MOLECULAR ONCOLOGY 4 (2010) 431–442

available at www.sciencedirect.com

www.elsevier.com/locate/molonc

Review

Pancreatic cancer stem cells – update and future perspectives

*Enza Lonardo, Patrick C. Hermann, Christopher Heeschen**

Clinical Research Programme, Spanish National Cancer Research Centre (CNIO), C/ Melchor Fernandez Almagro 3, 28029 Madrid, Spain

ARTICLE INFO

Article history:
Received 1 March 2010
Received in revised form
1 June 2010
Accepted 1 June 2010
Available online 9 June 2010

Keywords:
Pancreatic cancer
Stem cells
Cancer stem cells
Tumour-initiating cell
metastasis
mTOR
Hedgehog
Gemcitabine
Targeted therapy

ABSTRACT

Solid tumours are the most common cancers and represent a major therapeutic challenge. The cancer stem cell hypothesis is an attractive model to explain the functional heterogeneity commonly observed in solid tumours. It proposes a hierarchical organization of tumours, in which a subpopulation of stem cell-like cells sustains tumour growth, metastasis, and resistance to therapy. We will present the most recent advances in the cancer stem cell field, with particular emphasis on pancreatic cancer as one of the deadliest human tumours, and highlight open questions and caveats to be addressed in future studies. There is increasing evidence that solid tumours including pancreatic cancer are hierarchically organized and sustained by a distinct subpopulation of cancer stem cells. However, direct evidence for the validity of the cancer stem cell hypothesis in human pancreatic cancer remains controversial due to the limitations of xenograft models but supportive data are now emerging from mouse models using related or different sets of markers for the identification of murine cancer stem cells. Therefore, while the clinical relevance of cancer stem cells remains a fundamental issue for this rapidly emerging field, current findings clearly suggest that specific elimination of these cells is possible and therapeutically relevant. Targeting of signalling pathways that are of particular importance for the maintenance and the elimination of cancer stem cell as the proposed root of the tumour may lead to the development of novel treatment regimens for pancreatic cancer. Here we will review the current literature on pancreatic cancer stem cells and the future perspective of this rapidly emerging field.

1. Pancreatic cancer in 2010 – still a devastating diagnosis

Pancreatic adenocarcinoma is the deadliest solid cancer and currently the fourth most frequent cause for cancer-related deaths. The disease is characterized by late diagnosis due to lack of early symptoms, extensive metastasis, and high resistance to both chemotherapy and radiation, respectively. The only effective treatment modality to date for pancreatic cancer represents a very invasive and complex surgical procedure, also known as Whipple procedure, for which only a limited number of patients with local disease is eligible (currently about 20% of patients diagnosed with pancreatic cancer) (Philip et al., 2009).

* *Corresponding author*. Clinical Research Programme, Stem Cells & Cancer Group, Spanish National Cancer Research Centre (CNIO), Madrid, Spain.
E-mail address: christopher.heeschen@cnio.es (C. Heeschen).

1574-7891/$ – see front matter © 2010 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.molonc.2010.06.002

Despite increasing research activities in the field of pancreatic tumour and vascular biology, there has hardly been any substantial progress with new therapies regarding clinical endpoints over the past decades. The latest advancement by introduction of the nucleoside-analogue and chemotherapeutic agent gemcitabine improved clinical response in terms of pain reduction and loss of weight (Burris et al., 1997). Gemcitabine has become the first-line chemotherapeutic agent in pancreatic cancer, utilized in locally advanced or metastasized disease and in all patients that cannot undergo surgery. Overall, however, with a 5-year survival rate of 1–4% and a median survival period of 4–6 months, the prognosis of patients with pancreatic cancer has remained extremely poor (Ahlgren, 1996; Jemal et al., 2004; Philip et al., 2009; Rosenberg, 1997; Rothenberg et al., 1996; Warshaw and Fernandez-del Castillo, 1992). The more recent addition of erlotinib to our armoury for fighting pancreatic cancer, the only other approved agent to gemcitabine, has not resulted in markedly improved survival (Moore et al., 2007). Therefore, new insights into the complex biology of pancreatic cancer are desperately needed to pave the way for the development of more effective treatments for these patients.

2. Cancer stem cell concept

At least two models have emerged trying to explain the heterogeneity and inherent differences in tumour-regenerating capacity: the clonal model, whereby mutant tumour cells with a growth advantage are selected and expanded during tumorigenesis (Nowell, 1976), and the hierarchical model, in which primary tumours and metastatic cancer are initiated by a subpopulation of cancer stem cells (Clarke et al., 2006). The acquisition of genetic alterations underpins the clonal model, but epigenetic modifications and the influence of the microenvironment are also likely to play important roles during progression. The clonal evolution may involve a stochastic component, whereby a distinct population of tumour cells acquires an appropriate set of somatic mutations and develops metastatic capability (Reya et al., 2001). The cancer stem cell (CSC) hypothesis could indeed be a relevant model to account for the functional heterogeneity that is observed in solid tumours (Visvader and Lindeman, 2008). It proposes a hierarchical organization of cells within the tumour, in which a subpopulation of stem-like cells is responsible for sustaining tumour growth and drive metastasis. One important implication of this organization is that CSCs are placed at the apex of this hierarchy and may represent the source of tumour relapse (Bonnet and Dick, 1997). It is important to note that the two models are not mutually exclusive, as CSCs themselves undergo clonal evolution, as shown for leukaemia stem cells (Barabe et al., 2007).

