control and Sdc-1 siRNA-treated Caco2 cells. Affymetrix gene array analysis was performed by our collaboration partners (Prof. Dr. G.W. Yip and colleagues) at the Department of Anatomy, National University of Singapore. The initial sample preparation steps and independent target confirmation were performed by Sampath Kumar Katakam**.** 22 genes were significantly upregulated and 48 genes downregulated in Sdc-1depleted cells by at least a factor of 1.5 (Fig.1.5A) Differentially regulated genes were placed into categories based on their Gene Ontology annotations (Fig.1.5B) and candidate genes potentially involved in Sdc-1 dependent phenotypic changes were further analysed. Notably, genes involved in cell adhesion, cell motility and integrin-mediated signaling were differentially regulated (Fig.1.5 B, C) in accordance with altered invasiveness of Sdc-1depleted cells and with a role of integrin-matrix-interactions in cancer stem cell function (Farahani E et al., 2014). By qPCR, we could independently confirm upregulation of the integrin subunit *ITGA2*, of the integrin substrate fibronectin, and of the RhoA modulator *ARHGAP28* in Sdc-1depleted cells (Fig.1.5 C). The retinoic acid inducible orphan receptor *GPRC5A/RAI3* is upregulated in colon cancer (Zougman A et al., 2013) and was found to by dysregulated upon Sdc-1 knockdown according to our Affymetrix screening. At the functional level, we were able to demonstrate that siRNA knockdown of *GPRC5A* significantly reduced the impact of Sdc-1 knockdown on cell proliferation (Fig.1.5 D). Importantly, flow cytometric analysis revealed a significantly increased expression of the active conformation of beta1 integrin in Sdc-1depleted colon cancer cells (Fig.1.5 E,F). As these data suggested an upregulation and increased activation of integrin-mediated signaling in Sdc-1 siRNA-treated cells, we investigated focal adhesion kinase (FAK) activation as a downstream readout of integrin activation. Western blotting revealed an increased phosphorylation of FAK in Sdc-1depleted Caco2 (Fig.1.5 G) and HT29 (Fig.1.5 H) cells. Interestingly, enzymatic degradation of heparan sulfate by heparinases also resulted in increased FAK activation, suggesting a potential role of the heparan sulfate chains of Sdc-1 in this process (Fig.1.5 I). As the active conformation of beta1 integrin was expressed more prominently in Sdc-1depleted cells (Fig.1.5 E,F), we next tested a potential influence on adhesion to its substrate fibronectin (upregulated in Sdc-1depleted cells). Adhesion of Sdc-1depleted Caco2 and HT29 cells to fibronectin was significantly increased compared to controls (Fig.1.5 J). Pretreatment of the cells with the peptide GRGDSP, which contains the RGD integrin binding site of fibronectin, abolished the differentially increased adhesion caused by Sdc-1 knockdown, thus demonstrating the importance for increased beta1 integrin activation on Sdc-1 modulated cell adhesion.

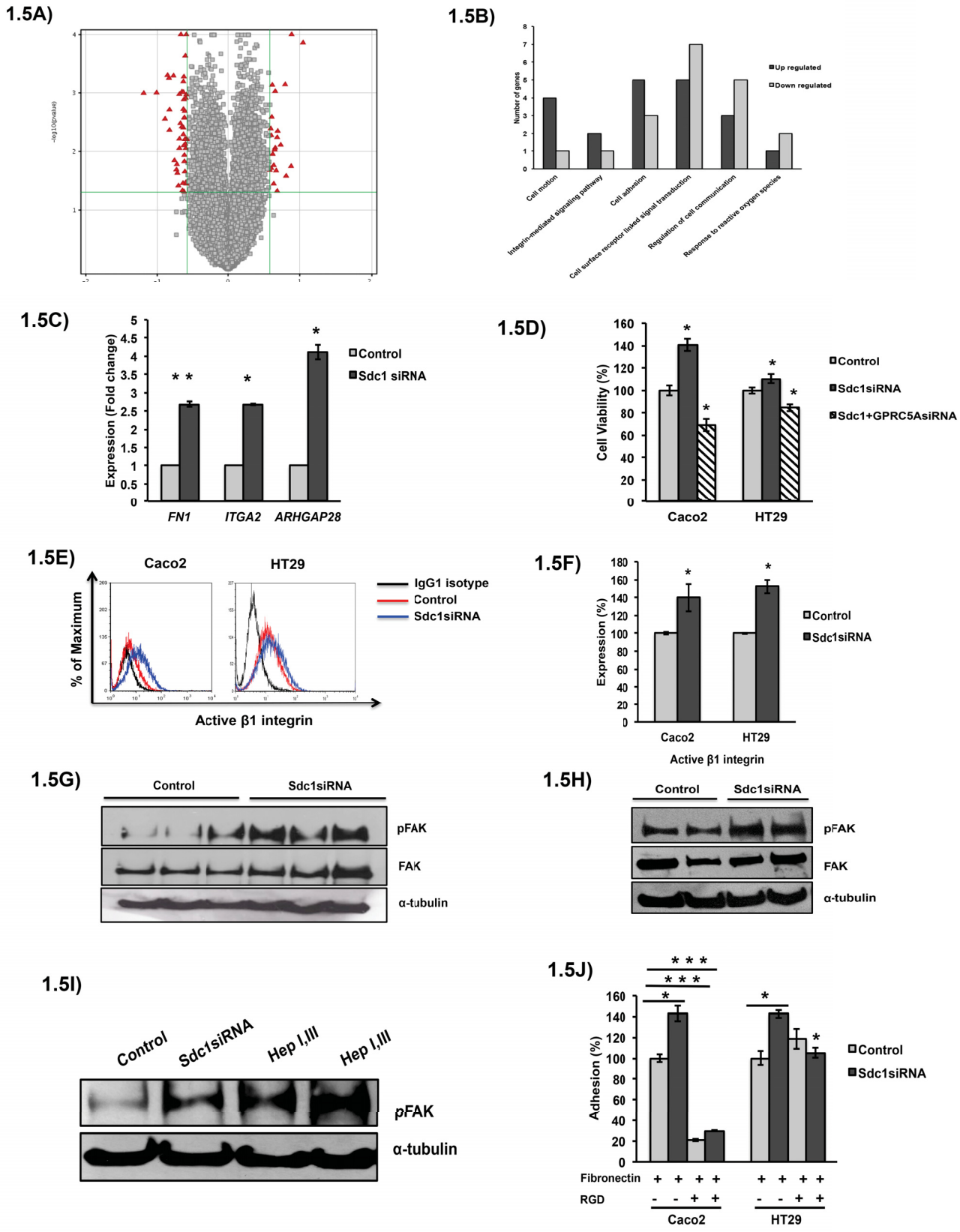


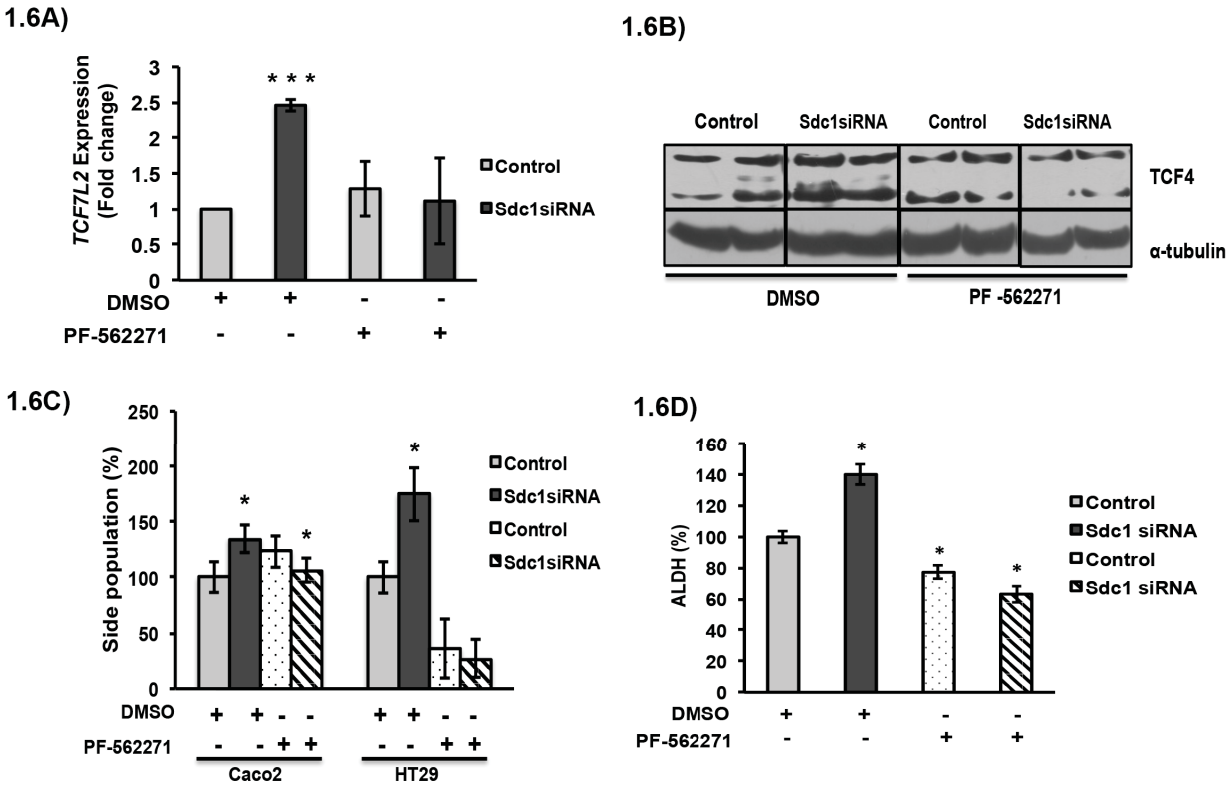
Figure 1.5. siRNA knockdown of Syndecan-1 results in activation and upregulation of integrin-associated pathways. A-C) Affymetrix microarray analysis for differential gene expression between control and Sdc-1 siRNA-transfected Caco2 cells. cDNA generated from control and

Sdc-1 siRNA-treated cells was subjected to Affymetrix microarray analysis. Differentially expressed genes were selected based on the criteria of $p < 0.05$ after Benjamini–Hochberg correction. (Fig.1.5A) Volcano plot of genes with at least a two fold change in expression levels in syndecan-1 silencing cells compared against control, with $p < 0.05$ (black triangles). (Fig.1.5B) Gene Ontology groupings of up- and downregulated genes detected in the Affymetrix microarray screen that have known functions relevant to the observed phenotypic changes (Fig.1.5C). Confirmation of differential gene expression of *ITGA2, FN1* and *ARHGAP28* by SYBR green qPCR analysis in Sdc-1 siRNA- vs. control-transfected Caco2 colon cancer cells. *$p < 0.05$, **$p < 0.01$, n=9, error bar = SEM. (Fig.1.5D) MTT assay. siRNA knockdown of GPRC5A abolishes the increase in cell viability caused by Sdc-1 siRNA knockdown. *$p < 0.05$, n=3, error bar = SEM (Fig.1.5E) Flow cytometric detection of the active conformation of human β1 integrin reveals the presence of higher amounts of active integrin in Sdc-1depleted cells. (Fig.1.5F) Quantitative analysis of active ß1 integrin expression reveals a 40% and 50% increase in Sdc-1depleted Caco2 and HT29 cells, respectively, compared to controls. n=3, error bar = SEM, *$p < 0.05$. Representative plot of flow cytometric analysis.Western blot analysis of FAK phosphorylation in Caco2 (Fig.1.5G) and HT29 (Fig.1.5H) cells shows increase in phosphorylation levels upon Sdc-1 knockdown in both the cell lines. Data shown are triplicates from a single experiment representative of three independent experiments. (Fig.1.5I) Treatment of control and Sdc-1 knockdown Caco2 cells with Heparinase I & III shows an increase in FAK phosphorylation levels. Data shown are duplicates from a single experiment representative of two independent experiments. (Fig.1.5J) Cell adhesion to the ECM substrate fibronectin is significantly increased upon Sdc-1 silencing. Interference with integrin-fibronectin interactions using the GRGDSP peptide (RGD) results in a strong inhibitory effect on both control and Sdc-1 depleted cells. N=18, Error bars = SEM, *** =$p < 0.001$, *= $p < 0.05$.

3.1.6 Increased FAK activation in Sdc-1depleted cells is mechanistically related to the augmented CSC phenotype.

Several reports have provided evidence for a role of beta1 integrin and FAK-mediated signaling in mediating EMT and stemness related cellular functions in breast cancer, squamous cell carcinoma, colon cancer and additional tumor entities (Luo M et al., 2009; Schober M et al., 2011; Ashton GH et al., 2010; Shibue T et al., 2009) As FAK activation was increased in Sdc-1 depleted cells, we hypothesized that a possible mechanistic link to the augmented stem cell phenotype in these cells. Application of the FAK inhibitor PF-562271 (Roberts WG et al., 2008) lowered the increased expression of *TCF7L2*/TCF-4 at the mRNA and protein levels in Sdc-1 depleted Caco2-cells

compared to controls (Fig.1.6 A, B). Notably, the increased SP observed in Sdc-1 depleted Caco2 and HT29 cells was abolished by FAK inhibitor treatment (Fig.1.6 C). Moreover, the differentially increased ALDH1 activity of Sdc-1 knockdown Caco2 cells could be blocked by PF-562271 (Fig.1.6 D). At the gene expression level, FAK inhibition abolished the Sdc-1dependent increase of *LGR5, EPCAM, CD133* and *NANOG* expression relative to controls (Fig.1.6 E). Finally, we could demonstrate that FAK inhibition significantly inhibited the substantial increase in the sphere formation capacity of Sdc-1 depleted Caco2 cells (Fig.1.6 F,G). Overall, our results show that Sdc-1 and FAK drive the self-renewal of colon CSCs. These results strengthen our hypothesis that the stem cell phenotype in colon cancer cells is augmented via a FAK-Wnt signaling axis, which is enhanced in the absence of Sdc-1.



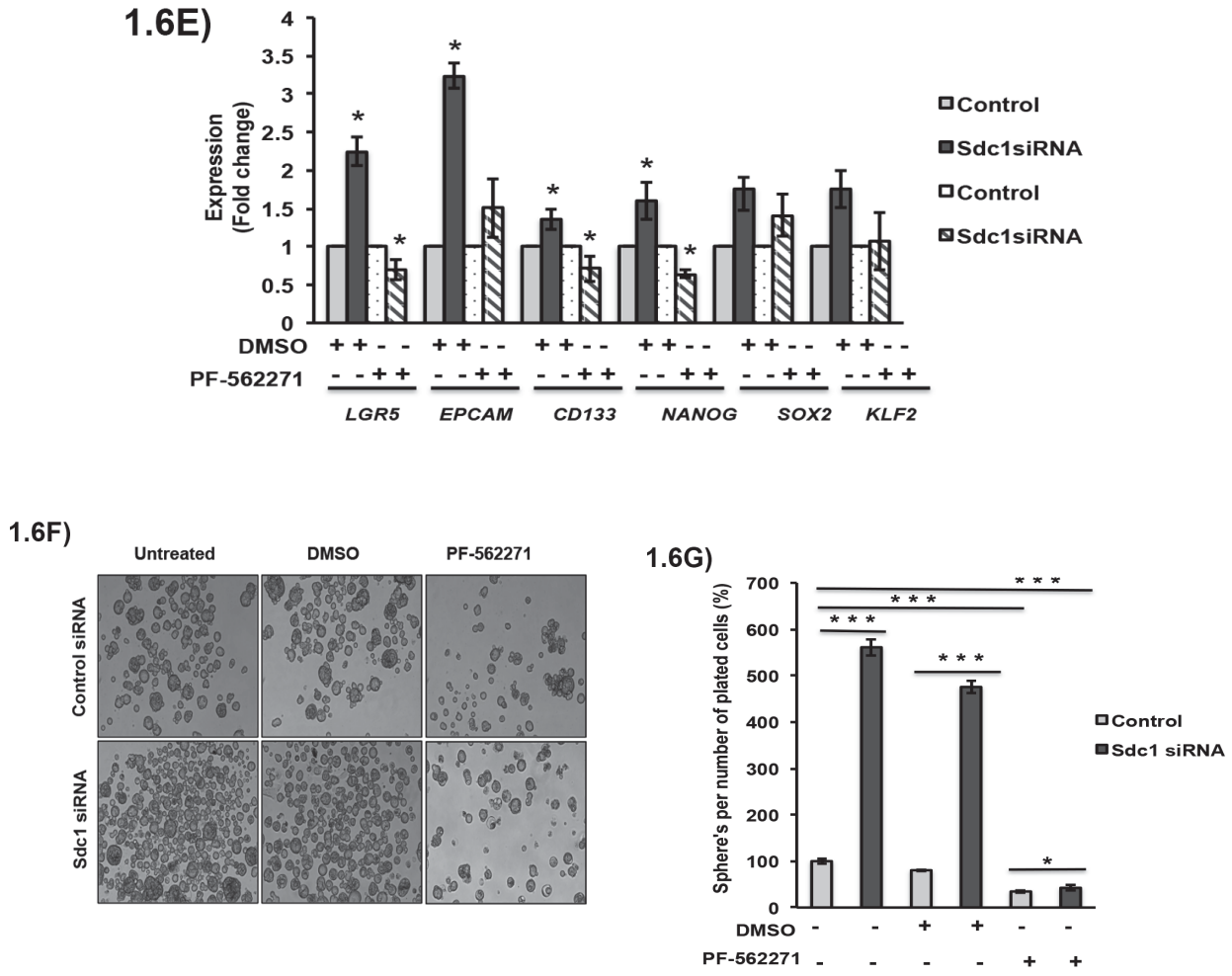


Figure 1.6. Impact of Sdc-1dependent FAK activation on the stem cell phenotype of human colon cancer cells. Pharmacological inhibition of FAK reduces Sdc-1 dependent upregulation of the transcription factor TCF4 (TCF7L2). (Fig.1.6 A) SYBR green qPCR analysis of *TCF7L2* expression in control and Sdc-1 depleted Caco2 cells subjected to treatment with or without 10ug/ml FAK inhibitor PF-573228 (1h). Sdc-1dependent upregulation of *TCF7L2* is reduced by FAK inhibition. ***p < 0.01, n=9, error bar = SEM. (Fig.1.6 B) Western blot analysis of TCF7L2 expression in Caco2 cells after 2h of treatment with 10ug/ml PF-573228 shows a decrease in expression levels upon Sdc-1 knockdown. Data shown are duplicates from a single experiment representative of three independent experiments. (Fig.1.6 C). FAK inhibitor treatment decreases the Sdc-1 dependent increase in the side population of Caco2 and HT29 cells. n=3-6 P=<0.05 error bars=SEM (Fig.1.6 D) FAK inhibitor treatment decreases the Sdc-1dependent increase in ALDH activity in Caco2 cells. n=3-6 P=<0.05 error bars=SEM (Fig.1.6 E). SYBR green qPCR analyis reveals that increased mRNA expression of *LGR5*, *EPCAM*, *CD133*, *NANOG, Sox2* and *KLF2* in Sdc-1deficient Caco2 cells is abolished by FAK inhibitor treatment. **p < 0.05,

n= 3-6, error bars= SEM. FAK inhibition abolishes the Sdc-1-dependent increase in sphere forming capacity of Caco2 cells. (Fig.1.6 F). Representative micrograph of spheres formed from Caco2 cells subjected to +/- Sdc-1 siRNA knockdown and +/- FAK inhibitor treatment. (Fig.1.6G). Quantitative analysis of sphere formation as shown in (Fig.1.6 F). Sdc-1 knockdown results in a significant ~5 fold increase compared to controls, while FAK inhibition completely suppresses sphere formation in both controls and Sdc-1depleted cells. ***$p < 0.001$, n=3-6, error bar = SEM.