Specifically, the cancer stem cell hypothesis suggests that a distinct population of cells with stem cell properties is essential for the development and perpetuation of various human cancers, including pancreatic cancer (Al-Hajj et al., 2003; Hermann et al., 2007). CSC are a population of cancer-initiating cells that usually constitute a small, but variable percentage of the total tumour mass, and that show three defining features: they are able to self-renew, to generate all the heterogeneous cell types a tumour contains, and are the only cells within a tumour that can give rise to tumours in secondary recipients. Usually, CSCs have additional characteristic traits like a distinct surface markers expression profile, and the capacity of asymmetric/symmetric cell division that allows the CSC population to maintain/expand itself, while at the same time generating the more differentiated progeny of tumour cells (Wicha, 2006). Intriguingly, CSCs are often resistant to standard therapy (Bar et al., 2007; Hermann et al., 2007,2008).

The concept of cancer stem cells was under debate for the past few years, but while the first evidence was shown early for leukaemia and myeloma (Bruce and Van Der Gaag, 1963; Park et al., 1971), their existence has now been validated in several solid tumours, such as breast cancer (Al-Hajj et al., 2003), glioblastoma (Singh et al., 2004), colorectal (Ricci-Vitiani et al., 2007), liver, (Ma et al., 2007) and pancreatic cancer (Hermann et al., 2007; Li et al., 2007). Apparently, several questions still remain to be addressed including the interpretation of data generated with different xenograft models (Quintana et al., 2008). In each of these studies, CSC were identified by a set of specific criteria in order to demonstrate their role in tumour initiation and progression (Clarke et al., 2006): the expression of surface markers depending on the tumour of origin, the capacity of self-renewal and differentiation, and the ability to recapitulate the phenotype of the original tumour using *in vitro* and *in vivo* tumourigenicity assays.

The CSC theory is an intriguing model to explain both the wide heterogeneity observed in an originally monoclonal tumour, and tumour relapse after treatment due to the presence of a therapy-resistant population. However, the application of this theory to the development of pancreatic cancer and the identification, quantification, and clinical relevance of pancreatic cancer stem cells is a controversial issue. In addition, the cell origin of pancreatic cancer stem cells still remains to be identified (Hermann et al., 2009). One possibility is that CSCs arise from somatic stem or progenitor cells with genetic alterations that lead to malignant behaviour. Another possibility would be that CSCs originate from the dedifferentiation of a lineage-committed cell that has (re-)acquired stem cell characteristics through mutation. This issue is further complicated by the current lack of convincing evidence for a stable stem cell population in the normal pancreas. The identification and precise investigation of a putative pancreatic stem cell including its niche using genetically engineered mouse models (GEMM) will hopefully add further insights to understand the origin of CSCs.

3. Pancreatic cancer stem cell markers and heterogeneity of pancreatic cancer cells

Putative pancreatic CSCs, for the first time defined by the simultaneous expression of CD44, CD24, and EpCAM (Li et al., 2007), are highly tumourigenic and possess the ability to both self-renew and to produce differentiated progeny that reflects the heterogeneity of the patient's primary tumour. However, it should be noted that in this first study, putative CSCs were compared to a population of cells that were negative for all three markers. Since EpCAM identifies epithelial cells within the tumour, the confinement to EpCAM negative cells as the control population may have been too restrictive, as

these cells should primarily represent non-epithelial inflammatory, stromal and vascular cells (Li et al., 2007). Using a different cell surface marker, Hermann et al. showed that CD133+ cells in primary pancreatic cancers and pancreatic cancer cell lines also discriminated cells with enhanced proliferative capacity (Hermann et al., 2007), which also show the defining CSC traits. Interestingly, they showed that the CD44 + CD24 + EpCAM+ subpopulation clearly overlaps with the CD133+ population.

In a more recent study, Mueller et al. also used CD133 to investigate for the first time a therapeutic strategy targeting this subpopulation of human pancreatic cancer cells that is highly enriched for tumour-promoting CSCs both in primary pancreatic cancer cells and the xenografted pancreatic cancer cell line L3.6pl (Mueller et al., 2009). Other distinctive markers have also been used for the characterization of CSCs: ALDH-1 (ALdehyde DeHydrogenase-1) has been shown to be associated with the tumourigenic cells in pancreatic cancer (Feldmann et al., 2007; Jimeno et al., 2009; Rasheed et al.), although more recent comprehensive investigations suggest an abundant expression of ALDH-1 in normal pancreas tissue (Deng et al.), which would disqualify ALDH-1 as a suitable marker for CSCs in humans. Moreover, side population (SP) cells, which exclude the DNA dye Hoechst 33342, proved to be cancer-initiating cells in several tumours (Hirschmann-Jax et al., 2004), but these data require further validation as the use of SP cells in gastrointestinal cancers has generated conflicting data (Burkert et al., 2008).

Apparently, a number of studies have published conflicting data on the expression of these markers for the identification of pancreatic CSCs, particularly for CD133, suggesting that the analyzed CSC populations are by no means pure and technical obstacles still remain. Importantly, it was reported that the use of different CD133 antibodies can translate into significantly different findings (Mueller et al., 2009). Two studies using different CD133 antibodies for histological analysis on pancreatic cancer tissue have led to opposing results with respect to the CD133 expression patterns (Immervoll et al., 2008; Maeda et al., 2008). Moreover, the differentiation of colon CSCs did not coincide with a change in CD133 promoter activity, mRNA, splice variants, protein expression, or even cell surface expression of CD133. In contrast, a change occurred in CD133 glycosylation suggesting that CD133 is expressed on both CSC and differentiated tumour cells, but is probably differentially folded as a result of differential glycosylation to mask specific epitopes (Kemper et al., 2010). In summary, CSCs can be reliably identified by AC133, which only binds to this modified form of CD133, but the use of this antibody should still be interpreted with caution as handling of the cells may affect the results. Interestingly, CD133 has also been used to distinguish different types of pancreatic cancers (Shimizu et al., 2009).

Although none of these markers appear to selectively characterize a pure population of CSCs, their use increases not only our knowledge about the biology of these cells, but also produces consistent data for a strong enrichment of CSC. Moreover, another important aspect to consider before the evaluation of data is the origin of the investigated sample, since the results obtained from fresh patient-derived samples could be significantly different from those obtained from established cancer cell lines. The analysis of clinical samples will certainly provide more relevant data, as established cell lines have adapted to *in vitro* culture conditions, and potentially no longer resemble their primary counterparts, especially after long-term passaging.

However, from a practical perspective the use of well-characterised stable cancer cell lines may still be important for some mechanistic work, since primary cells are usually much more difficult to handle (e.g. low transfection efficiency, reduced viability in culture conditions) (Hermann et al., 2007). However, the most important aspect for testing the CSC hypothesis and potential targeted therapeutics is the use of optimized preclinical model systems. The current gold standard for this is the investigation of primary patient-derived xenografts, since these xenografts closely reflect the heterogeneity of mutations and cellular composition of the original primary tumours. Recent data provide further experimental proof for a close relationship between primary tumours and their corresponding xenografts (Ding et al., 2010). Interestingly, this study also demonstrated that xenografts bear a signature very similar to metastatic lesions suggesting that these secondary tumours are formed by only a small cell population within the primary tumour that is also more potent in engrafting in immunocompromised mice.

A strong inter-individual heterogeneity concerning the expression patterns of markers that have been used for the enrichment of CSC has been reported that may be related to differences in tumour stages (Hermann et al., 2009; Mueller et al., 2009) but may also relate to the digestion and subsequent culturing of the primary tissue. It may indeed also indicate that the CSC hypothesis is not a universal model for all individual patient samples. Whether an individual tumour follows the CSC model or not may depend on whether the initializing mutation occurred in the stem cell compartment or in more differentiated progenitor cells but it may switch to follow the clonal evolution model during tumour progression and metastasis (Shmelkov et al., 2008).

4. Cancer stem cells are likely to change during progression

CSCs are defined by their self-renewal and differentiation capacities. Normal stem cells accomplish these two tasks by asymmetric cell division, a process where the stem cell divides to generate one stem cell bearing self-renewal capacity and one daughter cell that subsequently differentiates (Fig. 1, middle and right-hand side). This modus maintains a stable number of stem cells. However, it has been shown that CSC numbers can increase markedly during progression requiring a more preferential symmetric division modus (Pece et al., 2010). A cell division can become symmetric in several ways: (i) the dividing cell resides within a polar environment and exposure to different environments induces alternative fates; (ii) genetic/epigenetic changes drive symmetric division (e.g. aberrant DNA methylation, abnormal RNA interference and chromatin remodelling) (Fig. 1, middle and right-hand side).

CSCs must have diverse self-renewal strategies that permit dynamic modulation of their numbers, cell proliferation