3.1.7 Sdc-1 siRNA knockdown modulates the stem cell phenotype, but not survival in response to irradiation.

We have previously shown that Sdc-1 siRNA knockdown increases the resistance of MDA-MB-231 breast cancer cells to radiotherapy (Hassan H et al., 2013). To investigate a potential link of the enhanced stem cell phenotype of Sdc-1depleted colon cancer cells to radiation resistance, we subjected Sdc-1 depleted and control Caco2 and HT29 cells to irradiation with a therapeutically relevant dose of 2 Gy. Side population analysis revealed a differential impact of Sdc-1 siRNA knockdown on Caco2 and HT29 cells under irradiation conditions: In both cell lines, irradiation increased the control cell SP, whereas Sdc-1 knockdown increased the SP even further (Fig.1.7A). qPCR analysis of stemness-related gene expression revealed an impact of irradiation on *LGR5* expression in control cells, and a substantial increase in Sdc-1depleted cells. Expression of *EPCAM, NANOG* and *KLF2* was not substantially altered by irradiation (Fig.1.7B). In accordance with an enhanced stem cell phenotype, an investigation of colony formation of Sdc-1depleted cells revealed an increased survival compared to controls. However, under irradiation conditions, survival was reduced both in control and Sdc-1 siRNA knockdown cells to a similar extent (Fig.1.7 C). As increased stemness has been linked to increased resistance to chemotherapy due to increased expression of multidrug resistance proteins (Ghisolfi L et al 2012; Richard V et al., 2013), we tested the impact of cisplatin and doxorubicine treatment on the viability of Sdc-1depleted Caco2 and HT29 cells. While Sdc-1 siRNA treatment did not alter sensitivity to cisplatin (results not shown), we observed an increased resistance to doxorubicine in Sdc-1 depleted HT29 cells (Fig.1.7 D).

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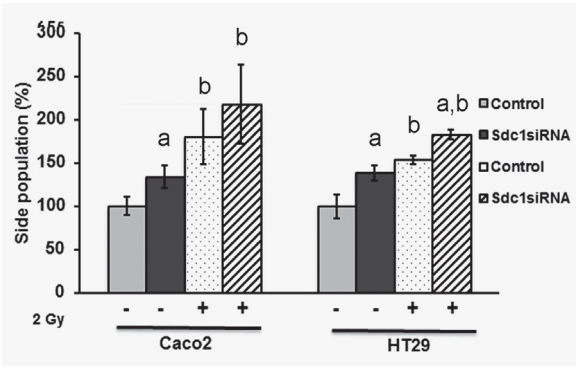

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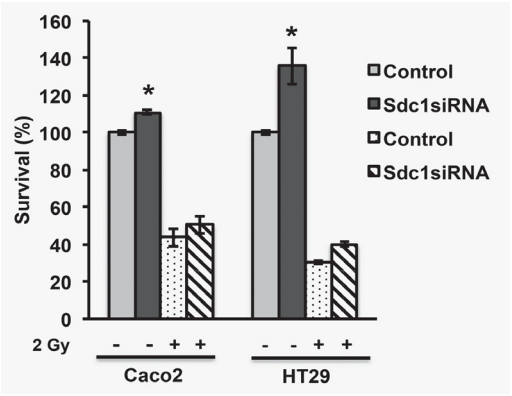

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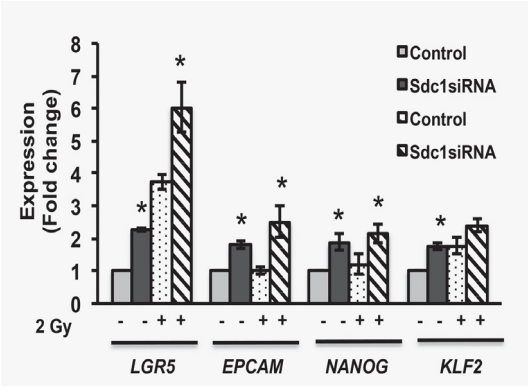

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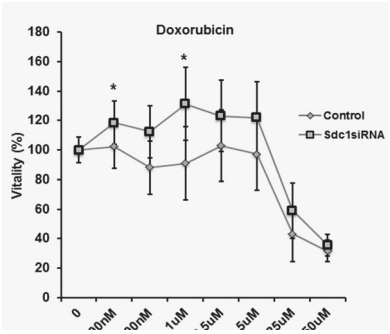
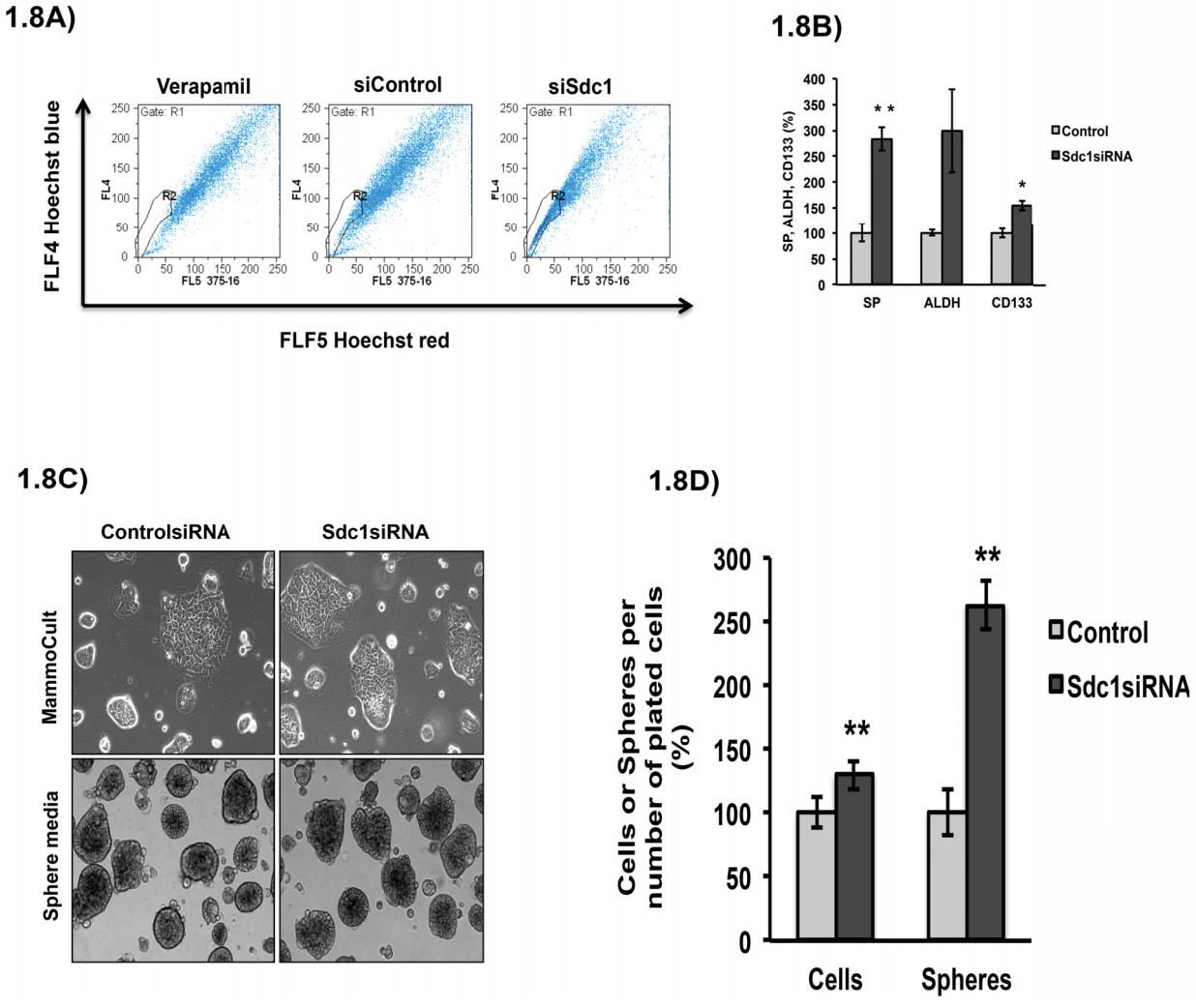


Figure 1.7. Sdc-1 depletion modulates changes in the colon cancer stem cell pool in response to irradiation. (Fig.1.7 A) Control and Sdc-1 siRNA-treated Caco2 and HT29 cells were subjected to irradiation of 2 Gy. After 4 h of radiation, the side population was analyzed by flow cytometry. a=$p<0.05$ compared to unirradiated control, b=$p<0.01$ compared to unirradiated control. $n\geq3$, error bars = SEM. (n=3-6, *=$P<0.05$B). (Fig.1.7 B) Radiation with 2 Gy induces significant increases in the expression of the stem cell markers *LGR5, EPCAM, NANOG*, and *KLF2*, as assessed by SYBR green RT-PCR (n=3-6, *=$p<0.05$). (Fig.1.7 C) Colony survival assay shows an increase in the survival rate upon Sdc-1 knockdown in both the cell lines Irradiation significantly reduces the colony survival rate independent of Sdc-1 (n=3-6, *=$P<0.05$, **=$P<0.01$). (Fig.1.7 D) Sdc-1 depleted HT29 cells shows increased resistance to doxorubicin in Sdc-1 depleted HT29 cells.

3.1.8 The impact of Sdc-1 depletion on the CSC phenotype is substantially enhanced by SP enrichment.

We next asked the question if the stemness-associated phenotype of Sdc-1 depleted colon cancer cells could be enhanced by sorting of the side population and subsequent culturing in specialized stem cell media. Upon SP enrichment and subsequent culturing in Mammocult media, we saw a significant increase in the SP levels and CD133 expression in Sdc-1depleted cells, whereas the increase ALDH activity was non statistically significant (p=0.13) (Fig.1.8 A,B).Consistent with this observation, by using two different kinds of media Mammocult media (adherent conditions) and sphere media (suspension), we observed an increase in the ability of the Sdc-1 depleted sorted cells to form tumorospheres when grown in suspension culture (Fig.1.8 C,D) indicating increased self-renewal (a fundamental characteristic of stem cells. In the cells cultured in Mammocult media we saw an increase in pFAK (Fig.1.8 E) and TCF4 (Fig.1.8 F) expression in Sdc-1 depleted SP sorted cells compared to SP control cells. And also we observed an increase in the number of colonies in irradiated Caco2 cells upon Sdc-1 siRNA knockdown, indicating that Sdc-1depletion renders SP-enriched cells more radioresistant (Fig.1.8 G).



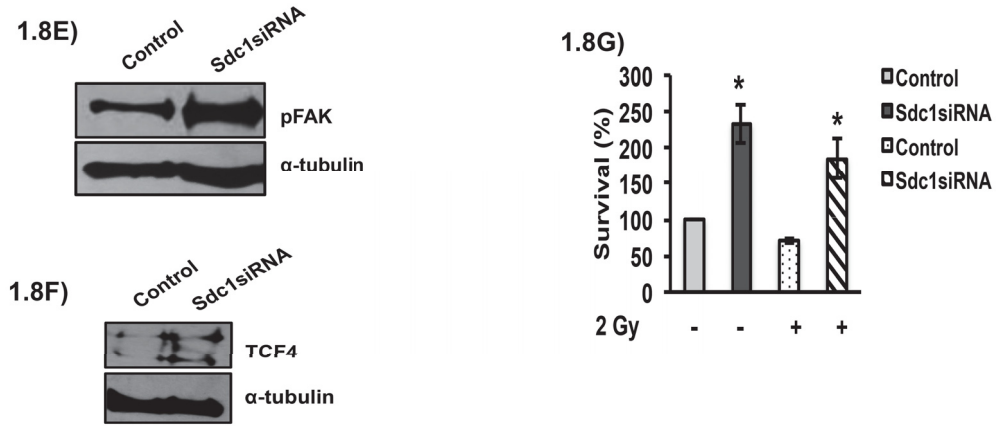


Figure 1.8. Side population-enrichment and culturing in MammoCult media enhance the cell phenotype of Sdc-1 silenced Caco2 cells. The side population of Caco2 cells was sorted by flow cytometer, cultured in MammoCult or Sphere media respectively and re-analyzed for stemness-associated parameters. (Fig.1.8 A, B) The side population and CD133 of Sdc-1 depleted Caco2 cells cultured in Mammocult shows a significant increase of ~3 and ~1.5 folds. *=p>0.05, n=3, error bars=SEM. (Fig.1.8 C) Sdc-1depleted side population enriched Caco2 cells showed increased cell numbers/plated cell in MammoCult media, and increased sphere numbers when cultured in sphere media. Representative morphology of Caco2 cells cultured in MammoCult or sphere media, respectively. (Fig.1.8 D) Quantitative analysis of cell and sphere numbers in SP enriched Sdc-1silenced and control Caco2 cells cultured in sphere or MammoCult media, respectively. *=p>0.05, n=3, error bars=SEM (Fig.1.8 E,F) Increased expression of pFAK and Tcf4 in SP-enriched Sdc-1depleted Caco2 cells cultured in Mammocult media as analyzed by western blotting. (Fig.1.8 G) Sdc-1depleted SP enriched Caco2 cells cultured in Mammocult media show a substantial increase in the colony survival rate, with an increased survival rate after irradiation compared to controls (compare with Figure 1 . 7C). Data are expressed as mean percentage +/- SEM relative to the controls (set to 100%) (n=3, *=P< 0.05, **=P< 0.01).

3.1.9 Loss of Sdc-1 increases the colon cancer tumorigenicity in vivo

To assess the loss of Sdc-1 response to tumorigenicity we implanted Sdc-siRNA and control siRNA treated HT29 cells in NOD/SCID mice. *In vivo* xenograft experiments were performed by our collaboration partners (Dr. Rolland Reinbold and colleagues) at the ITN, Segrate, Milan, Italy, with help by Sampath Kumar Katakam. We found an increase in tumor growth, suggesting that loss of

Sdc-1 significantly increased tumorigenicity *in vivo* which could be becuase of the presence of tumor initiating cells (Fig.1.9).

1.9)

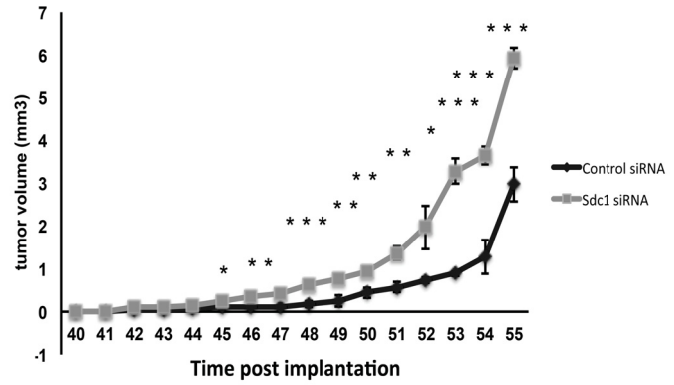


Figure 1.9. Sdc-1 depletion increases colon cancer tumorigenicity in vivo. Control and Sdc-1 siRNA-treated HT29 cells were injected into *NOD/SCID mice. Increase in tumor growth was observed from day 48 to day 55.*(n=3,**=P< 0.01***=P<0.001).

3.2 Part 2: Functional interplay of heparanase and Syndecan-1 in colon CSCs.

3.2.1 Loss of Sdc-1 regulates the expression of HPSE.

During tumor progression, specific sets of genes control the regulation of heparan sulfate proteoglycans and their associated enzymes. (Yang Y et al., 2007; Peretti T et al., 2008). Previously, we showed that expression of HS3ST2 (Vijaya Kumar A et al., 2014) and HS2ST1 (Vijaya Kumar A, 2014) to have a profound effect on breast cancer cell malignancy. Gene array analysis and confirmatory qPCR of Sdc-1siRNA transfected MDA-MB-231 breast cancer cells previously showed an increase in the transcription levels of *HPSE* compared to the controls (Ibrahim et al., 2012). Transcriptional studies show that loss of Sdc-1 (Fujiya M et al., 2001; Lundin M et al., 2005; Wang H et al., 2010) and enhanced expression of *HPSE* (Lerner I et al., 2011; Doviner V et al., 2006; Nobuhisa T et al., 2005) correlates with tumor growth, invasion metastatic potential, and reduced postoperative survival of cancer patients (Mikami S et al., 2001). With these changes in the expression patterns of Syndecan-1 and heparanase, we hypothesized that loss of Syndecan-1 may regulate the heparanase levels in colon cancer cells. To test our hypothesis, we used human Caco2 cells as an in vitro system. Interestingly, it was previously shown that induction of oncogenes Ras and SRC in Caco2 cells showed a decrease in the expression of Syndecan-1 and an increase in the heparanase activity. (Lévy P et al., 1997). In a first step we attempted to verify altered expression by quantitative real-time PCR. We found that knockdown of Syndecan-1 in Caco2 and COLO205 cells upregulated the mRNA expression of heparanase, whereas this effect was not seen in HT29 cells (Fig 2.1A). When Syndecan-1 was overexpressed ectopically, heparanase expression was also markedly rescued in Caco2 and HT29 cells (Fig 2.1B) These results suggested that HT29 might employ a different mechanism contributing to heparanase-promoted cell invasion. Syndecan-1-depleted Caco2 cells were next tested for heparanase activity using an assay based on the measurement of heparanase-induced degradation of biotinylated-HS fragments (b-HS). In this assay, heparanase degrades a b-HS fragment, which is specifically designed so that it does not bind to FGF when degraded by heparanase, thus resulting in a lower strepatavidin-HRP signal. By this method, we were able to distinctly measure a 34% increase in the activity of Sdc-1siRNA Caco2 cells compared to controls, whereas in HT29 cells, Sdc-1siRNA showed no significant difference in heparanase activity (Fig 2.1C) Thus, heparanase activity correlated with Syndecan-1 expression in

Caco2, but not in HT29 cells. To test if the Sdc-1-dependent regulation of heparanase expression occurred at the transcriptional level, we performed a *HPSE* promoter luciferase reporter assay (Baraz L et al., 2006). We found that the promoter activity was significantly increased in Sdc-1 siRNA-treated cells compared to the control siRNA (Fig 2.1D). To test if the transcriptional increase of heparanase has a role in cancer cell invasiveness, we performed matrigel invasion chamber assays. Consistent with previous findings, knockdown of Sdc-1 expression dramatically increased the cell invasiveness. The influence of heparanase in contributing to invasiveness was determined by treating the Sdc-1siRNA and control siRNA transfected cells with an inhibitor that targets heparanase activity, SST0001. Previous reports have highlighted the therapeutic role of this non-anticoagulant N- acetylated glycol split heparin in myeloma (SST0001, a Chemically Modified Heparin, Inhibits Myeloma Growth and Angiogenesis via Disruption of the Heparanase/Syndecan-1 (Joseph P et al., 2011). Interestingly, a decrease of ~50% in colon cancer cell invasion was observed upon SST0001 inhibitor treatment, suggesting that the increase in heparanase expression contributes to the increased invasion of Sdc-1 knockdown cells (Fig 2.1E). To test a possible influence of *MMP*'s in the Sdc-1-mediated invasion phenotype, we performed gelatin zymography using the conditioned media obtained from the invasion assay. We did not observe any significant difference in the activity of *MMP2* and *MMP9,* suggesting that the influence of heparanase in invasion of Sdc-1-depleted cells is indeed stronger than a possible effect of gelatinolytic MMPs (Fig 2.1F). qPCR analysis revealed no strong effect on the expression of several MMPs, whereas TIMP1 expression was decreased (Fig 2.1G). Overall, the moderate transcriptional up- and downregulation of several proteolytic factors was not uniform and did not indicate a preferential direction. Taken together, these results indicated that decreased expression of Syndecan-1 regulate the activation of heparanase transcription, playing a critical role in the promotion of invasiveness of colon cancer cells.