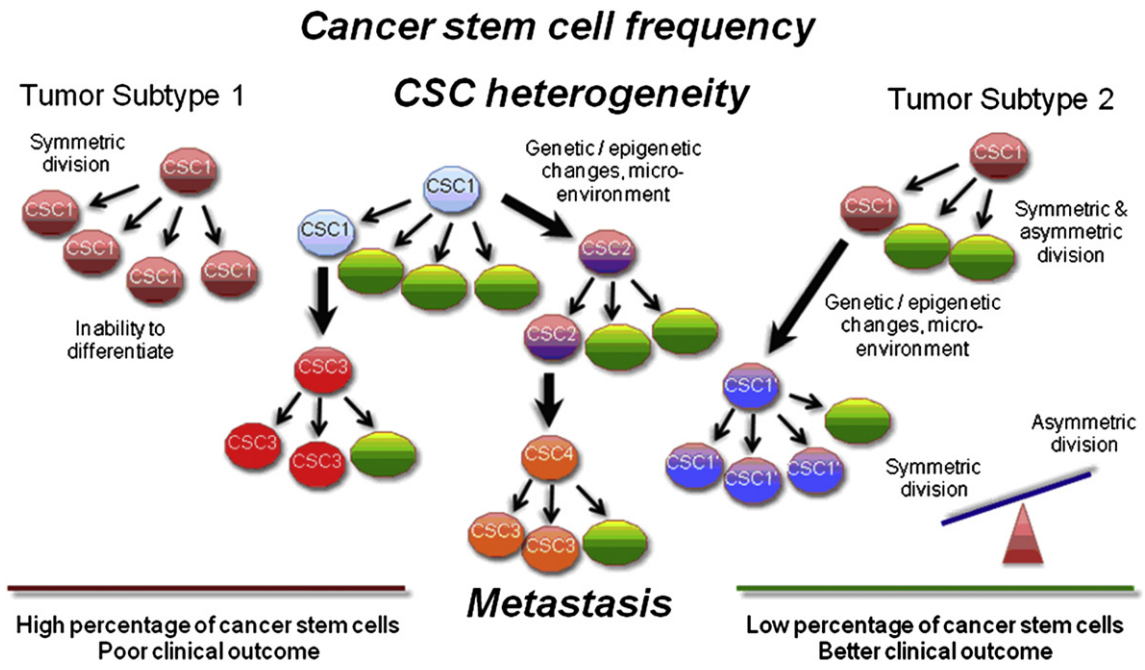

Figure 1 – Frequency and genetic heterogeneity of CSCs. The number of cancer stem cells has been linked to prognosis, but this may not represent a fixed stage as the frequency of CSCs may increase during progression due to genetic/epigenetic as well as environmental changes.

and tumour relapse. It is currently unknown whether CSC homeostasis, which readily establishes after *in vivo* implantation of highly purified CSCs, is maintained by asymmetric divisions, or by a strategy that uses symmetric divisions to balance CSCs and more differentiated progeny. CSCs use symmetric divisions at a much higher rate as compared to their normal counterparts to expand their pool or to generate more differentiated progeny (Pece et al., 2010). Symmetric divisions are defined as the generation of daughter cells that are destined to acquire the same fate (Fig. 1, left-hand side). Indeed, Pece et al. recently reported that a different ratio of asymmetric versus symmetric division as compared to normal stem cells is causal for the increasing numbers of CSC in G3 versus G1 tumours, which at least in part could explain the biological and clinical heterogeneity of breast cancers at different stages (Pece et al., 2010). CSC are defined by their 'potential' to generate CSC and differentiated daughter; in other words, a pool of CSC with equivalent developmental 'potential' may produce only new cancer stem cells in some divisions and only more differentiated progeny in others. Furthermore, symmetric division may confer developmental plasticity, increased growth and enhanced regenerative capacity as well as an inherent risk of cancer (Morrison and Kimble, 2006). It is intriguing to speculate that CSCs are able to use either symmetric division only, or a combination of symmetric and asymmetric division (Fig. 1, right-hand side). The preferential modus may be determined by the sustained activation of different developmental cascades such as certain hormones, growth factors (fibroblast growth factor, epidermal growth factor), and signalling pathways (sonic hedgehog, Wnt/β-catenin and/or notch), which are involved in the strict control of self-renewal and differentiation of CSC.

5. Migrating cancer stem cells and metastasis

Metastasis remains the major cause of mortality in pancreatic cancer patients, and there is currently no curative treatment for metastatic pancreatic cancer. Not all pancreatic cancer cells within a tumour bear the same metastatic potential, and only a small subset of cells home to specific sites in the body. Metastasis is a multi-event process that involves the invasion of cancer cells from primary neoplasms, followed by their dissemination through lymphatic or blood vessels, and finally the establishment of micrometastases in secondary sites that culminate in established metastases at distant tissues/organs. Although there is a large mass of apparently highly heterogeneous cancer cells in the primary tumour, the molecular events leading to metastasis at distant sites consist of a series of sequential transforming events that most likely only a few tumour cells can accomplish (Fig. 2).

Intriguingly, the CSC concept also bears implications for the development of metastasis: If indeed only CSCs are capable of initiating tumour growth, then it seems likely that these cells also play an important role in the metastatic process. In line with this hypothesis, a specific set of surface markers and genes in pancreatic cancer stem cells have been associated with the event of distant metastasis. A detailed characterization of CD133+ pancreatic cancer stem cells and the critical involvement of a distinct subset responsible for tumour metastasis has been demonstrated by Hermann et al. (2007). The authors identified a subpopulation of CD133+/CXCR4+ cells, which displayed a high migratory activity towards gradients of the CXCR4 ligand SDF-1. The migration of these cells could be inhibited *in vitro* by anti-CXCR4 neutralizing antibodies, and the small molecule inhibitor AMD3100. *In vivo* experiments with sorted