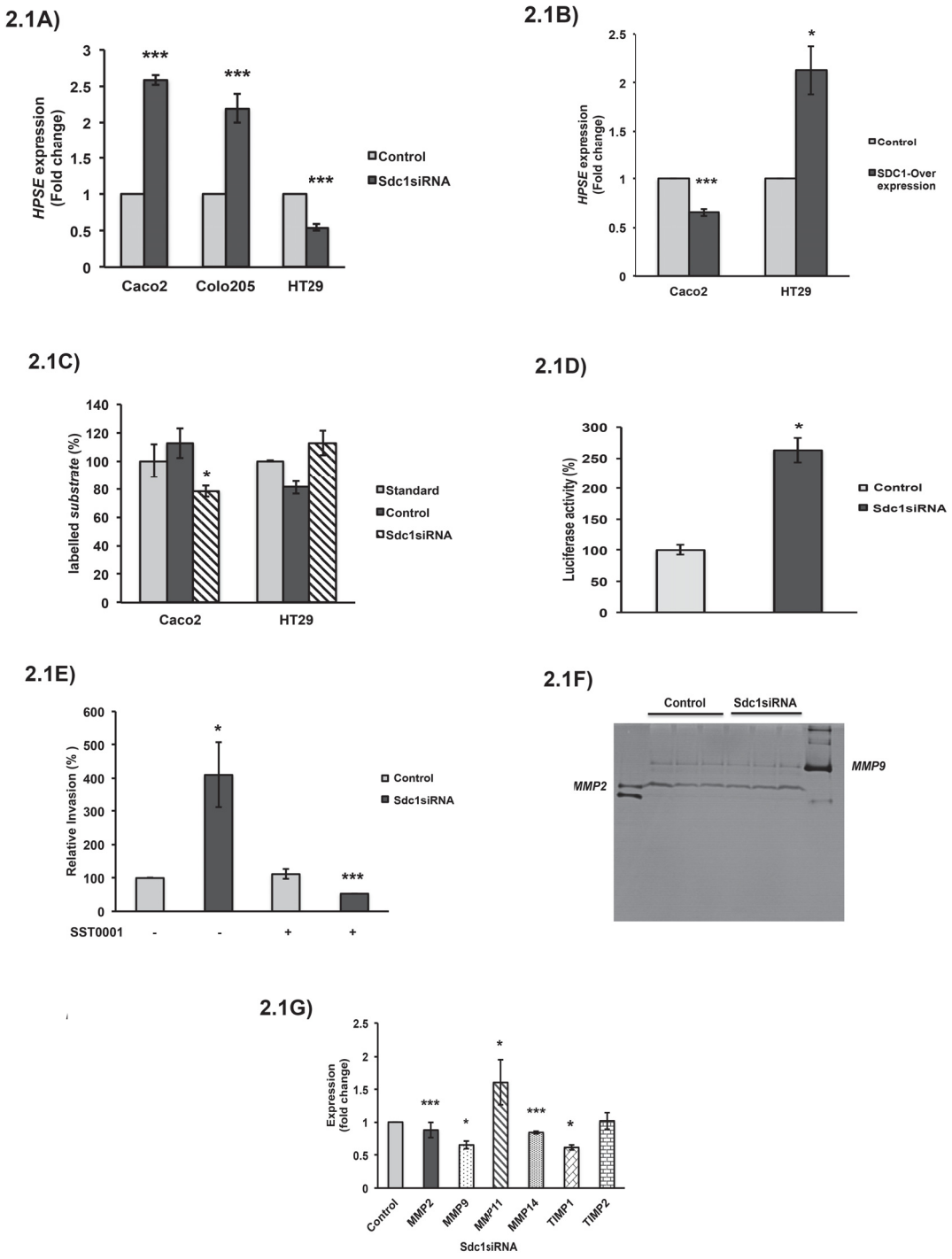


Figure 2.1. Sdc-1 depletion increases HPSE expression.

(Fig 2.1A) TaqMan® qPCR analysis of *HPSE* expression with Sdc-1KD in Caco2, COLO 205 and HT29 cells shows a highly significant increase of 2.6, 2.2 folds in Caco2 and COLO 205, respectively while in HT29, a significant decrease of ~50% in *HPSE* expression was observed compared to controls. N=9, error bars=SEM, *=P<0.05, **=P<0.01, ***=P<0.001

(Fig 2.1B) qPCR analysis of *HPSE* expression with Sdc-1 over expression in Caco2 shows a significant decrease of 35% whereas in HT29 cells showed a significant increase of 2.1 folds compared to controls. N=9, error bars=SEM, *=P<0.05. (Fig 2.1C) HPSE activity was determined by comparison with known standard concentrations using heparan degrading enzyme assay kit (Takara Mirus Biomedical Inc., Madison, WI). Sdc-1siRNA KD showed a significant increase of 34% in the activity of *HPSE* compared to the controls in Caco2 cells whereas Sdc-1siRNA treatment in HT29 caused no significant difference in the activity. Data are representative of three independent experiments performed. N=3, error bars=SEM, *=P<0.05. (Fig 2.1D) Heparanase activity was determined by luciferase assay using the heparanase promoter (*HPSE*-LUC). The relative light units in each sample were normalized against beta-galactosidase activity, measured by a colorimetric assay. Sdc-1KD showed a significant increase of 2.5 folds in heparanase activity compared to controls. N=15, error bars=SEM, *=P<0.05. Each transfection was carried out in triplicate and the data are representative four independent experiments performed. (Fig 2.1E) Sdc-1KD significantly increased invasion by 4.1 folds in Caco2 cells compared to controls whereas a highly significant decrease of ~50% in invasion was observed upon SST0001 inhibitor treatment. Representative images of stained matrigel matrix filters. t=72hrs. Data are representative of one of the three independent experiments performed. N=4, error bars=SEM, *=P<0.05, ***=P<0.001. (Fig 2.1F) Gelatin zymography showed no difference in the activity of gelatinolytic proteases *MMP2* and *MMP9* suggesting the influence of *HPSE* in invasion. Data shown are triplicates from a single experiment representative of three independent experiments. (Fig 2.1G) TaqMan® qPCR analysis of MMPs and TIMPs in Sdc-1 silenced Caco2 showed no difference in the mRNA expression of MMP2, MMP9, MMP14 and TIMP2 whereas MMP14 showed a slight increase in the expression and TIMP1 showed a significant decrease of 31% . Data are expressed as mean percentage +/- SEM relative to the controls (set to 100%) (n=3-5, *=P< 0.05, **=P< 0.01).

3.2.2 EGR1 regulates HPSE promoter activity in Sdc-1 depleted cells.

As described in the Figure 2.1D, Sdc-1 depletion positively regulates *HPSE* expression at the transcriptional level. To determine the molecular mechanisms, we first searched the *HPSE* promoter sequence for potential transcriptional regulators of in the 1.9-kB region of the *HPSE* promoter utilized in our reporter assay. Using bioinformatics search engines (Genomatix, TRANSFAC) we identified and selected potential transcription factors (NF-κB, *SNAIL, EGR1*) predicted to bind to

the *HPSE* promoter. In fact, it is known from previous studies that NF-κB and *EGR1* contribute to the regulation of the *HPSE* promoter (Meirovitz A et al., 2011). To test if these transcription factors regulate the expression of *HPSE* in Sdc-1 depleted colon cancer cells, we performed promoter reporter assays. Transactivation studies using an NF-κB expression vector co-transfected with a reporter construct containing the 1.9-kB region of *HPSE* promoter showed a decrease in the activity in Sdc-1 depleted cells (Fig 2.2A). Supporting western blot analysis demonstrated a decrease in the expression of NF-κB in Sdc-1 depleted cells (Data not shown), suggesting that NF-κB may indeed act as a repressor regulating the *HPSE* promoter in Sdc-1 depleted cells. Next we checked the influence of *SNAIL*, using a *SNAIL* expression vector, co-transfected with a reporter construct containing the 1.9-kB region of *HPSE* promoter. We observed an increase in the promoter activity in Sdc-1 depleted cells and control cells (Fig 2.2B). Next we identified designated *EGR1* binding sites in the 1.9-kB region of the *HPSE* promoter using bioinformatics analyses (see above). The *EGR1* transcription factor is an important regulator of heparanase promoter activity. *EGR1* was shown to control heparanase expression acting as either activator or repressor of heparanase transcription, depending on the cell and tissue type (de Mestre AM et al., 2005). qPCR analysis demonstrated an increase in the expression of *EGR1* and another heparanase regulator, *COX2* in Sdc-1 depleted cells (Fig 2.2C). It is well known that *EGR1* up-regulates *HPSE* transcription in breast, prostate, and colorectal tumor cell lines, whereas in melanoma cells it represses the activity (de Mestre AM et al., 2005). To further investigate the expression of *EGR1* at the protein level, we performed an immunoprecipitation (IP) assay using the anti-EGR-1 antibody. As shown in Fig 2.2D, we saw an increase in the expression of *EGR1* in Sdc-1siRNA- compared to control siRNA-treated cells. To test the role of *EGR1* in Sdc-1-dependent *HPSE* regulation, we performed a double knockdown experiment using Sdc-1 and *EGR1* siRNA. While we saw an increase in *HPSE* activity in Sdc-1depleted cells, the double knockdown of Sdc-1 and *EGR1* resulted in decreased *HPSE* activity (Fig 2.2E). To test if this regulation was truly occurring at the promoter level, we performed a luciferase assay using 1.9-kB region of *HPSE* promoter with Sdc-1siRNA and double knock down of Sdc-1 and *EGR1*. The results were in accordance with the RT-PCR data, showing an increase in the promoter activity in Sdc-1 depleted cells, whereas double knock down of Sdc-1 and *EGR1* resulted in decreased activity compared to the respective controls (Fig 2.2F).

Altogether, these results support the notion that *EGR1* acts as a trans-activator in driving *HPSE* promoter activity in Sdc-1-depleted Caco2 cells.

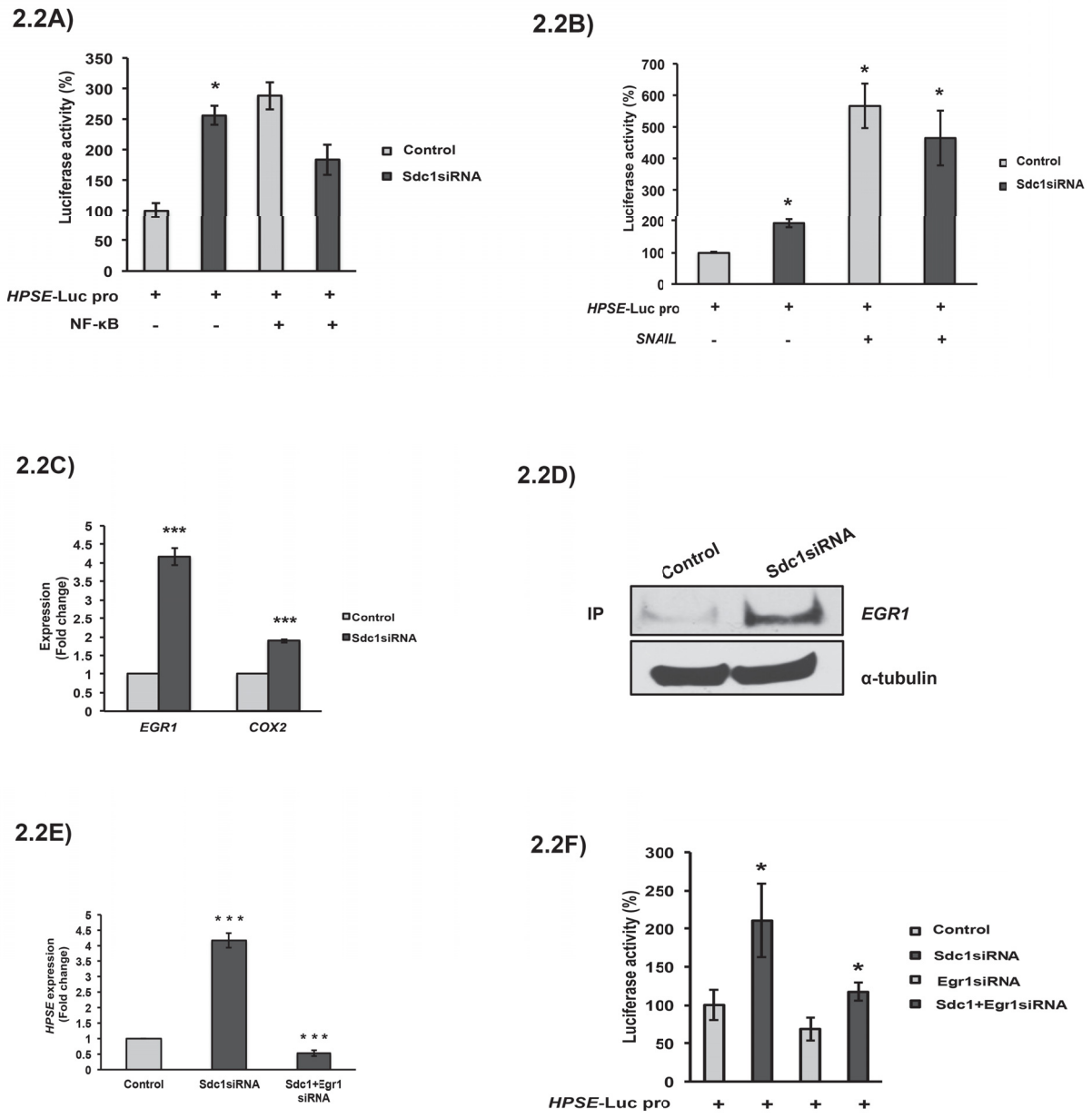


Figure2.2 Sdc-1 depletion increases HPSE expression through EGR1.

(Fig 2.2A) An expression vector encoding human NF-κB was co-transfected with the *HPSE* promoter (*HPSE*-LUC), at a ratio of 1:2. Luciferase light units and beta-galactosidase activities were measured 48h later. Sdc-1KD showed a significant increase of 2.2 folds in heparanase activity compared to the control, whereas co-transfection with NF-κB showed an increase in the activity compared to its corresponding untreated controls. N=7, error bars=SEM, *=P<0.05. (Fig 2.2B) An

expression vector encoding human Snail was co-transfected with the *HPSE* promoter (*HPSE*-LUC), at a ratio of 1:2. Luciferase light units and beta-galactosidase activities were measured 48h later. Sdc-1KD showed a significant increase of 2 folds in heparanase activity compared to controls whereas co-transfection of *SNAIL* showed a significant increase in the activity in both control and Sdc-1KD compared to the control. N=7, error bars=SEM, *=P<0.05. (Fig 2.2C) SYBR green PCR analysis of *EGR1 and COX2* expression with Sdc-1KD in Caco2 cells shows a highly significant increase of 4 and 2 folds respectively compared to controls. N=9, error bars=SEM, ***=P<0.001. Data are representative of three independent experiments performed. (Fig 2.2D) Protein homogenates were incubated with 2.5 µl anti-Egr-1 antibody as indicated, the Egr-1/antibody complex collected by protein A-Sepharose and solubilized. Equal aliquots were electrophoresed through SDS-PAGE and immunoblotted with anti-Egr-1 antibody. Immunopreciptation analysis showing the increased expression of *EGR1* upon Sdc-1KD compared to the controls. Data shown are singlet, representative of three independent experiments. (Fig 2.2E) SYBR green qPCR analysis of *EGR1* expression with Sdc-1KD in Caco2 cells shows a highly significant increase of 4 folds whereas upon Sdc-1siRNA and Egr1siRNA, there is a 40% decrease compared to controls. N=9, error bars=SEM, ***=P<0.001. (Fig 2.2F)Sdc-1KD showed a significant increase of 2 folds in *HPSE* promoter (*HPSE*-LUC) activity compared to controls whereas with double knock down of Sdc-1 and EGR1 showed a decrease in the activity compared to corresponding controls. With the Sdc-1 and EGR1 double knock down there is a significant decrease in the activity compared to Sdc-1siRNA control. N>4, error bars=SEM, *=P<0.05.

3.2.3 EGR1/FAK cross-talk regulates the expression of HPSE in Sdc-1-depleted cells.

We next wanted want to look into the molecular consequences of altered *HPSE* expression in Sdc-1 knock down cells. Previously, we saw that loss of Syndecan-1 in Caco2 cells resulted in changes in integrin activity with a concomitant increased phosphorylation of FAK (see results, Part 1). To test if FAK might be contributing to the *HPSE* regulation in Sdc-1-depleted cells, we used a FAK inhibitor (PF-562271). Treatment of Sdc-1 siRNA and control siRNA transfected cells with FAK inhibitor decreased the expression of *HPSE* as analyzed by qPCR (Figure 2.3A). Further, we checked the influence of FAK inhibition on the enzymatic activity of *HPSE*. Treatment with PF-562271 showed only a minor decrease in *HPSE* activity in Sdc-1-depleted cells compared to the controls, suggesting that the regulation occurs predominantly at the mRNA level (Figure 2.3B). It is observed that integrin-dependent signals are involved in regulating *EGR1* expression. Moreover, *EGR1* transcription has been previously linked to Erk1/2 MAPK activity acting on the *EGR1*

promoter (Cabodi S et al., 2009). We therefore asked if FAK may also be involved in *EGR1* regulation. As analyzed by qPCR a moderate decrease in the expression of *EGR1* was observed upon FAK inhibitor treatment compared to untreated Sdc-1 KD cells (Figure 2.3C). To determine which signal acts first in this regulatory axis, we performed a double knockdown experiment using *Sdc-1* and *EGR1* siRNAs. Western blot analysis of Sdc-1 KD cells revealed an increase in FAK phosphorylation while using siRNA targeting *EGR1*, a decrease in FAK phosphorylation was observed compared to Sdc-1siRNA alone. A further decrease in phosphorylation levels was evident upon Sdc-1 and *EGR1* double KD (Figure 2.3D). This result suggests that loss of Sdc-1 increases the expression of *EGR1*, which further regulates phosphorylation of FAK, probably in an indirect manner. These data indicate that the classic focal adhesion pathway is involved in transcriptional activation of heparanase through the transcription factor *EGR1*.