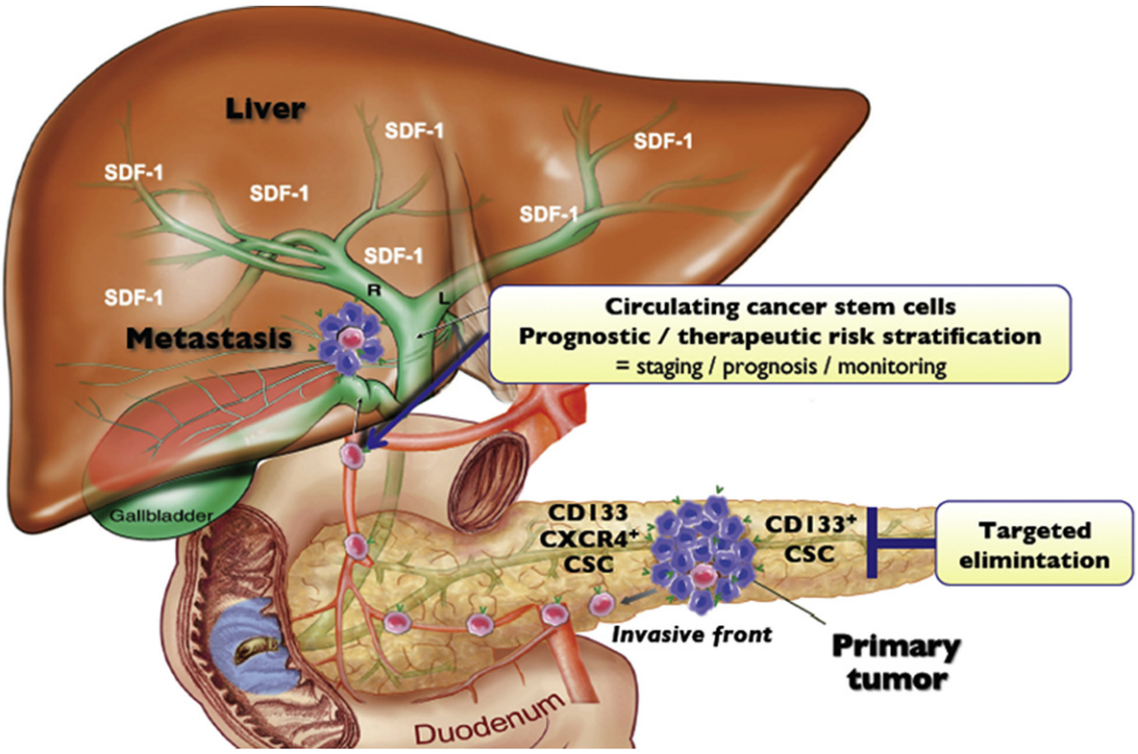

Figure 2 – Distinct populations of pancreatic CSC. A subpopulation of migrating cancer stem cells, identified by additional expression of CXCR4 can be detected in the invasive front in the pancreas as well as in the circulating blood. Detection of these circulating CSC could serve as prognostic and therapeutic biomarker.

CD133+/CXCR4+ cells demonstrated that the co-expression of this receptor is essential for the generation of liver metastasis (Hermann et al., 2007). These results clearly show that CXCR4, which was originally identified due to its role in leukocyte trafficking, is also implicated in metastasis of malignancies like breast (Muller et al., 2001) and pancreatic cancer (Hermann et al., 2007; Mueller et al., 2009). Consistently, Nakata et al. has demonstrated that CCR7 expression is correlated with lymph node metastasis in pancreatic cancer (Nakata et al., 2008).

It is possible that the tumourigenic CSC can acquire a migrating phenotype during an epithelial-mesenchymal-transition process (EMT) in primary neoplasms, which would enable them to spread to distant sites. Recently, Wellner et al. suggested that the EMT-inducer ZEB1 supports metastasis not only by promoting tumour cell mobility and dissemination, but also by maintaining a stem cell phenotype through inhibition of miR200 family members, which is necessary for the formation of metastases from disseminated tumour cells (Wellner et al., 2009). These results suggest that the metastatic process and the migratory capacity of pancreatic cancer stem cells is not random, but depends on the specific expression pattern of chemokine receptors and adhesion molecules on these cells and the presence of their respective ligands in the hosting tissues.

6. Xenograft models versus genetically engineered mouse models

Several mouse models have been developed to study cancer cell biology (Fig. 3). These models were developed to investigate the factors involved in malignant transformation, invasion and metastasis, as well as to examine response to therapy. One of the most widely used models is the human tumour xenograft. In this model, human tumour cells or tissues are transplanted, either under the skin or into the organ type from which the tumour originated, into immunocompromised mice that do not reject human cells. The mice most frequently used as xenograft recipients are athymic nude mice, severely compromised immunodeficient (NOD/SCID) mice, or NOG/SCID mice with an additional loss of NK-cell function (Quintana et al., 2008). Usually, these xenografting models show reliable tumourigenicity, and enable us to expand precious primary tumour tissues as well as study tumour biology and treatment response *in vivo* (Jimeno et al., 2009). However, the individual mouse models each bear advantages and risks: While athymic nude mice and NOD/SCID mice may underestimate the number of tumour-initiating cells (Quintana et al., 2008), the NOD/SCID Il2rg$^{-/-}$ mice utilized by Quintana et al. for studying the CSC hypothesis provide a very permissive environment, having virtually no remaining immune response, which could actually overestimate the tumourigenic potential of the investigated cells. Another caveat in determining the number of tumour-initiating cells in immunocompromised mice is the co-injection of Matrigel™, since the growth factors contained further influence the permissiveness of the immediate host environment. In our opinion, it would be very interesting to see the tumourigenic activity of CSCs in NOD/SCID Il2rg$^{-/-}$ mice revisited in other solid tumours, since the results observed by Quintana et al. may also be related to the advanced stage of