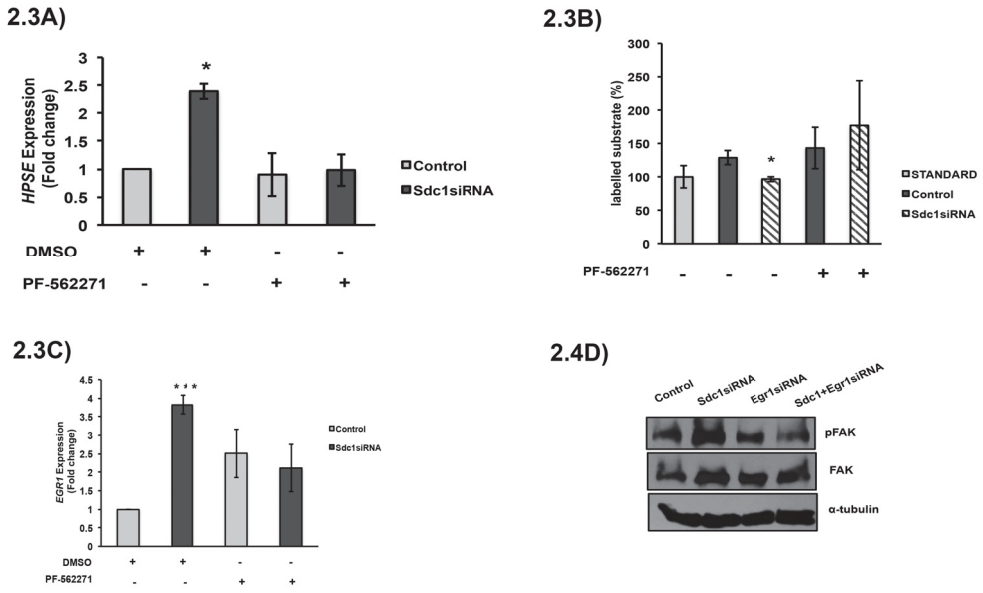


Figure 2.3. An EGR1-FAK feedback loop is involved in regulating HPSE expression in Sdc-1 depleted cells.

(Fig 2.3A) TaqMan® qPCR analysis of *HPSE* expression in Sdc-1KD Caco2 cells showed an increase of 2.4 fold whereas treatment, with 10ug/ml PF-573228 inhibitor for 1hr decreased the expression compared to the control. N=4, error bars=SEM, *=P<0.05. (Fig 2.3B) Sdc-1KD cells show a significant increase of 31% in heparanase activity. Note that the reduction of labeled substrate corresponds to an increase in enzyme activity. With PF-573228 inhibitor treatment for 6hrs the activity decreased compared to the control. N=3, error bars=SEM, *=P<0.05. (Fig 2.3C)

Upon Sdc-1KD, a significant increase of 3.8 fold in the expression of EGR1 was observed, while PF-573228 inhibitor treatment for 1hr decreased its expression. (Fig 2.3D) Western blot analysis of Sdc-1 KD cells showed an increase in FAK phosphorylation, while using siRNA targeting Egr1, a decrease in FAK phosphorylation was observed compared to Sdc-1siRNA. A further decrease in phosphorylation levels was evident upon Sdc-1: Egr1 double KD. Data shown are representative of three independent experiments.

3.2.4 Role of the Syndecan-1 and heparanase axis in cancer stem cell signatures.

We next wanted to address the question whether the Sdc-1-*HPSE* regulatory mechanism affects the phenotype and functional properties of Cancer stem cells. In the first part of the results section, we had demonstrated that Sdc-1 regulates the stem cell population in colon cancer cells. So to this end, we tested the hypothesis if increased expression of *HPSE* controls the stemness in Sdc-1-depleted cells. Flow cytometric analysis had previously shown an increase in the SP phenotype with Sdc-1 silencing in Caco2 and HT29 (Results, Part 1). Interestingly, treating Caco2 cells with the *HPSE* inhibitor SST0001 effectively inhibited the side population compared to untreated Sdc-1 KD cells, while only a slight decrease in the SP was observed in inhibitor-treated Sdc-1 KD HT29 cells compared to untreated controls (Fig 2.4A). To further identify the stem cell targets, we screened for a panel of genes and we found that *VIM*, *LGR5*, *CD-133*, *NANOG* and *SOX2* were upregulated upon Sdc-1 KD, whereas upon SST0001 treatment this upregulation of stemness-associated genes was abolished (Fig 2.4B).

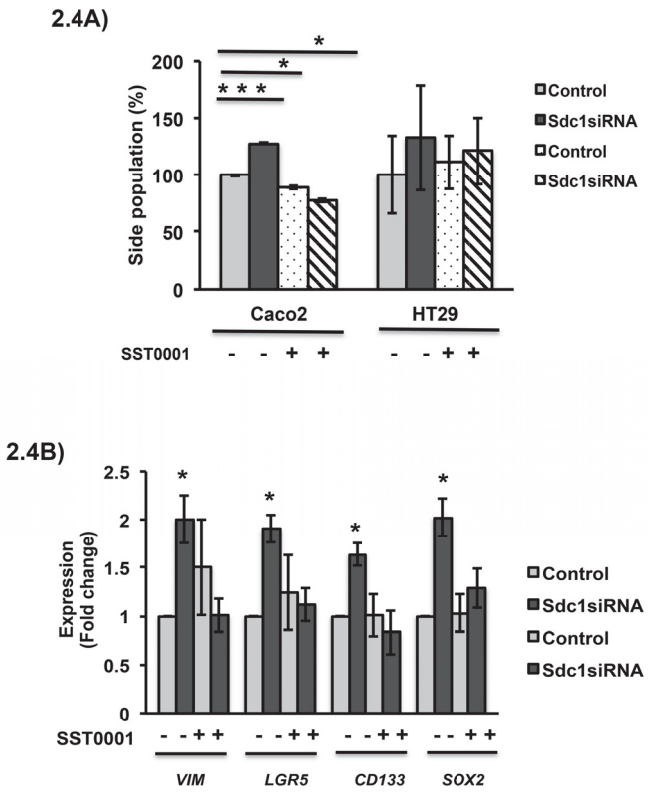


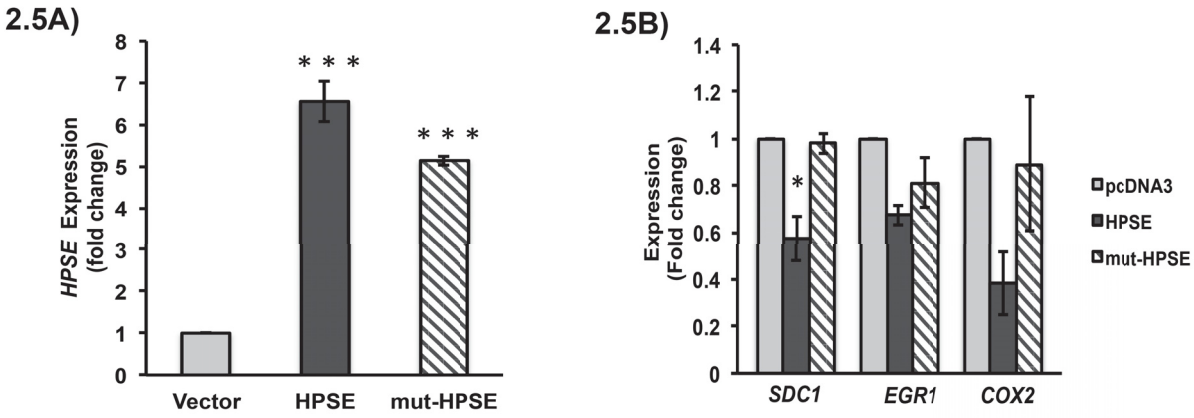
Figure 2.4 Inhibition of HPSE abolishes the Sdc-1-dependent enhancement of a colon CSC phenotype.

(Fig 2.4A) Quantitative SP analysis revealed a 30% and 35% increase in Sdc-1-depleted Caco2 and HT29 cells compared to siRNA controls. Treating Caco2 cells with *HPSE* inhibitor (SST0001) for 1 hr effectively inhibits the side population by 50% compared to Sdc-1KD treated and its corresponding untreated control. An 11% decrease in the SP was observed in inhibitor treated Sdc-1 KD HT29 cells compared to its corresponding untreated control. N=3, error bars=SEM, *=P<0.05, **=p≤0.01, error bars=SEM. (Fig 2.3B) Increased mRNA expression of *VIM*, *LGR5*, *CD-133*, *NANOG* and SOX2 in Sdc-1 deficient Caco2 cells was observed by SYBR green qPCR analysis, while treatment with SST0001 abolished this increase. N= 3-6 , error bars= SEM.

3.2.5 Enhanced HPSE expression decreases Sdc-1 expression.

To further evaluate the role of heparanase in colon cancer, we performed gain-of-function studies by generating transfected cell pools that stably express human heparanase (*HPSE*) and enzymatically inactive mutant heparanase (mut-*HPSE-dominant HPSE*). We confirmed increased expression levels of heparanase by qPCR (Fig 2.5A). Next, we sought to determine whether

upregulation of heparanase influences the expression of genes that are involved in the Sdc-1dependent regulation of heparanase. As heparanase is firmly related to the process of syndecan-1 shedding (Mahtouk K et al., 2007) we checked the expression of Syndecan-1 by qPCR and we found a decrease in its expression. While two other heparanase regulators, *EGR1* and *COX2* (Lerner I et al., 2011) were decreased in the heparanase overexpressiong clones, these data did not reach statistical significance (Fig 2.5B). The decreased syndecan-1 expression was further confirmed by flow cytometry in heparanase overexpressing cell clones. This finding may support the concept that heparanase sheds the Syndecan-1 ectodomain, an important trigger of the switch from autocrine to paracrine signaling (Wang H et al., 2001)(Fig 2.5C). In addition to its catalytic activity, heparanase promotes the expression of other glycosaminoglycan processing enzymes. For example, it was shown that heparanase influences the expression of HS editing enzymes of the Sulf family (Escobar Galvis ML et al., 2007; Hammond E et al., 2014). When we examined *HPSE* and mut-*HPSE* clones by qPCR analysis, we observed an increase in the expression of *SULF1* and *SULF2* and a decrease in the expression of *HS2ST1* relative to vector controls (Fig 2.5D). It has been observed that these genes are involved in enzymatic modification of HS, promoting tumor growth and progression by enhancement of protumorigenic signaling events (Lai JP et al., 2008; Uchimura K et al., 2006; Vijaya Kumar A, 2014).



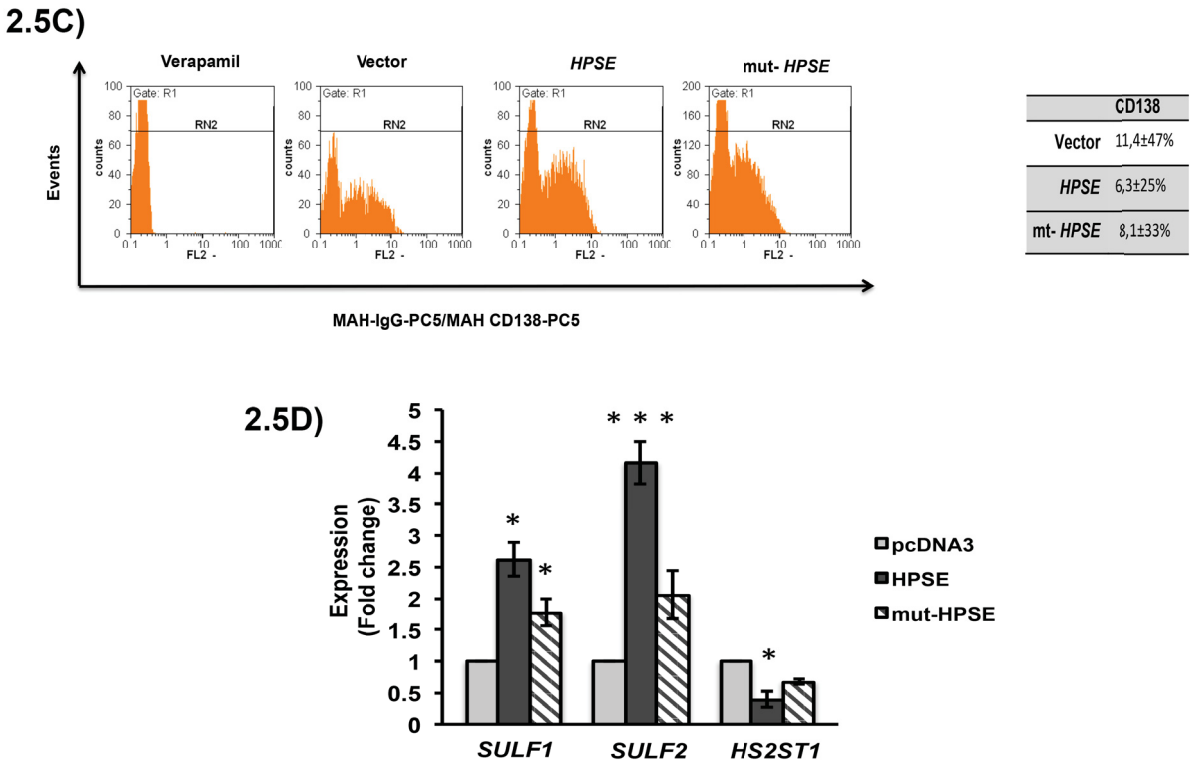
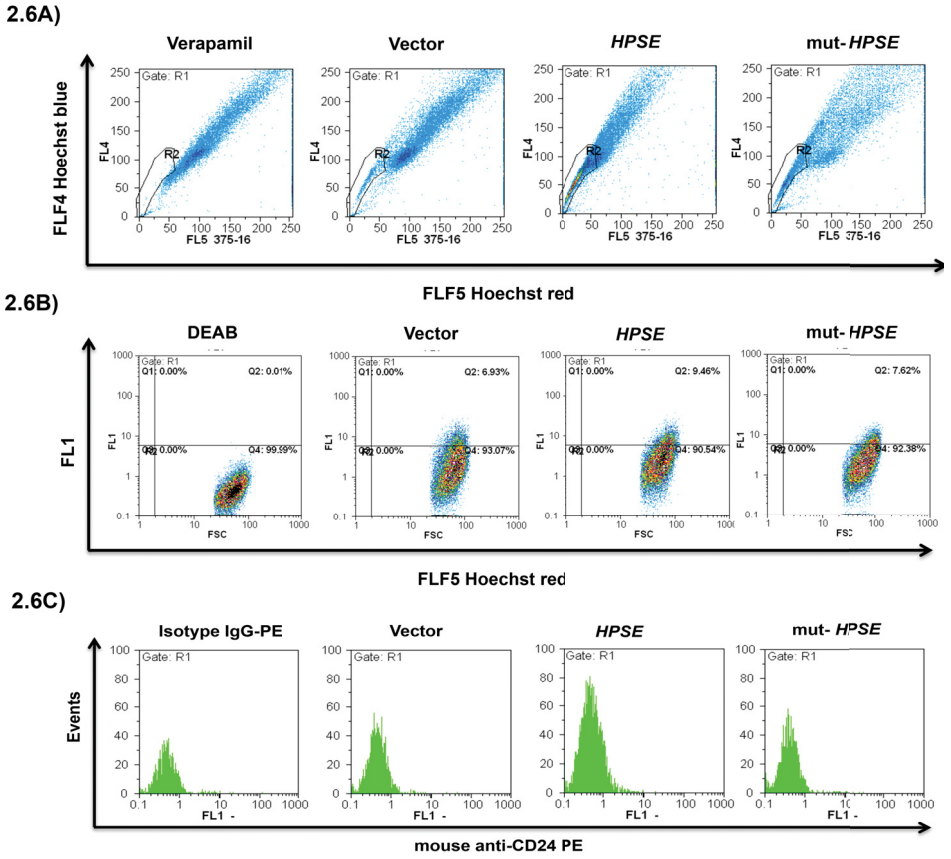


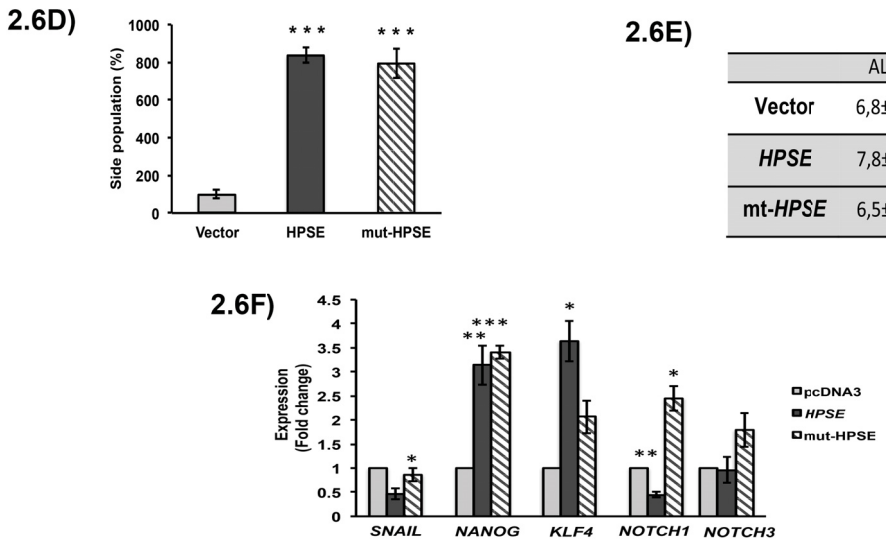
Figure 2.5. Increased expression of HPSE decreases Sdc-1 expression.

(Fig 2.5A) Caco2 cells were stably transfected with pcDNA 3.1 vector, *HPSE* and mut- *HPSE* cDNA. Confirmation of increased expression by TaqMan® qPCR analysis showed a highly significant increase of 6.5 and 5 fold expression of *HPSE* and mut-*HPSE* compared to the vector (pcDNA3). N=9, error bars=SEM, ***=P<0.001. (Fig 2.5B) *HPSE* induces a significant decrease of 40% in the expression of Sdc-1 while there is no significant effect on *EGR1* and *COX2* expression. Mut-*HPSE* did not alter the expression of Sdc-1, *EGR1* and *COX2* as assessed by TaqMan® and SYBR green RT-PCR (n=3-6, *=P<0.05,). (Fig 2.5C) Representative result displaying flow cytometric analysis of CD138 (Sdc-1) expression. *HPSE* overexpression in Caco2 cells induced a decrease in the expression of Sdc-1 by 35% compared to vector control. (Fig 2.5D) *HPSE* over expression in Caco2 cells induced a significant increase in the expression of sulfatases, *SULF1* and *SULF2* by 2.5 and 4.3 folds respectively and a decrease of 60% in the expression of a HS sulfotransferase, *HS2ST1* compared to vector control. mut-*HPSE* overexpression in Caco2 resulted in significant increase in the expression of *SULF1* by 1.8 fold while no significant effect was observed in *SULF2* and *HS2ST1* expression at mRNA level. (n=3-6, *=P<0.05, **=P<0.01).