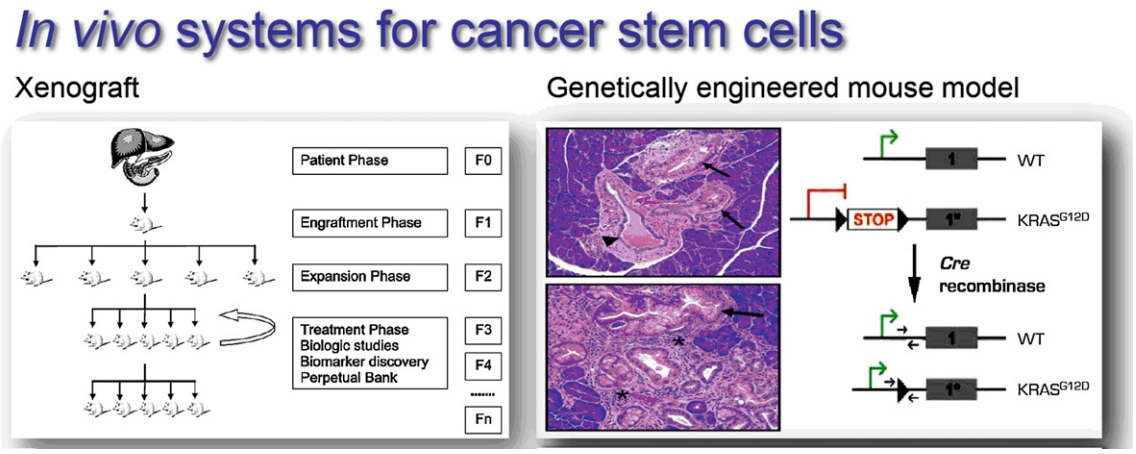

Figure 3 – Two different animal models for *in vivo* investigations – xenografts of freshly isolated human pancreatic cancer and genetically engineered mouse models ((Jimeno et al., 2009, Hingorani et al. 2003)).

the utilized primary tumours, which were either stage III or stage IV, or may actually be an observation specific for melanoma.

Therefore, further studies in other tumour entities and/or models will be necessary to clarify this important point. In this regard, genetically engineered mouse model (GEMM) do not only enable us to look in great detail into the relevance of different genes during tumourigenesis, progression, and metastasis but also to study the CSC hypothesis in a syngeneic setting. The genetic profile of these mice is altered, so that one or several genes thought to be involved in transformation or malignancy are mutated, deleted or overexpressed. In these models, the effect of the induced genetic alteration(s) is studied over time. While these models certainly present an interesting platform to investigate response to different treatments, their main advantage is that we can reproduce genetic mutations often seen in humans (e.g. Kras, p53), and identify key steps in disease progression on a genetic basis. Interestingly, the oncogenic mutation of Kras, an event observed in approximately 85% of human tumours, was shown to lead to the expansion of progenitor cells and malignant transformation in zebrafish (Park et al., 2008). GEMM bear full immune competence and have the potential to reproduce specific genetic abnormalities that are present in human tumours. In contrast to xenografting studies, GEMM are extremely useful for long-term studies of tumourigenesis and progression, and to explore therapeutic approaches at various stages of tumour development. However, the disadvantages of GEMM are that the complexity and heterogeneity of human tumours cannot be reliably mimicked so that the relevance of treatment response for the human setting remains questionable.

Human tumour xenografts, if used at early passages, still closely represent the complexity of genetic and epigenetic variations that exist in the original human tumour, and thus they can be used to identify novel therapeutic approaches. Especially in tumour entities, where tissue samples are scarce, multiple therapies can be tested on the same tissue after the *in vivo* expansion of tumour tissues. Furthermore, results from xenografting studies can usually be obtained within a few weeks, making xenografts a useful tool for high-throughput screening efforts. In most cases, the xenografted cells or tissue pieces are implanted subcutaneously. However, even though orthotopic tumour models are more time consuming and can sometimes be technically challenging, orthotopic implantations should be preferred whenever possible, since the tumour can then be investigated within its normal (micro-)environment. For therapeutic studies, this represents a great advantage, since questions of drug delivery and biodistribution can be assessed in a more relevant setting. While the lack of immune response against tumour cells in immunocompromised mice certainly represents a major drawback, it efficiently rules out confounding factors in drug response studies. Therefore, even though xenograft models are sometimes regarded as inferior to the GEMM models, it must be kept in mind that they bear the advantage of looking into human tumour biology if combined with early passages of primary tissue samples. In summary, both xenografts and GEMM models have their strengths and limitations, it must be the scientist who has to choose the appropriate model depending on the question he would like to address.

7. Cancer stem cell signalling as new targets for novel treatment modalities

Despite great efforts, pancreatic cancer continues to be one of the deadliest cancer-related diseases in the world (Philip et al., 2009). Therefore, the development of novel therapeutic strategies is an issue of outstanding significance to finally improve the currently devastating prognosis. Different groups have demonstrated that CSCs in pancreas (Hermann et al., 2007; Hong et al., 2009; Jimeno et al., 2009) and brain tumours (Bao et al., 2006) are responsible for the resistance to conventional anti-cancer therapies such as chemotherapy and radiation. In the case of pancreatic cancer, cell cycle analyses of CD133 + CSC have proven that while these cells stop proliferating under the influence of the cytotoxic agent gemcitabine, they did not undergo apoptosis, and as soon as gemcitabine was withdrawn, these cells immediately started to repopulate