3.2.6 Heparanase expression regulates the cancer stem cell phenotype.

To better understand the potential role of heparanase in controlling the stemness of colon cancer cells, we analyzed the cancer stem cell properties by flow cytometry(Figure 2.6A). Hoechst 33342 dye exclusion assays showed an increase of the SP in *HPSE*- and also interestingly in mut-*HPSE*-overexpressing clones compared to vector controls (Figure 2.6D). We next analyzed other colon cancer stem cell-associated parameters, the activity of ALDH isoform 1 (ALDH1) and the colon cancer surface marker CD133 (Figure 2.6B and 2.6C). We only detected a minor increase of these parameters in *HPSE*- and mut-*HPSE* overexpressing clones, while interestingly, we saw an increase in CD24 expression in *HPSE* overexpressing clones compared to vector (Figure 2.6E). In order to obtain a more detailed view of the possible roles of *HPSE* in cancer stemness, we examined the expression profile of various genes involved in colon CSC functions. Strikingly, we saw a significant increase in the expression of *NANOG* and *KLF4* in *HPSE*-transfected clones whereas mut-*HPSE* overexpressing cells showed an increase in *NOTCH1* and *NOTCH3* expression (Figure 2.6F). These results collectively provided further evidence that *HPSE* is involved in regulating stem-like characteristics of colon cancer cells.





	ALDH	CD133	CD24
Vector	6,8±37,2	6.04±1,9	1,9±41
HPSE	7,8±42,2	7,2±2,2	4,0±77
mt-*HPSE*	6,5±33,1	7,7±2,4	1,8±62

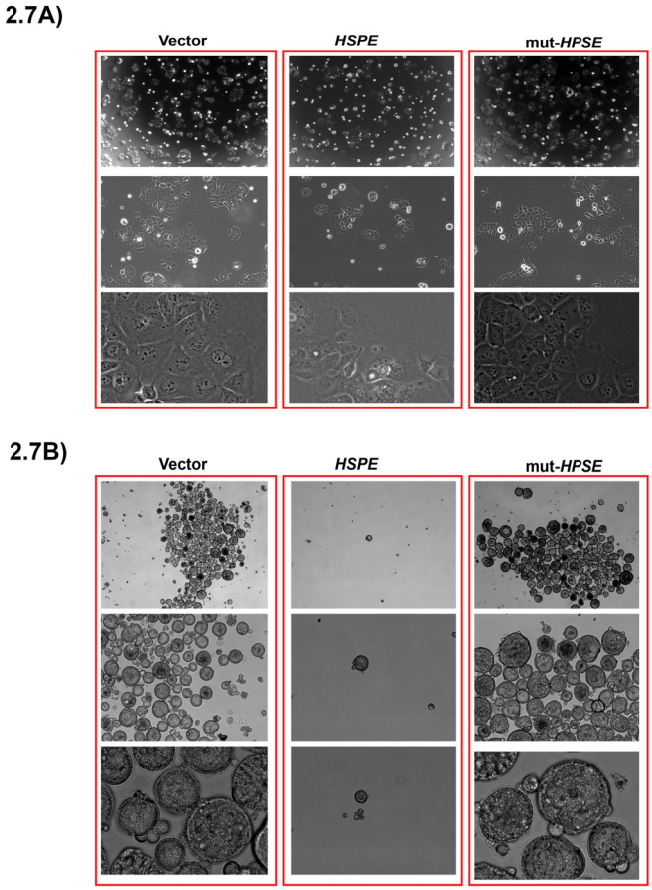
Figure 2.6. Upregulation of HPSE enhances the colon cancer stem cell phenotype.

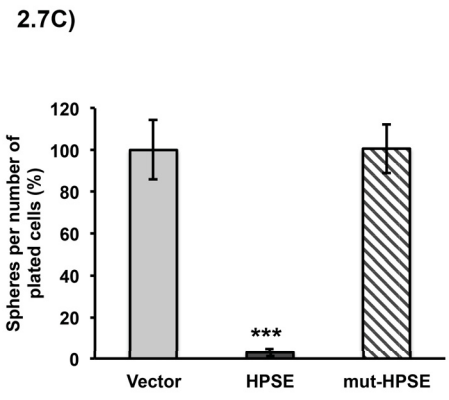
(Fig 2.6A, 2.6B and 2.6C) Representative result displaying the side population phenotype, ALDH activity and CD133 expression. (Fig 2.6D) Quantitative analysis of the SP revealed a 8.2 fold and 8 fold increase in *HPSE* and mut-*HPSE* overexpressing clones, respectively. n≥3, error bars=SEM, ***=P<0.001. (Fig 2.6E) Fluorescence intensity of ALDH and CD133 did not show a difference, whereas CD24 revealed a 4 folds increase in *HPSE* compared to vector and mut-*HPSE* over expressing clones. n≥3, error bars=SEM. (Fig 2.6F) SYBR green qPCR analysis. *HPSE* overexpression in Caco2 induced a significant increase in the mRNA expression of *NANOG* and *KLF4* by 3.2 and 3.6 folds, respectively, while no significant effect was observed in *SNAIL*, *NOTCH1* and *NOTCH3* compared to vector control. mut-*HPSE* over expression in Caco2 resulted in significant increase in the expression of *NANOG* and *NOTCH1* by 3.4 and 2.5 folds while no effect was observed in *SNAIL*, *KLF4* and *NOTCH3* expression at mRNA level. N=9, error bars=SEM, *=P<0.05, **=P<0.01, ***=P<0.001.

3.2.7 Enhanced heparanase decreases the sphere formation ability in colon cancer cells.

In striking contrast to the phenotypic marker characterization, the *HPSE*-overexpressing cells failed to form spheres (Figure 2.7B and 2.7C). In contrast, in cells overexpressing the dominant-negative mutant of *HPSE,* we observed no difference compared to vector controls (Fig 2.7C)**.** Therefore, the enzymatically active form of *HPSE* may remodel the ECM and in turn the cells may lose the ability to generate spheres under suspension culture conditions. Having found that,

we attempted to further investigate this effect by using heparanase inhibitor (SST0001) in untransfected Caco2 and HT29 cells, followed by sphere formation assays (Fig 2.7D). Surprisingly, we saw a decrease in sphere formation with SST0001 in both Caco2 and HT29 cells at the physiological expression levels of heparanase.





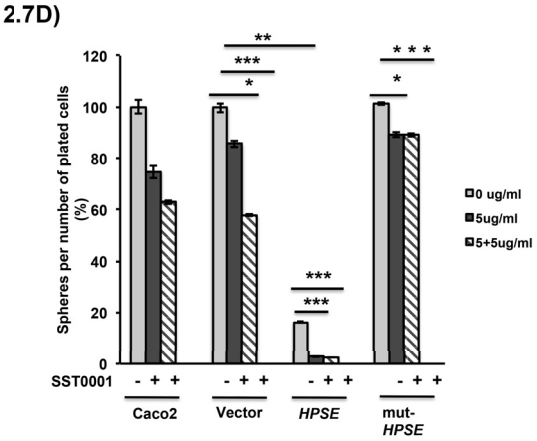


Figure 2.7. Increased expression of enzymatically active HPSE decreases colonosphere formation.

(Fig 2.7A) Representative results displaying the morphology of vector, *HPSE* and mut-*HPSE transfected* Caco2 cells undeer adherent culture conditions. (Fig 2.7B) Representative figure displays the spheres generated from the cells shown in panel 2.7A after 10 days. *HPSE* overexpression decreases the formation of spheres compared to vector and mut-*HPSE* cells. (Fig 2.7C) Quantitative analysis of the sphere formation efficiency in *HPSE* over expressing Caco2 resulted in a highly significant decrease in the spheres by 97% compared to control vector and mut-*HPSE*. N=9, error bars=SEM, *=P<0.05, **=P<0.01, ***=P<0.001. (Fig 2.7D) Quantitative analysis of the sphere formation efficiency after *HPSE* inhibitor (SST0001) treatment. Caco2 and Vector controls revealed a 25% and 20 % decrease in spheres at 5ug/ml inhibitor concentration. Treating the cell with (5ug/ml) inhibitor and further adding the inhibitor (5ug/ml) to the generated spheres showed a 40% decrease in sphere formation in both Caco2 and vector. . Treating the cells with *HPSE* inhibitor in *HPSE* over expressing cell completely abolished sphere formation. mut-*HPSE* showed a 10% decrease in spheres. N=9, error bars=SEM, *=P<0.05, **=P<0.01, ***=P<0.001.

3.2.8 Heparanase expression enhances WNT signaling and Integrin activation.

In an extension of this work, we wanted to look for the intracellular signaling pathways involved in heparanase-induced stem cell properties. As Wnt/b-catenin signaling is a potential direct downstream target for colon cancer stemness, we wanted to investigate the influence of Wnt signaling regulation in *HPSE* overexpressing cells. To test this hypothesis, we analyzed the SP in the presence or absence of a WNT inhibitor (IWAP2). Analysis of the side population in *HPSE*- and mut-*HPSE*- overexpressing Caco2 cells showed an increase of 1.8 and 1.6 fold compared to the vector controls. In contrast, treatment of the cells with WNT inhibitor (IWAP2) inhibited the side population in *HPSE* overexpressing Caco2 cells compared to the untreated *HPSE* overexpressing cells (Fig 2.8A). Furthermore, by western blot analysis we saw an increase in the expression of the Wnt-related transcrioption factor *TCF4* in *HPSE* overexpressing cells compared to vector controls and mut-*HPSE* cells (Fig 2.8B). This indicates that activation of the Wnt signaling is crucial for enhancement of stemness activities in *HPSE* overexpressing cells. To further identify the intracellular signaling pathway underlying this phenotype, we sought to investigate the involvement of integrins. Western blot analysis furthermore revealed an increased

phosphorylation of FAK as a readout of increased integrin activation (Fig 2.8C) (Zetser A et al., 2003).

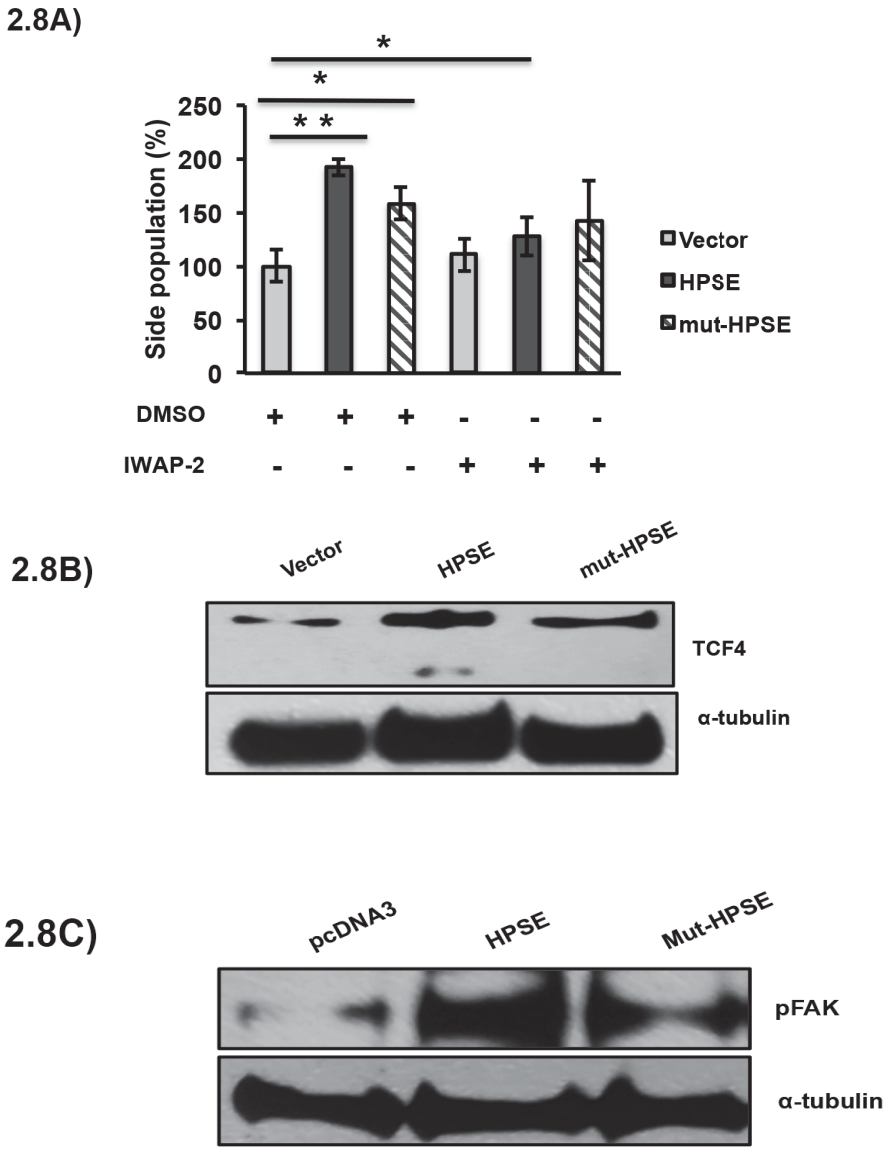


Figure 2.8. Enhanced HPSE expression modulates cancer stem cell like cells through Wnt and FAK signaling.

(Fig 2.8A) Analysis of the side population in Caco2 *HPSE* and mut-*HPSE* overexpressing cells showed an increase of 1.8 and 1.6 fold compared to the vector control. Treating the cells with WNT inhibitor (IWAP2) for 1 hr effectively inhibited the side population by 64% in *HPSE* overexpressing Caco2 cells compared to the untreated *HPSE* overexpressing cells, while no significant difference was observed in mut-*HPSE* over expressing clones. **=$p \leq 0.01$, $n \geq 3$, error bars=SEM. (Fig 2.8B) Western blot analysis showing the increased expression of TCF7/L2 in

HPSE over expression Caco2 cells compared to the vector control and mut-*HPSE*. Data shown are representative of three independent experiments. (Fig 2.8C) Western blot analysis showing the increased expression of pFAK in *HPSE* overexpressing Caco2 cells compared to the vector control and mut-*HPSE*. Data shown are representative of three independent experiments.

3.2.9 Influence of heparanase expression on the radio- and chemosensitivity of colon cancer cells.

As FAK activation has been linked to radiation resistance (Eke I et al., 2012) ,we further investigated the anchorage-independent growth properties of *HPSE* overexpressing cells following irradiation by colony formation assays. We saw a decrease in the number of colonies in *HPSE* and mut-*HPSE* overexpressing cells compared to vector controls. Irradiation with 2Gy showed a slight decrease in colony survival compared to the non-radiated controls meaning that *HPSE* overexpressing cells are more resistant to radiation in comparison to irradiated vector controls (Fig. 2.9A). In colon cancer, even though most of the chemotherapeutic regimens are principally sensitive, drug failures occur in highly metastatic cancers, which is attributed to therapeutic resistance (Dalerba, P et al., 2007)As therapeutic resistance has been linked to CSC properties such as high expression of multidrug resistance proteins, we wanted to investigate the chemo resistance properties of the *HPSE* overexpressing cells. When we treated these cells with paclitaxel and cisplatin, surprisingly mut-*HPSE* overexpressing cells showed increased resistance to paclitaxel and cisplatin, whereas *HPSE* overexpressing cells did not show a clear effect compared to vector (Fig 2.9B and 2.9C). Taken together, our data suggest that *HPSE* overexpression is associated with changes in the resistance of colon cancer cells to chemo- and radiotherapy, involving a differential role for the enzymatic activity of *HPSE*.

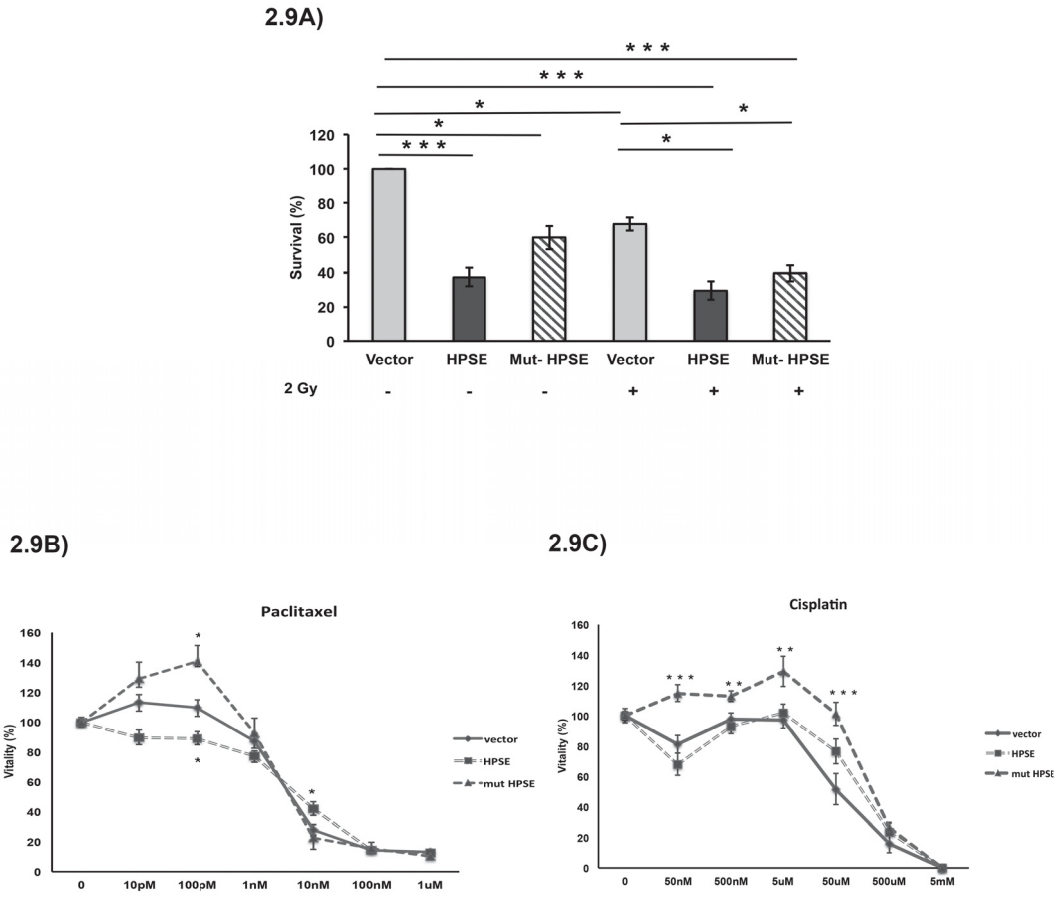


Figure 2.9: Increased expression of HPSE influence radio- and chemo- resistance Of colon cancer cells.

(Fig 2.9A)Colony survival assay showed a highly significant decrease of 63% in the survival rate in *HPSE* while only a 40% decrease was observed in mut-HPSE over expressing Caco2 cells compared to vector control. Irradiation of Caco2 cells with 2Gy showed a profound decrease in the colony survival compared to the non-radiated ones. *HPSE* overexpressing Caco2 cells upon irradiation showed a further significant decrease in the survival rate of 21% compared to its non-radiated counterpart. N=9, error bars=SEM, *=P<0.05, **=P<0.01, ***=P<0.001. (Fig 2.9B and 2.9C) *HPSE* overexpression slightly increases the sensitivity to the chemotherapeutic drug paclitaxel, whereas mut-*HPSE* overexpression increases resistance to both paclitaxel and cisplatin relative to vector controls. N=9, error bars=SEM, *=P<0.05, **=P<0.01, ***=P<0.001.

4 Discussion:

4.1 *Role of Sdc-1 in colon cancer stemness.*

In this study, we have demonstrated a novel function of the HSPG Sdc-1 as a regulator of a colon CSC phenotype. siRNA-mediated knockdown of Sdc1 resulted in an increase in phenotypic stem cell markers (including the SP, ALDH, *CD133*, *LGR5, SOX2, NANOG*), acquisition of an EMT-like phenotype, and in enhanced stemness-associated properties including colony formation ability, sphere formation, and increased tumor growth in a xenograft model. These stemness-associated alterations were associated with changes in clinicopathologically relevant parameters, including an increase in invasiveness (partially linked to EMT), increased proliferation and tumor growth in vivo (potentially linked to a higher proliferative potential), increased resistance to chemotherapy (known to be associated with the SP phenotype (Richard V et al., 2013) and increased resistance to radiation (Ghisolfi L et al., 2012) in the SP-enriched population of Sdc1-depleted cells. Assisted by a transcriptome-wide analysis of Sdc1-depleted Caco2 cells, we were able to attribute the enhanced CSC phenotype to increased signaling through several pathways. While some aspects such as changes in cell viability could be functionally linked to lesser known factors such as GPRC5A (Fig.5D) (Zougman A et al., 2013), two major pathways appear to be closely linked to the Sdc1-dependent enhancement of the CSC phentoype, namely Wnt signalling and enhanced integrin/FAK activation. In deadly, remedyless metastatic cancers, the gain of oncogenic mutations and the loss of tumor suppressors lead to the oncogenic hindrance that controls the regulation of several cancer signaling pathways for example, Wnt (TCF4), Hedgehog (GLI code) and Notch, with differential regulation of cancer stemness signatures. Several publications have established and confirmed that canonical Wnt signaling is an important gate keeping pathway in the regulation of CSC function in colon (Fevr T et al., 2007; Guo H et al., 2014) . The key switch of theWnt signaling pathway is regulated depending on the tumor stage, primary growth and metastasis. In colon cancer, hyperactive canonical Wnt signaling is mainly involved in promoting tumor-initiating cells (tumor stemness) with complex oncogenic events. Wnt signaling regulated stemness associated genes induce a reprogramming events in cancer that promotes high invasion and metastasis (Fevr T et al., 2007; Vermeulen L et al., 2010; Ragusa S et al., 2014;). Cells with the highest Wnt activity were found to define colon CSCs (Vermeulen et al. 2010), whereas single Lgr5-positive stem cells were capable of building crypt-villus structures in vitro even in the absence of a mesenchymal niche

(Sato T et al., 2009). In fact, the Wnt/β-catenin pathway regulates growth and maintenance of colonospheres (Kanwar SS et al., 2010; Yeung TM et al., 2010). Notably, inhibition of the Wnt signaling pathway via silencing of beta-catenin was shown to decrease the chemotherapy-resistant side-population colon cancer cells (Chikazawa N et al., 2010), underscoring the clinicopathological significance of this pathway. Recent results indicate that post-translational glycoprotein modifications regulate colon CSCs and colon adenoma Progression in in a genetically altered mouse model of colon cancer through altered Wnt receptor signalling (Guo H et al., 2014). Data from model organisms such as Drosophila have indicated an important role for HS in mediating Wnt signalling (Pataki CA et al., 2015). Notably HSPG of the Syndecan and Glypican families have been identified as Wnt coreceptors (Niehrs C et al., 2012). However, their individual roles appear to be context-dependent, as data from Syndecan-1-deficient mice suggest that this proteoglycan is required to maintain a Wnt-responsive mammary progenitor cell population (Alexander CM et al., 2000), whereas Syndecan-4 is apparently capable of inbibitng Wnt/beta catenin-signaling through regulation of LRP6 and R-spondin 3, as determined by gain- and loss-of-function experiments in mammalian cell lines and Xenopus embryos (Astudillo P et al., 2014). The possibility of shedding of syndecans adds further complexity, as it has been shown that shedding of Syndecan-1 in cancer cells can switch syndecan-dependent signalling responses to members of the glypican family (Ding K et al., 2005). Finally, we have recently shown that specific alterations in the sulfation pattern of HS, such as increased 3-O-sulfation can lead to an upregulation of TCF4, a transcription factor downstream of Wnt, with a resulting change in proliferation and invasiveness of breast cancer cells (Vijaya Kumar et al., 2014), indicating that changes in the fine structure of Syndecan-1 HS can also have an impact on the signalling response. While all of these results underscore the importance of Syndecan-1 in Wnt signalling in an oncological context, our data indicate that downregulation of colon cancer cell-autonomous Syndecan-1 leads to enhanced Wnt signalling. This finding may be at least partially due to upregulation of the Wnt coreceptor LGR5, resulting in enhanced activation of TCF4. Moreover, the increased SP in Sdc-1-depleted cells would apparently be associated with a relative increase in Wnt signalling in the overall cell population, as stated above. Apart from an increased activation of the Wnt signaling pathway, we detected enhanced integrin activity in Sdc-1-depleted colon cancer cells, which was due to increased gene expression (*ITGA2*) and activation (beta1 integrin), respectively. Via specific interactions with ECM ligands such as fibronectin or laminins, these dimeric transmembrane receptors mediate cell adhesion and motility (Harburger DS et al., 2009) Indeed, *ITGA2* enhances the metastatic activity of colon

cancer cells (Ferraro A et al., 2014; Chin SP et al., 2015). It was reported that *PHLDA1*, a candidate marker for epithelial stem cells in the human intestine, is regulate by modulation of ITGA2 expression levels. (Sakthianandeswaren A et al., 2011.) In association with the beta1 subunit, alpha 2 -integrin expression increased tumorigenicity and loss of the differentiated epithelial phenotype in colon cancer. (Kirkland SC et al., 2008) (Okazaki K et al., 1998). Particularly β1 integrins are key regulators of proliferation and homeostasis in the intestine which influence signaling pathways relevant to stem cell function, oincluding the hedgehog pathway. (Jones RG et al., 2006) In addition, they promote metastatic behaviour in colon cancer cells (Fujimoto K et al., 2002; Shibue T et al., 2013). Interestingly, it was also reported that TCF-4, which was dysregulated in our experimental system upon Sdc-1-siRNA knockdown, mislocalizes in the intestinal epithelia of the β1 integrin–deleted mice (Jones RG et al., 2006). These data provide another link between ß1 integrin function and the Wnt-signaling pathway, and suggest a crosstalk of the signaling pathways affected by Sdc-1-depletion in colon cancer cells. The demonstration that E-cadherin, which is also dysregulated upon Sdc-1-depletion, acts as a ligand for alpha2beta1 integrin further supports this view (Whittard JD et al., 2002)

Finally, enhanced expression of the ECM ligand fibronectin in Sdc-1-depleted Caco2 cells may have enhanced signaling via these pathways, thus enhancing both cell motility and stem cell properties. An important downstream signal transducer of integrin signaling is focal adhesion kinase, which was recently shown to co-immunoprecipitate with syndecan-1 in human breast cancer cells (Ibrahim SA, et al. (2012)). Consistent with previous findings in breast cancer cells (Ibrahim et al. 2012), and with increased integrin activation in our cells, we could detect enhanced activation of FAK upon Sdc-1-depletion in Caco2 cells. Notably, enzymatic removal of HS also enhanced FAK signaling (Fig. 1.5E), suggesting a role of the HS chains of Sdc-1- in this process. These findings are of clinicopathological relevance, as overexpression of FAK has been demonstrated in several tumor entities, including colon cancer (Lark AL et al., 2003). Using a pharmacological inhibitor of FAK, we could block several of the phenotypic changes caused by Sdc-1siRNA depletion, suggesting that increased FAK activation may be a pivotal point in the Sdc-1-dependent signaling network. Notably, these changes are probably not only linked to enhanced invasive behaviour and resistance to radiation (see Hassan H et al., 2013, for discussion), but also to the CSC phenotype and properties: FAK was shown to be linked to the Wnt pathway and to regulated intestinal regeneration and tumorigenesis. It was required down-stream of Wnt/c-Myc signaling to induce AKT-mTOR signaling pathways and

promote intestinal tumorigenesis in mice following Apc tumor-suppressor loss (Ashton GH et al., 2010). Also, FAK regulates expression of WNT3a in human breast cancer cells, where down-regulation of FAK with siRNA caused decreased WNT3a transcription and increased WNT3a protein levels (Fonar Y et al., 2011). In addition, downregulation of FAK ctivity with an autophosphorylation inhibitor in colon cancer cells decreased transcription of the Wnt (co)receptors Frizzled and LRP5 and increased transcription of the WNT pathway inhibitor, Dickkopf-1 (DKK1), demonstrating that FAK also acts upstream of WNT pathway (Fonar Y et al., 2011). Recently, targeted deletion of FAK in mammary epithelium was shown to suppress mammary tumorigenesis demonstrating that FAK plays a significant role in the maintenance of mammary cancer stem cells (Luo M et al., 2009). Using the mammary cancer stem cell markers aldehyde dehydrogenase, CD24, CD29 and CD61 it was shown that down-regulation of FAK reduced the stem cell pool, self-renewal sphere formation and migration of mammary cancer stem cells in vitro (Luo M et al., 2009). FAK kinase activity preferentially regulated proliferation and tumor sphere formation of luminal progenitors, while scaffolding function of FAK was required for regulation of the basal mammary stem cells (Luo M et ala., 2013) Furthermore, down-regulation of another stemness-ssociated pathway, the Notch pathway, by silencing of Notch 1 expression caused a decrease of FAK and downstream AKT phosphorylation in MDA-231 breast cancer cells, which decreased cell migration and invasion (Wang J et al., 2011), demonstrating cross-talk of Notch and FAK signaling. These reports show novel functions of FAK in cancer stem cells and cross-talk with main cancer stem cell signaling pathways, and also demonstrate that both functions of FAK, kinase-dependent and kinase-independent (scaffolding function) are critical for cancer stem cell functions and tumorigenesis. Another level of regulation, has been suggested based on studies on the pluripotency-associated transcription factor Nanog, which was also found to be dysregulated in Sdc-1-depleted cells in our study. Nanog was recently shown to induce FAK promoter activity (Ho B et al., 2012) and increased Nanog protein expression caused increase of FAK expression and induced tumorigenesis (Lin YL et l., 2011). Both Nanog (Lin T et al., 2005) and FAK (Golubovskaya V et al., 2004) transcription are repressed by p53. It was shown that inactivation of p53 increased induced pluripotent stem cell generation (Krizhanovsky V et al., 2009; Hong H et al., 2009; Kawamura T et al., 2009; 99–102), and p53 mutations and inactivation correlated with FAK overexpression in tumors (Golubovskaya VM et al., 2008) The detailed molecular mechanisms of FAK and WNT interaction and cross-signaling pathways in cancer stem cells need to be elucidated in more detail, however, our data support the notion of a crosstalk of these pathways.

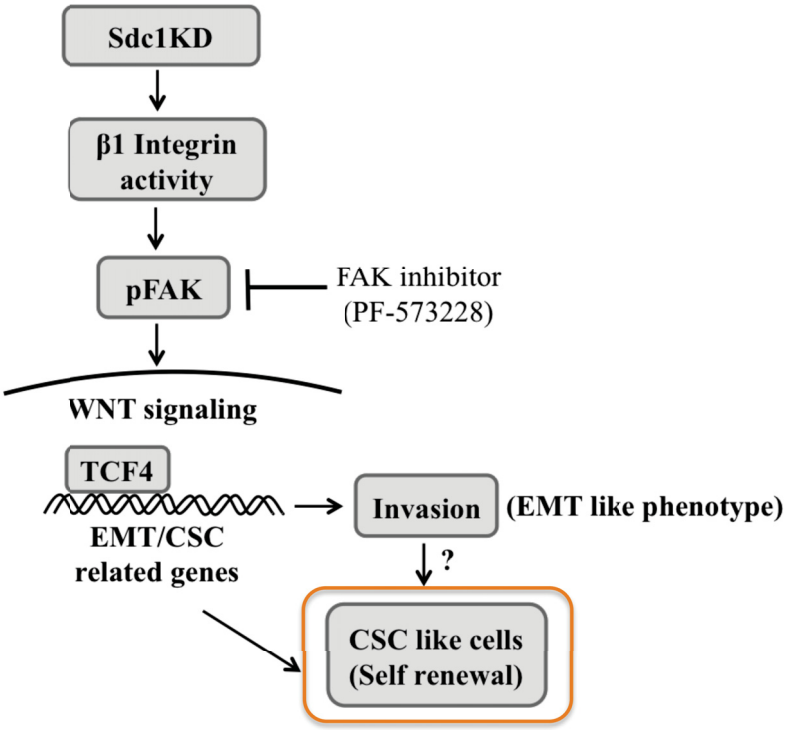


Figure. Role of Sdc-1 in cancer stemness - Proposed model.

Based on our presented data, we hypothesize that, in colon cancer cell lines, down-regulation of Sdc-1 could activate EMT like conditions to promote tumor cell invasion, further regulating the stem cell like population. Loss of Sdc-1 activates β 1 integrin with an increases in focal adhesion kinases (pFAK), could activate EMT like conditions to promote tumor cell invasion, further regulating the stem cell like population. Loss of Sdc-1 activates β 1 integrin with an increases in focal adhesion kinases (pFAK), which inturn induces TCF4 expression promoting FAK: WNT signaling axis, driving EMT like state with an increase in invasion and further pushing the cells to stem cell like state. Using a FAK specific inhibitor (PF-573228) there is an decrease in WNT signaling with an decrease in CSC signatures in a coordinated manner.

4.2 The *Sdc-1- HPSE axis in colon cancer invasion and stemness.*

Further on to what have been discussed so far about the importance of syndecan-1 in cancer stem cell properties, the second part of the project was focused on the interplay between syndecan-1 and heparanase in invasion and associated stemness. HSPGs are essential major components of the extra cellular matrix and cell surfaces, which fabricate the dynamic network in normal tissue architecture and homeostasis (Bernfield et al. 1999). In the tumour microenvironment, dysregulated expression of HSPGs and their processing enzyme heparanase has been reported in numerous tumor entities (Yip GW et al. 2006 Götte and Yip, 2006). With reference to the clinical data, transcriptional reduction of Syndecan-1 and increase in heparanase expression has been observed in several cancers particularly in colon cancer enhancing tumorigenesis, invasion, and metastasis (Vishnu C. Ramani 2013; Mikami S 2001:Karene Mahtouk 2007; Yang Yang 2007; Anurag Purushothaman 2011). This demonstrates the relevance, but also the complexity of altered expression of HSPGs and their processing enzymes in clinico-pathological settings. The underlying molecular mechanism of this regulation remains poorly investigated. This project mainly focused on the understanding of the interplay between syndecan-1 and heparanase and the possible molecular signaling routes. We report for the first time a new mechanism in which loss of Syndecan-1 regulates the expression of heparanase at the transcriptional level. This observation reveals new possibilities to understand the long term driven functions of syndecan-1 and heparanase in malignancies.

Our results mainly emphasize that (i) loss of syndecan-1 enhances the transcriptional regulation of heparanase (ii) this enhanced heparanase expression increases the invasion which is reversed by targeting heparanase, (iii) a molecular cross talk between EGR1 and activation of FAK upon loss of syndecan-1 which collectively drives the heparanase expression, (iv) that this expression further boosts colon cancer stem cell properties, (iv) that over expression of heparanase enhances the colon cancer stemness with their extra cellular remodeling properties, and (v) that the active form of the enzyme, heparanase inhibits the colonosphere formation ability. These results adds to the findings of the first part of this study on syndecan-1 and its role in colon cancer stemness. Upon downregulation of syndecan-1, we observed an increase in heparanase expression in Caco2 and COLO 205, whereas in HT29 cells its expression decreased, as measured by qPCR. The underlying causes for the decrease in HPSE expression in HT29 cells with loss of syndecan-1 are unknown, but may be due to the difference in oncogenic mutations between Caco2 and HT29 (Ahmed D et al., 2013) In contrast, a rescue experiment

with the over expression of syndecan-1 in Caco2 showed a decrease in heparanase expression but an increase in HT29, which is further supported by heparanase activity assay. These observations indicated the existence of strong feed back mechanism. We furthermore observed an increase in heparanase promoter activity upon syndecan-1 KD in Caco2. Co-transfection of Caco2 cells with NF-κB further increased the heparanase promoter activity. Even though NF-κB regulates the heparanase expression positively in some contexts (Elkin et al., 2011; Deepak et al., 2015; Jin-Min Wu, 2005), NF-κB showed a negative regulation of HPSE promoter activity with loss of syndecan-1 in Caco2 cells. Notably, it was shown that increase in expression of syndecan-1 regulates the NF-κB expression (Min CK et al., 2009) whereas loss of syndecan-1 showed decrease in its expression (Götte et al., 2013). This may indicate a potential role of syndecan-1-dependent NF-κB regulation in heparanase transcription. In support of this, we have also seen a decrease in the NF-κB expression with the loss of syndecan-1 (data not shown). Interestingly, we found that *SNAIL* coexpression increased heparanase promoter activity, which could explain the EMT driven properties of heparanase. Also it is observed that *SNAIL* represses the expression of syndecan-1 (Contreras et al., 2014). So it could be that in the presence of low levels of syndecan-1, *SNAIL* positively regulates heparanase expression. As bioinformatic analyses reveal that there are putative binding sites for *SNAIL* in both syndecan-1 and heparanase promoters, it is tempting to suggest an active role of *SNAIL* in unknown feedback mechanism of syndecan-1 and heparanase expression. Also, increase in the *EGR1*, an early growth response gene expression with the loss of syndecan-1 correlates with the increase in HPSE expression. Based on mutagenesis and trans-activation studies it was shown that *EGR1* binds to the heparanase promoter and up-regulates *HPSE* transcription in colon cancer cells (Hulett *et al.,* 2005). In colitis and the associated tumorigenenic *in vivo* models it was also shown that EGR1 acts as a potent inducer of heparanase in colonic epithelium tumor cells (Elkin et al., 2011). Together with these observations our results further support that *EGR1* directly regulates HPSE transcription. Increase of invasion in syndecan-1 KD cells may be due to the degradation of HS chains, which impairs cellcell contact and cell-matrix adhesion interactions. Heparanase acts as a key in controlling the cell barrier with its HS degradative and syndecan-1 sheddase activity (Purushothaman A et al., 2008), making it worthy to think that absence of syndecan-1 at the cell membrane will favour proinvasive conditions. Several pharmacodynamic studies has been demonstrated that SST0001, a potent HPSE inhibitor to have potential anti-tumor activity in different cancer models. (Joseph P. Ritchie 2011: Anurag Purushothaman 2011) As heparanase has multiple functions in the tumor microenvironment it is worthy to think that SST0001

decreases the invasion of syndecan-1 depeleted cells through an unknown cascade of signaling events (G. Cassinelli 2013). Regarding additional factors, it was reported that EGR1 regulates the HGF-induced cell invasion through the coordination of MMPs in HCC cells (Evin Ozen 2012). So the increase in EGR1 expression also may contribute to the increase in invasion in syndecan-1 depleted cells. However, at least in syndecan-1-depleted Caco2 cells, we observed only moderate changes in MMP expression and activity, suggesting that HPSE regulation may be the more important mechanism. At least the increase in invasion upon loss of syndecan-1 could be because of EGR1 expression. Moreover, it has been previously identified that heparanase regulates HGF expression (Ramani VC et al. 2011). So it could be possible that syndecan-1 loss increases heparanase expression and in turn this heparanase may regulate the HGF signaling in a feed back loop to control invasion of tumor cells. Furutre studies elucidating the interplay of Syndecan-1 and HGF in this context appear worthwhile.