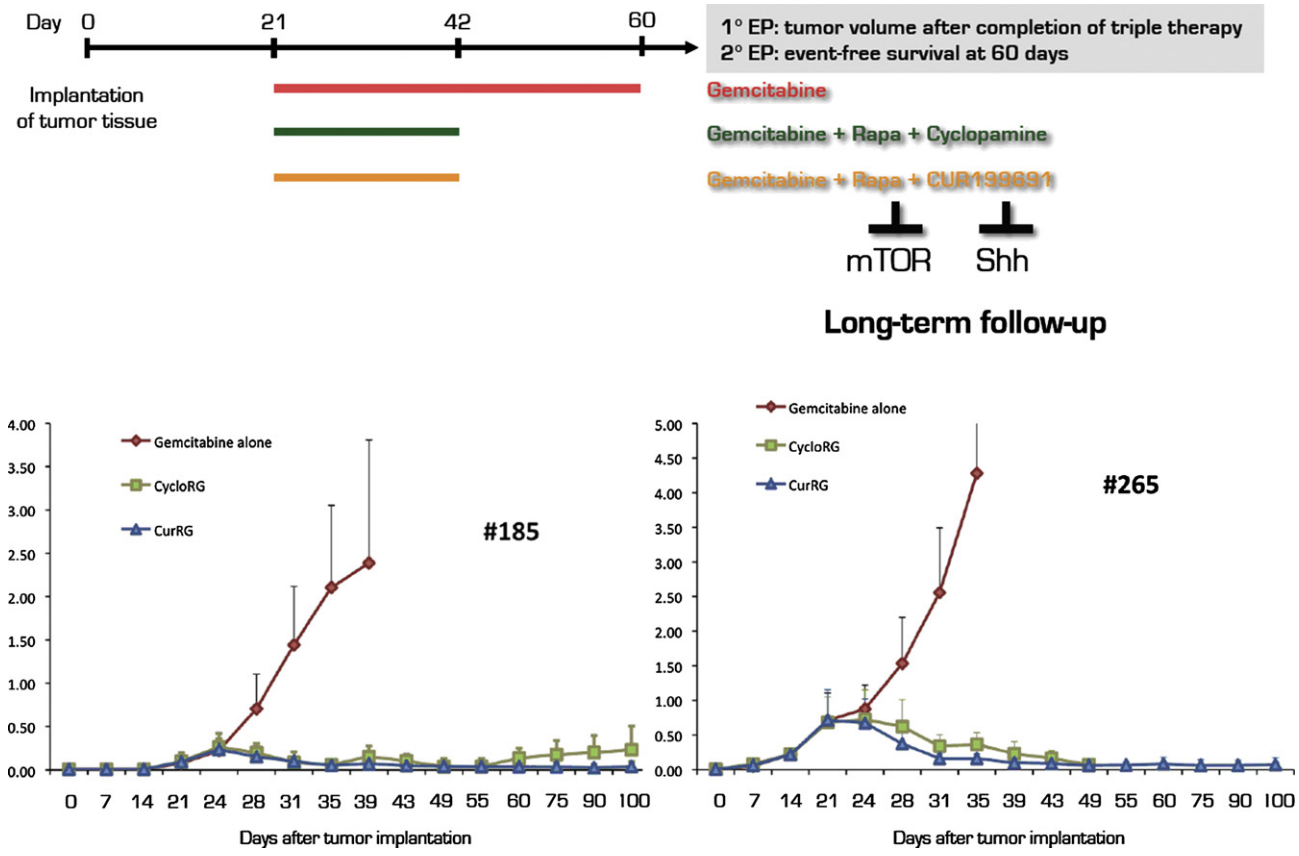

Figure 4 – Study design of combination therapy for treatment of mice bearing primary pancreatic cancers. Mice were followed for 100 days for relapse of cancers.

the cancer (stem) cell pool. In contrast, the more differentiated CD133 negative cells representing the vast majority of the tumour cell population became apoptotic after the application of gemcitabine. Thus it is becoming increasingly obvious, that it is only the more differentiated tumour cells that can be targeted with standard therapy, even resulting in a selection process for undifferentiated tumour-initiating and metastasis-propagating cancer stem cells.

Since these CSCs have been shown to exclusively generate tumours in secondary recipients (Hermann et al., 2007; Li et al., 2007), the question arises if these undifferentiated cancer cells represent a new and important target for novel therapeutic approaches, finally making it possible to target the real root of pancreatic cancer. In our opinion, there are mainly two possible ways of depleting cancers for their CSC populations: either finding therapeutic agents that selectively kill these cells, or those that drive CSCs into differentiation, thus making them susceptible to standard (chemo-)therapy. While it may be of great interest to inhibit CSC function, practically doing so may turn out to be rather complicated as specific stem cell features distinct from normal stem cells need to be defined.

Stem cells in general, but especially cancer stem cells possess distinctive traits that provide them with higher resistance levels against classic cytotoxic agents: They strongly express ABC membrane transporters that can exclude toxic substances from the cell (Goodell et al., 1996), have an extraordinarily high capacity to repair DNA damage, display a reduced immunogenicity, and have inherent anti-apoptotic properties (Visvader and Lindeman, 2008). Most importantly, however, the tumour compartment may contain a quiescent subpopulation of CSCs, which could evade the effects of most cytotoxic drugs at least in part due to lack of proliferation. Indeed, quiescence protects the stem cell compartment from most injuries and ensures its functionality during their long lifespan. As a consequence, quiescent stem cells have been shown to survive conventional cancer chemotherapy and radiation (Wilson et al., 2008). Moreover, Pece et al. demonstrate the isolation of stem cells from cultured mammospheres, on the basis of their ability to retain the lipophilic dye PKH26 as a consequence of their quiescent nature. By using markers associated with the signature of this PKH26-positive cells the authors prospectively isolated stem cells from the normal gland and from breast tumours (Pece et al., 2010).