Due to the loss of HSPG, epithelial cells lose their cell polarity, gain migratory and invasive properties via the process of epithelial to mesenchymal transition (EMT). Although underlying molecular processes remains poorly understood, it is observed that loss of syndecan-1, expression ehances formation of lamellipodia with an increase in invasive capabilities (Ibrahim et al. 2012). It is also observed that HSPG bind to several EMT-inducing factors, like TGF-b. So enhanced expression of heparanase may shed these bound HSPG's which may further enhance EMT-like conditions (Kirkbride, K.C. et al. (2005). Also, a shift of syndecan-1 from epithelial cell surface to stroma might indicate the antimetastatic effects of syndecan-1 at the cancer cell surface where the loss of its expression can promote EMT (Mennerich, D. et al. (2004). Generation of the soluble form of syndecan-1 due to heparanase activity (Yang Y et al., 2007), may enhance EMT-associated signaling. This explains the maintenance of cell–cell contact by transmembrane syndecan-1 and the loss of its expression or shedding into its soluble form by heparanase and proteases initiates EMT properties (Nikolova, V. et al., 2009). It is observed that EGR1 mediates upregulation of Slug expression,and downregulates E-cadherin, which consequently increases the invasive capability (Cheng JC et al., 2013). We cannot rule out the possibility that SNAIL represses the expression of syndecan-1 in a similar fashion to that of E-cadherin. Our results also clearly indicated that SNAIL increases heparanase promoter activity, which may further drive the EMT process (Cano A 2002). A mountain of evidence shows that these EMT-like conditions promote proliferation, metastasis, chemo-, immune- and radiotherapy resistance, all of which are relevant to cancer stem cell properties (Nieto MA et al.2012).

As we previously observed in the first part of this work, the enhanced activation of integrins caused by loss of Syndecan-1 results in the increase of focal adhesion kinase activation. Therefore, we thought that FAK might be involved in the regulation of heparanase expression. Blocking FAK auto- phosphorylation with its potent inhibitor PF562271 decreased the heparanase activity in the absence of syndecan-1. It was previously reported that a heparanase receptor activates PI3K-AKT pathway (Anjum Riaz 2013), but it is not clear whether phosphorylated FAK activate the heparanase receptor. Moreover, the authors observed that PF562271 effectively abolished heparanase-induced AKT activation (Anjum Riaz 2013). This is consistent with our results, where FAK inhibitor abolishes heparanase activity moderately. Notably, activation of b1 integrins concomitant with the loss of syndecan-1 may also be involved in the expression of *EGR1*. It was reported that integrin/EGFR cross-talk dependent adhesion signals regulate the *EGR1* expression (Cabodi S et al., 2009). So, it could be that upon syndecan-1 loss, the beta1 integrin complex on the plasma membrane maytrigger the expression of the EGR1 through adhesion dependent signals, which would further lead to the activation of FAK. Integrin-dependent adhesion plays a role in Egr-1 regulation (Cabodi S et al., 2009). Overall, these data demonstrate that upon syndecan-1 loss, EGR1/pFAK cross-talk is required for expression of heparanase through a novel regulatory signaling cascade. In addition to this, we also showed that heparanase inhibitor decreased the side population in syndecan-1 depleted cells making it evident that loss of syndecan-1 increases the expression levels of HPSE. Whereas in HT29, the SST0001 didn't show a clear effect on side population of syndecan-1 depleted cells, which could be explained by the fact that syndecan-1 loss decreases the heparanase levels in HT29 cells. Supporting to the SST0001 effect on side population of syndecan-1 depleted Caco2 cells, we saw a decrease in the invasion associated gene (VIM) and known stem cell markers (*VIM, LGR5, CD133, SOX2*) that are know to be involved in mediating cancer stemness. These results for the first time showed a novel role of heparanase inhibitor SST0001 on side population levels. As SST0001 decreased invasion in syndecan-1 depleted cells, it is possible that genes involved in invasion may also further regulate the side population or it could be that SST0001 is directly acting on the genes associated with stemness. At least an effect with SST0001 on vimentin expression indicates that the process of invasion is also linked to cancer stemness. This is an interesting objective to further understand the molecular mechanism involved in two important coordinated mechanisms of cancer recurrence- invasion and cancer stemness.

Forced expression of heparanase decreased the expression of syndecan-1 and also two no effect with genes involved in heparanase regulation, Egr1 and Cox2. Also, as analyzed by flow cytometry, a decrease in syndecan-1 expression in clones with high heparanase expression indicates that heparanase may shed the syndecan-1 at the membrane, whereas the clones expressing the enzymatically inactive mutant form of heparanase didn't show a strong decrease of syndecan-1.These results indicates the presence of a strong feed-back loop of syndecan-1 and heparanase regulation in tumor malignancy. Strikingly, we report a role of heparanase in regulating cancer stem cell properties for the first time. The significant increase in the side population as a result of heparanase overexpression provides further evidence for the multifunctional role of heparanase in the tumor microenvironment. High levels of heparanase were associated with decreased *SNAIL* expression. Even though co-expression studies with SNAIL showed an increase in heparanase promoter activity, *SNAIL* has no influence on heparanase expression if cells already express high levels of heparanase as evident from the qPCR. Notably, at mRNA level we saw a high increase in the expression of *NANOG* and *KLF4* in both heparanase and mutant heparanase clones. Increase in *NOTCH1* and *NOTCH3* in mutant heparanase expressing cells could explain the reasons for the observed increase in the side population in dominant negative clones (Bu P, 2013). We saw a marked increase in ALDH and *CD133* in heparanase high clones. ALDH (Shenoy A, 2012) (Emina H. Huang 2009) and *CD133* (Elsa N Garza-Treviño 2015) (Sanchita Roy 2012) are the widely accepted markers for colon cancer stemness along with other markers like CD24 (Ke J, 2012), CD44 etc., Particularly, CD24 is very high in heparanase overexpressing clones. Heparanase expression is increased in human malignancies and in several xenografts models of human colon, breast, lung, prostate, pancreas and ovarian tumors (McKenzie E, 2000). So during the progression of the primary tumor heparanase, by promoting autocrine and paracrine signaling functions, appears to initiate non-stem cell epithelial cells to develop into tumor-initiating cells via the re-expression of stem cell markers, including pluripotency-associated transcripton factors. t is very well described that deeply invading colon carcinoma cells express high levels of heparanase (Friedmann, Y., et al. 2000) and that the CSCs have post-EMT cell characteristics (Borovski T 2011). Dissociation of these invading cells from the basement membrane alters the dynamic cell-ECM interactions and the resulting signals to generate the characteristics of cancer stem cells via the process of epithelialmesenchymal transition (EMT). In fact, the observed over expression of SNAIL, and the associated increases in the heparanase promoter activity in syndecan-1 depleted cells may further drive the heparanase activity can trigger shedding of syndecan-1, which also acts as EMT

marker in some cancers.(Poblete CE et al., 2014; Zeisberg M et al., 2009). Notably, it is reported that heparanase is involved in a type 2 EMT process in which kidney tubule epithelial cells develop into myofibroblasts (Masola V, 2012). These data suggest that heparanase also may also influence the EMT process in a variety of tumor cells.

It is very well described that heparanase is involved in metastatic potential of human tumor cells (Vlodavsky, I, et al 1999: Hulett, MD, et al 1999; Nakajima, M 1988: Vlodavsky, I, et al 1994). Heparanase cleaves the HSPG, at the subendothelial basement membrane in turn facilitating initiation of a metastatic cascade and also tumor angiogenesis. Cleavage of HSPG by matrix degradation enzymes — MMPs and heparanase facilitate extravasation of bloodborne tumor cells by modulating growth factor activity and bioavailability for angiogenesis sprouting. (Israel Vlodavsky 2001). It has been estimated that during the process of metastasis few unique cells successfully colonize to the secondary site and spread the disease to distant organs and these as seeds for the secondary tumor growth formation, which is a major reason for tumor recurrence (Croker AK 2008; Li F2007; Chambers AF 2002; Pantel K2004). Stem cells and metastatic cancer cells share common properties that are crucial for this process. The expression of heparanase correlates with the metastatic potential of tumor cells and our results underscore the importance of heparanase in cancer stem cell propoertiess. As heparanase expression increases from early stage of human colon cancer (well-differentiated) to poorly differentiated colon carcinoma, our results may indicate that heparanase expressing cells may dedifferentiate to acquire a stem cell- like state. So it could be possible that high heparanase expression changes cell plasticity in poorly differentiated tumors. This may aid the conversion of de-differentiated like state with embryonic stem cell like signatures, with consecutive self-renewal of the proliferating cells (Schwitalla S 2013). This is supported by our finding of an, increased side population in cells expressing the dominant negative form of mutant heparanase. Indeed there are indications for a cell adhesion dependent function of enzymatically inactive heparanase (Goldshmidt et al., 2003; Vlodavsky I 2011; Levy-Adam et al., 2008). This mutant form of heparanase promotes cell attachment and Rho activation with the coordinated phosphorylation of signaling molecules like Akt and Src (Anjum Riaz 2013). Strikingly, in the sphere formation assay we saw a drastic decrease in sphere formation in heparanase high cells. This result supported by previous observations showing that inhibition of N-linked glycosylation using tunicamycin decreased sphere numbers (GG Jinesh et al 2013), which underscores the role of glycosylation in this process. While HS is attached to core proteins via O-glycosidic rather than N-glycosidic bonds, one could nevertheless envisage that high heparanase expression will cleave

heparan sulfate, which disrupts ECM dynamics at cell surfaces and basal membranes, thus causing the inability to form 3D spheres. Importantly, sphere formation is a process involving cell to cell contact. Each sphere contains a group of cells inside, so due to loosening of cell-cell contacts either via HS-ligand interactions or via downregulation of cadherins, a decrease of spheres may occur in heparanase overexpressing clones. It could also be that heparanase overexpressing clones can survive in adherent conditions, whereas in suspension culture they lose the property of survival due to loss of cell- cell contact. Future studies addressing these potential mechanisms are worthwhile. Notably, dominant negative heparanase clones formed spheres equal to that of vector controls, suggesting that the active heparanase indeed inhibits the formation of spheres due to changes in HS structure. Surprisingly, we also observed a decrease in the formation of spheres upon treatment of Caco2 control cells with the heparanase inhibitor SST0001. This may indicate~~s~~ that inhibition of physiological expression levels of heparanase could decrease~~s~~ sphere forming abilities mediating a decrease in cancer stemness, e.g. via a reduction in the side population, whereas with a high heparanase expression, cells may lose their cell to cell contact and therefore decreases the formation of spheres. Notably, in mutant form of heparanase even after treatment with SST0001, no effect on spheres was observed. It has to be noted that SST0001 binds at active site of the enzyme whereas in mutant heparanase clones, due to mutation at active site, SST0001 inhibitor did not show any effect. Slight decrease in spheres with the SST0001 treatment indicates it could be because of physiological expression of active heparanase in mutanat heparanase clones.

A range of signals have been shown to regulate the tumor initiating stem cell capacities of colon cancer, including Wnt pathway (Tesshi Yamada 2000). We observed a high expression of TCF4 in heparanase over expressing clones. Our results furthermore showed a decrease in the side population upon incubation with the Wnt inhibitor, IWAP2 (Chikazawa N 2010). IWAP2 inhibits the palmitylation of Wnt proteins thereby blocks Wnt secretion and activity (Chikazawa N, 2010). Our results, plays an important role in formation of the side population. Also it is observed that MDR genes acts as target for the TCF4/β-catenin complex. (Tesshi Yamada 2003). e observed an increase in the autophosphotylation of FAK in colon cancer cells over expressing heparanase. Increased pFAK in high heparanase cells might be involved in adhesion-dependent signaling. Even though the clear molecular mechanism of heparanase in adhesion dependent signaling is not clear, it could be that heparanase degrades the ECM, which changes the adhesion properties or integrin activation. By this way, it may cause a similar situation as in

Sdc-1-knockdown cells, or as in cells treated with bacterial heparitinases, both of which showed increased FAK phosphorylation. Also it was shown that latent heparanase binds to the heparanase receptor regulating the AKT signaling. It is also observed that FAK inhibitor blocks heparanase induced Akt signaling (Riaz A et al.,2013), suggesting that FAK and heparanase act as a feed back regulation to activate Akt signaling. As observed in the first part of the thesis, the increase in pFAK in heparanase over expressing cells may also contribute to cancer stemness. As there is a strong feed–back mechanism between FAK and TCF4 according to our data, it is important to consider that they could act synergistically to regulate cancer stem cell properties. Moreover FAK and TCF4 can act independently to drive cancer stemness. It is also possible that FAK and TCF4 could compensate for each other in promoting genes involved in cancer stemness signaling. Thus, the observed increase in pFAK in heparanase over expressing cells may involves ECM remodeling and associated altered adhesion properties, which indicates an additional notion of heparanase functions in invasion, metastasis associated colon cancer stem cell signaling.

Closely linked to the previous observations is our finding that heparanase oveexpressing cells show increased beta1-integrin activation (O Goldshmidt, 2003), which along with the observed increased FAK activation indeed suggests that heparanase is involveds in adhesion-dependent signaling. The colony formation assay resulted in a decrease in the number of colonies respective to vector and mutant heparanase, even though it was previously reported that heparanase promotes colony formation, which could be due to different experimental systems. (Cohen-Kaplan V 2008). We have also seen a decrease in the proliferation in heparanase overexpressing cells compared to the vector controls. This result supported that the sheddase activity of heparanase might be contributing to the extra cellular matrix remodeling. The colony formation assay may vary between the knockdown vs over expressing cells depending upon the physiological expression levels of heparanase. Also its functions may be different depending on the *in vitro* vs. *in vivo* studies, which involve additional cell types and noncell autonomous effects. It was observed that high *CD133* and *CD44* expression, but not *CD24*, in colon cancer contributes to increased radio resistance (Sara Häggblad Sahlberg 2014). This supports our observation where we have seen high *CD24* expression in heparanase overexpressing cells compared to vector controls. Irradiated heparanase overexpressing cells showed more radioresistance compared to untreated controls. In addition to this, we have also observed an increase in chemoresistance with paclitaxel and cisplatin treatment in mutant heparanase compared to vector and heparanase overexpressing clones, (Kobayashi Y 2011)

whereas in heparanase over–expressing cells the effect of cisplatin is not clear. As discussed previously, the mutant form of heparanase is involved in adhesion dependent signaling which in turn may promote chemoresistance of cancer cells by increasing the side population. It is also important to consider that side population is controlled by several additional factors like genetic alterations, the ECM niche microenvironment, micro RNA's, stem cells and its quiescent vs active state (Vinitha Richard, 2013; K. Moitra 2011). As measured by ALDH activity, heparanase cells didn't show high activity. So it could be possible that the combination of side population and ALDH may have strong synergistic effects on chemoresistance of tumor-initiating cells (J. Hilton, 1984; C. P. Huang, 2013). Altogether, the nature of drug resistance of tumor-initiating cells is multifactorial, with various signaling pathways and complex mechanisms that could fine-tune chemosensitivity.

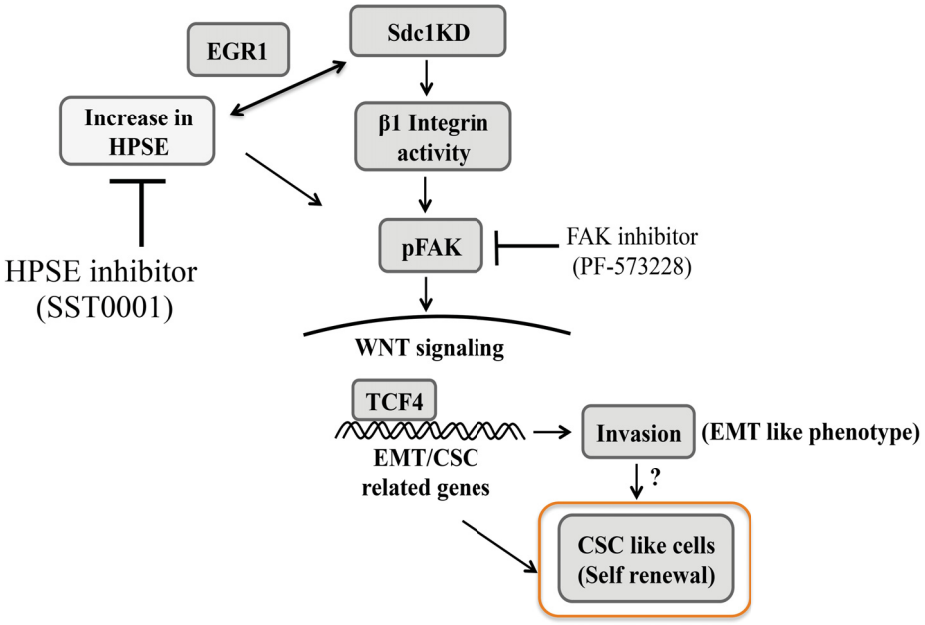
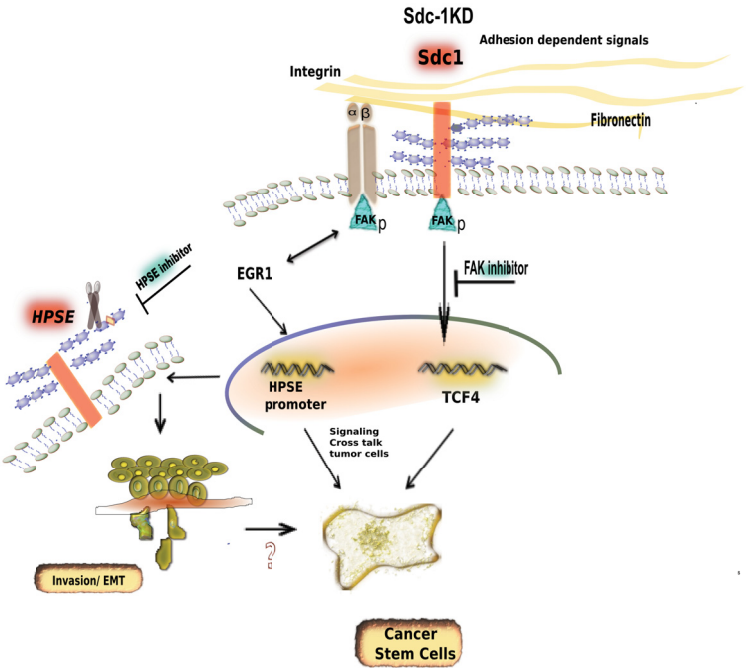


Figure. Sdc-1 and HPSE axis in colon cancer invasion and stemness - Proposed model:
Based on our presented data, we hypothesize that, in Caco2, down-regulation of Sdc-1increases *HPSE* expression through transcription factor EGR1, which could activate EMT like conditions to promote tumor cell invasion, further regulating the stem cell like population. Interplay

between of Sdc-1 and *HPSE* increases focal adhesion kinases (pFAK) levels. Over expression of *HPSE* inturn increases stem cell like signatures though FAK and WNT signaling axis. Using a *HPSE* specific inhibitor (SST0001) there is an decrease in invasion and CSC signatures.

To summarize, we have shown for the first time the involvement of the heparan sulfate proteoglycan Sdc-1 and HPSE in colon cancer stem cell properties. siRNA mediated depletion of Sdc-1 increased the stem cell phenotype based on *in vitro* sphere-forming assays and flow cytometry-based assays (side population (SP), ALDH and CD133). In addition, we observed an increase in matrigel invasiveness linked to EMT-inducing conditions. Mechanistically, upon Sdc-1 depletion we observed the activation of β1 integrin with an increase in adhesion to fibronectin. Focal adhesion kinase was strongly activated following Sdc1 knockdown, suggesting that Sdc-1 may be linked to integrin-induced actin remodeling. Importantly, with Sdc-1 knockdown, Wnt signaling is enhanced which in turn induceds TCF4 expression promoting the FAK:WNT signaling axis. The increase in SP, CD133 and colonospheres, which could be blocked using a FAK specific inhibitor. From the first part we conclude that loss of Sdc-1 co-operatively enhances activation of integrins, focal adhesion kinase and WNT, which then generates signals for increased invasiveness and cancer stem cell properties. Interestingly, sorted Sdc-1 depleted SP and enriched population showed a furthern increase in colonospheres, and colony formation assays revealed increased resistance of Sdc1-depleted cells to irradiation. Importantly, Sdc-1 depleted HT29 cells showed an increase in the tumor size in an *in vivo* mouse model. In the second part of the study, Sdc-1 loss was shown to enhances HPSE promoter activity through the transcription factor EGR1 and FAK axis. Importantly, HPSE inhibitor (SST0001) abolisheds the Sdc-1 induced invasion. Stable over expression of heparanase increased cancer stem cell properties (SP,; ALDH, CD24) through the activation of Wnt signaling. Notably, the sphere formation ability was lost, which could be due to ECM degradation by heparanase Strikingly, at normal physiological conditions, a HPSE inhibitor decreased sphere formation. We hypothesize that loss of Sdc-1 cooperatively enhances activation of integrins and focal adhesion kinase, which then generates signals for increased invasiveness, cancer stem cell properties and resistance to irradiation. A graphical representation of the key findings is shown in the figure.



4.3 ***Schematic diagram of the overall summary:***

Syndecan-1 modulates cell-matrix adhesion and signaling via FAK and EGR1 resulting in transcriptional regulation of proinvasive factor HPSE leading to increased invasiveness, promting cancer cancer stem cell properties.

4.4 Conclusion.

This work investigates the molecular impact of Sdc-1 and HPSE in colon cancer progression. Our findings provide a deeper insight into the co-operative regulation of Sdc-1 and HPSE and its downstream signaling loops involved in the cancer stem cell phenotype. What makes this study particularly interesting is how these two gene products cooperates in regulating the cancer stem cell signatures. Our data on the loss of Sdc-1 and increase in HPSE levels correlated with enhanced invasive and cancer stem cell properties, which suggested that Sdc-1 may play a negative role and heparanase acts as a positive driving force in colon cancer progression. While most of the mechanistic work presented in this study addressed the influence of Sdc-1 and HPSE on cell-autonomous properties of colon cancer cells, future studies need to extend this work to more complex in vivo models such as xenograft studies. Although the molecular complexity is challenging to understand the interdependent functional phenotype of syndecan-1 and heparanase, it is understood that loss of syndecan-1 increases the integrin activities and accelerates invasion and cancer stemness whereas heparanase, with several mechanisms (sheddase, autocrine-paracrine signaling and adhesion properties) in turn enhances the invasive properties and cancer stem cell signatures. Our results provided an important molecular evidence for novel mechanisms underlying the aggressive behavior of colon cancer at the highly metastatic stage. Finally, we provide for the first time, the evidence for an involvement of heparanase in cancer stem cells, and for a feed back regulation of Sdc-1 and HPSE in EMT- driven colon CSC properties. Our results supported new possibilities of Sdc-1 and HPSE to be used as tumor specific stem cell markers and molecular targets in colorectal cancer. Our findings may provide a novel concept to target a stemness-associated signaling axis as a therapeutic strategy to reduce metastatic spread and cancer recurrence.

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6. Abbreviations:

µg	Microgram
µl	Microlitre
µM	Micromolar
µm	Micrometer
6-OST	6-O-Sulfotransferase
A	Absorbance
ABCG2	ATP- binding cassette sub-family G member 2
AMP	Adenosine monophosphate
ANX	Annexin
APS	Ammonium persulfate
ALDH	Aldehyde dehydrogenase
ATF-2	Activating transcription factor-2
ATP	Adenosine tri phosphate
APC	Adenomatous polyposis coli
BCA	Bicinchonic acid
BCL2	B-cell lymphoma/leukemia-2
BMPs	Bone morphogenetic proteins
BRAF	Rapidly accelerated fibrosarcoma
bp	Base pairs
BSA	Bovine Serum Albumin
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
cm	Centimeter
CSC	Cancer stem cells
CMV	Cytomegalovirus
COL1A2	Collagen type I A2
COX-2	Cyclooxygenase-2
cRNA	Complementary RNA
CS	Chondroitin sulphate
CRC	Colorectal cancer

CDK8	Cell division kinase protein kinase 8
DAPI	4', 6'-diamidino-2-phenylindole
DCIS	Ductal breast carcinoma in situ
DMEM	Dulbecco’s modified Eagle’s medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DPBS	Dulbecco’s phosphate buffered saline
DS	Dermatan sulphate
dsDNA	Double-stranded DNA
dsRNA	Double-stranded RNA
DTT	1,4-Dithiothreit
E.coli	*Escherichia coli*
E-cad	E-cadherin
ECM	Extracellular matrix
EDTA	Ethylendiamintetracetic acid
EGFR	Epidermal growth factor
EHS	Engelberth-Holm-Swarm
ELISA	Enzyme-linked immunosorbent assay
EMP2	Epithelial membrane protein 2
EMT	Epithelial mesenchymal transition
ES	Embryonic stem cells
ER	Estrogen receptor
Erk1/2	Extracellular signal-regulated kinase 1/2
ERM	Ezrin, Radixin, Moesin
EXT	Exostose
EGR1	Early growth response protein 1
FAK	Focal adhesion kinase
FN	Fibronectin
FCS	Fetal calf serum
FGF	Fibroblast growth factor
Fig	Figure

GAGs	Glycosaminoglycans
GAIP	Gα-interacting protein
Gal	Galactose
GALT	Galactosyltransferase
GAP	GTPase-activating factor protein
GDI	Guanine dissociation inhibitor
GEF	Guanine exchange factor
GLCAT	Glucuronyltransferase
GlcNAc	N-acetylglucosamine
GPCs	Glycosylphosphatidylinositol -anchored glypicans
GPI	Glycosylphosphatidylinositol
h	hour
HPSE	Heparanase
HRP	Horseradish peroxidase
HS	Heparan sulfate
HSPG	Heparan sulfate proteoglycan
IVT	*In vitro* transcription
JAM-A	Junctional adhesion molecule A
JHDM1D	Jumonji C domain-containing histone demethylase1homolog D
JMD	Juxtamembrane domain
JNK	c-Jun NH2-terminal kinase
kDa	Kilodalton
KLF4	Krueppel-like factor 4
KS	Keratan sulfate
l	Litre
LB	Luria-Bertina
LGR5	Leucine- rich repeat containing G-protein coupled receptor 5
M	Molar
mA	Milliampere
MAGUK	Membrane associated guanylate kinase
MAPK	Mitogen-activated protein kinase
mg	Milligram

min	Minute(s)
miRNA	MicroRNA
ml	Millilitre
mM	Millimolar
MMP	Matrix metalloproteinase
MPP4	Membrane palmitoylated protein-4
mRNA	Messenger RNA
MT1-MMP	Membrane type 1 matrix metalloproteinase
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide
MCS	Mesenchymal stem cells
NADPH	Nicotinamide adenine dinucleotide phosphate
NDST	N-deacetylase/N-sulfotransferase
NF-kappaB	Nuclear factor kappaB
iPSCs	Induced pluripotent stem cells
OD	Optical density
OCT4	Octomer binding transcription factor 4
PAGE	Polyacrylamide gel electrophoresis
PAI	Plasminogen activator inhibitor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDK1	Phosphoinositide dependent kinase 1
PDZ	PSD-95, a postsynaptic protein; Discs-large, a *Drosophila* septate junction protein; and ZO-1, a tight junction protein
PGE_2	Prostaglandin E_2
PGs	Proteoglycans
PI3K	Phosphatidylinositol-3-kinase
PKB	Protein Kinase-B
PKCα	Protein kinase Cα
PLA_2	*Phospholipase A2*
PMA	Phorbol 12-Myristate 13-Acetate
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog

qPCR	Quantitative PCR
RAS	Rat Sarcoma
Rho	Ras homologue gene family member
RIPA	Radio-Immunoprecipitation Assay
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
ROCK	Rho-associated protein kinase
rpm	Rotations per minute
RPMI	Roswell Park Memorial Institute
RGD	Arg-Gly-Asp
RQ	Relative quantification
rRNA	Ribosomal RNA
RT	Room temperature
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription PCR
s	Second(s)
SCID	Severe Combined Immunodeficiency mice
Sdc	Syndecan
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SP	Side population
SI	Structural index
siRNA	Small interfering RNA
SLRPs	Small leucine-rich proteoglycans
ssDNA	Single-stranded DNA
Sulfs	Sulfatases
Shh	Sonic hedgehog
SNAIL	Zinc figure protein
SOX2	SRY (Sex determining region Y)- box2
SULF	Sulfatase
Taq	Thermus aquaticus

TA	Transit amplifying cells
TBS	Tris buffered saline
TEMED	N,N,N,N-tetra methylene diamine
TGF-β	Transforming growth factor-β
TIMP	Tissue inhibitor of metalloproteinases
TLR	Toll-like receptor
TWIST	Twist related transcription factor
TNF-α	Tumor necrosis factor-alpha
Tpl2	Tumor progression locus 2
TRITC	Tetramethylrhodamine isothiocyanante
TSP-1	Thrombospondin-1
uPA (PLAU)	Urokinase-type plasminogen activator
uPAR	Urokinase-type plasminogen activator receptor
UTR	Untranslated region
UV	Ultraviolet
v/v	Volume/Volume
VEGF	Vascular endothelial growth factor
Wnt	Wingless-type
w/v	Weight/Volume
^{o}C	Degrees centigrade
ZEB1	Zinc finger E-box- binding homeobox-1

7. Acknowledgment:

I would like to express my sincere gratitude to all the people who have contributed to this thesis directly or indirectly:

To begin with, it is with immense gratitude that I acknowledge my PhD supervisor Prof. Dr. Martin Götte. Your patience, support, insight and excitement for the work, encouraged me especially when things weren't working so well, gave me hope and confidence. Your belief in my abilities made it possible for me to tackle a project that was a challenge scientifically and emotionally. You provided the freedom for developing and performing scientific research, which is very important for a PhD student to develop innovation, responsibility and critical thinking. Thank you for the opportunity to support me for the scientific visits to Brazil, Italy and Egypt, which helped me to develop professionally and intellectually. Thank you for your support to collaborate with different groups I am interested in. You never said no for anything. With your support and patience I considered research as a lifestyle rather than a scientific job.

I extend my deepest gratitude to Prof. Bruno Moerschbacher, for being my thesis reviewer and for the great opportunity to be the part of MCGS. I admire and appreciate the work you have put in over many years to initiate the success of MCGS. I consider it as a great honor to be a part of your MCGS crew. You have been a great example for the passion towards research. The theme of MCGS - interdisciplinary scientific networking helped me to develop skills to network for scientific collaborations.

I am deeply grateful to Dr. Burkhard Greve for being my thesis reviewer. Thanks a lot for your constant support, guidance to use the flow cytometry facility. I cannot begin to describe how fortunate I feel to have had the pleasure to work with you. I owe thanks to Annette van Duelmen, Franz-Josef Wischmann, Timo Braun for their support to work in your group. Thanks Timo, its b'coz of you that I developed my teaching skills.

I would like to thank Dr. Rolland Reinbold (Italy) for getting me interested in cancer stem cells and for broadening my scientific horizons. Your encouragement, tolerance and patience means a lot to my PhD journey.

I would like to express the deepest appreciation to Dr. Tomo saric, for giving me the opportunity to work in your laboratory at the University of Köln, Germany. Thank you Narsimha for having taught me reprogramming techniques.

Prof. Mauro Pavao (Brazil), thanks for sharing your knowledge on glyco community. I treasure our many intellectual discussions and many things out of science.

I am forever indebted to Birgit for always being patient and supportive. You are the prime example for me to maintain constant energy levels. I would like to thank Sherif and Hebba for their kindness during the scientific visit to Egypt.

I would like to recall and express the deepest appreciation to Dr. Sreenivas, bachelor's teacher. It's just his motivation wherever I am today.

I would like to thank Prof. Dr. Israel Vlodavsky and Dr. Michael Elkin (Israel), and Dr. Alessandro Noseda (Sigma Tau) for providing essential reagents and helpful comments on the heparanase part of this thesis.

My parents, for their unconditional support. 'Thanks' would be too small for all the sacrifices and compromises they made to support me. Dady, its your hardwork to support my education financially right from childhood. What else I can pay in return? It's the only distance that has separated us.

This page would be incomplete without my wife, Archana it's just your support and patience over the past seven years that has kept me going. Let me also thank you for the time you invested in proofreading my thesis. Your willingness to take care our personal life made me to contribute more time on my professional development. I am truly blessed to have you as my life partner. I can't find any words to express; I just want to say ***"Thank you"*** *– I'M COMING!!!*

8. Appendix:

Publications and presentations:

1.Publications:

Ibrahim SA, Hassan H, Vilardo L, **Sampath Kumar Katakam**, Kumar AV, Kelsch R, Schneider C, Kiesel L, Eich HT, Zucchi I, Reinbold R, Greve B, Götte M. Syndecan- 1 (CD138) modulates triple-negative breast cancer stem cell properties via regulation of LRP 6 and IL-6-mediated STAT3 signaling. PLoS One 2013; 8: e85737.

Kumar AV, **Sampath Kumar Katakam**, Urbanowitz A-K, Götte M. Heparan sulfate a regulator of leukocyte recruitment in inflammation Current Protein and Peptide Science, 2015, 16,77-86.

Manuscript under revision:

Vijaya Kumar A, **Sampath Kumar Katakam**, Dorothe Spillmann , Björn Kemper, Burkhard Greve , Mauro S.G. Pavão, Francisco M. Goycoolea, Martin Götte. Heparan sulfate 2-O sulfotransferase-dependent signaling pathways determine breast cancer cell viability, cell-matrix interactions and invasive behavior. (Manuscript under revision in the *Journal of Biological Chemistry*)

Sakshi Singh, Ashish Singh, Raja Rajkumar, **Sampath Kumar Katakam**, Subburaj Kadarkarai Samy, Sheikh Nizamuddin, Amita Singh, Sheikh Shahnawaz, Vidya Peddada, Vinee Khanna, Pandi Selvam Veeraiah, Gyaneshwer Chaubey, Lalji Sing, Kumarasamy Thangaraj. Dissecting the influence of Neolithic demic diffusion on the Indian Y- chromosome pool through the J2-M172 haplogroup. (Manuscript under revision in *Scientific Reports*)

Manuscripts in preparation:

Sampath Kumar Katakam, Dorothe Spillmann , Björn Kemper, Burkhard Greve , Stefano Molgora,Valeria Tria, Zucchi I, Reinbold R, Martin Götte. Role of Sdc1 in the tumorigenicity of the stem cell like population in the colon cancer progression. (Manuscript to be submitted)

Sampath Kumar Katakam, Dorothe Spillmann , Björn Kemper, Burkhard Greve , Stefano Molgora ,Valeria Tria, Zucchi I, Reinbold R, Michael Elkin, Israel Vlodavsky, Martin Götte. Interplay between Sdc1 and HPSE and the influence of stemness in colon cancer progression. (Manuscript to be submitted)

Kumar AV, **Sampath Kumar Katakam,** Reinbold R, Burkhard Greve, Martin Götte. Contribution of heparan sulfate sulphotransferases (HS2ST1 and HS3ST2) to breast cancer stemness. **(**Manuscript in preparation)

Scientific Talks:

Interplay between Sdc-1 and HPSE in colon cancer pathogenesis. Program of Glycobiology, Institute of Medical Biochemistry, Federal University of Rio de Janerio, Brazil. 26.08.2013

Contribution of Sdc-1 to colon cancer stem cell properties.Retreat meeting of the Stem Cell Network NRW, Herne, Germany. 11-12.04.2014.

Interplay between Sdc-1 and HPSE in the tumorigenicity of the stem cell like population within colon cancer cell lines. 22nd Symposium on Glycosaminoglycans. Villa Vigoni, Menaggio, Italy. 18-20.09. 2014. *DFG travel fellowship recipient.*

Interplay between Sdc-1 and HPSE in the tumorigenicity of the stem cell like population. 2ndSymposium on Novel insights into cancer biology: new targets and therapeutic approaches, Cairo University, Egypt. 16-17.11.2014.

Posters:

- Interplay between Sdc-1 and HPSE in colon cancer pathogenesis. FEBS-MPST 2013, Kos, Greece. 26.09.-01.10.2013.

- Role of Sdc-1 in colon cancer stem cell properties. 7th International Meeting of the NRW Stem Cell Network, Cologne, Germany, 21.-22.04.2013.

- Role of Sdc-1 in the tumorigenicity of the stem cell like population within colon cancer cells. Retreat meeting of the Stem Cell Network NRW, Herne, Germany. 11. – 12.04.2014.

Role of Sdc-1 in the tumorigenicity of the stem cell like population within the colon cancer cell lines EMBO Conference Stem Cells in Cancer and Regenerative Medicine, 9.-12.10.2014, EMBL Heidelberg, Germany.

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(Unterschrift)