Therefore, if CSCs are the aim of new therapeutic approaches, all these traits and defence mechanisms need to be overcome. One of the most promising approaches to target stem cells is certainly the inhibition of stem cell-associated pathways (e.g. sonic hedgehog, mTOR, notch, BMI, BMP). Further therapeutic targets may represent specific enzymes (e.g. telomerase), membrane transporters (e.g. ABC transporters), or RNA translation (Ji et al., 2009). A publication by Feldmann et al. recently described increased sonic hedgehog (Shh)

activity in pancreatic cancers (Feldmann et al., 2007), which spurred interest in this pathway in the context of CSCs. Shh signals via inhibition of the transmembrane receptor patched, which again inhibits smoothened in the absence of Shh. Patched inactivation after binding of Shh leads to an activation of Smoothened, which in turn leads to transcription of the Gli protein family target genes.

Although several genetically engineered mouse models have been established to investigate a causative role of Hh signalling in pancreatic tumorigenesis, none of these distinguished paracrine versus autocrine canonical Hh signalling in pancreatic cancer. Yauch et al. recently demonstrated a paracrine requirement for the Hh pathway in xenograft models of pancreatic cancer, where Hh ligand is produced by tumour cells and the pathway is activated by the adjacent stroma (Yauch et al., 2008). To address whether a paracrine Hh signal is present in autochthonous mouse pancreatic tumours, and to test if epithelial cancer cells is competent to transduce the Hh signal, Tian el al. used an oncogenic form of smoothened to activate the pathway cell autonomously (Tian et al., 2009). These data indicated that Hh signalling is restricted to tumour stroma, contradicting previous reports that suggest a key role for ligand-driven epithelial Hh signalling in tumour cell growth. However, these studies are consistent with the observation that a subpopulation of CSCs, which do not express markers of epithelial differentiation, rely on Hh signalling (Feldmann et al., 2007; Li et al., 2007). Therefore, more work is needed to comprehensively define the precise mechanism by which Hh pathway activation in stromal cells may also generate a microenvironment providing a CSC niche in pancreatic cancer.

Interestingly, after *ex vivo* pre-treatment of pancreatic cancer cells with the naturally occurring Shh inhibitor cyclopamine, a decline in CSC content was observed but surprisingly this did not translate into reduced tumourigenic activity of pancreatic CSCs in a single-agent therapy (Mueller et al., 2009). Interestingly, however, cyclopamine alone significantly decreased the metastatic activity of the treated cells as compared to treatment with gemcitabine alone. This is compatible with earlier publications indicating an anti-metastatic effect of cyclopamine in an orthotopic mouse model of pancreatic cancer, using different pancreatic cancer cell lines (Feldmann et al., 2007). Interestingly, a combined treatment with simultaneous application of cyclopamine and gemcitabine completely eliminated the CD133 + CXCR4+ migrating cancer stem cell population, which has been demonstrated to be exclusively responsible for the metastatic spread of pancreatic cancers (Mueller et al., 2009).

Since Shh inhibition alone was not able to eliminate the CSC population completely, additional target populations have been investigated. Mueller et al. were able to show that CD133+ cells in pancreatic cancers show particularly high activity for mTOR signalling (Mueller et al., 2009). The mammalian target of rapamycin (mTOR) is a serine/threonine kinase, which belongs to the phosphatidylinositol 3-kinase (PI3K) superfamily, and is the target of a widely branched signalling pathway that activates mTOR among other downstream effectors (Inoki et al., 2005). Interestingly, it was recently demonstrated in the haematopoietic system that deletion of the upstream signalling molecule Pten leads to depletion of normal stem cells, while also resulting in an expansion of leukaemia-initiating cells (Yilmaz et al., 2006). Most of these effects were due to mTOR signalling, as a natural mTOR inhibitor, rapamycin, did not only deplete leukaemia-initiating cells but also restored normal hematopoietic stem cell function. Furthermore, mTOR signalling was recently demonstrated to be essential for the survival and proliferation of breast cancer stem cells (Zhou et al., 2007). For the first time it now seems that it may become possible to distinguish between normal stem cells and CSCs, rendering a targeted therapy even more selective, and potentially less harmful for normal stem cells within the human body. This would of course be a prerequisite for the clinical application of new treatment modalities.

For pancreatic CSCs, the authors were able to demonstrate that single-agent therapy with rapamycin alone resulted in a significant decrease in CD133 + CSCs. However, inhibition of the mTOR pathway by rapamycin was not sufficient to eliminate CSCs completely. Only combined inhibition of these two pathways by cyclopamine and rapamycin, together with gemcitabine, resulted in the desired targeting of the CSC. This triple (CRG) therapy resulted in a significant depletion of the pancreatic CSC pool. Implantation of cells that were pre-treated *ex vivo* demonstrated that tumourigenic activity was completely abrogated. In a clinically more relevant setting, the authors then investigated the effects of the triple therapy on established pancreatic cancers utilizing patient-derived pancreatic cancer tissues (Fig. 4) (Mueller et al., 2009). For the first time, the authors were thus able to show that a multimodal therapy, involving the inhibition of two relevant stem cell pathways and additional chemotherapy, represents a very promising approach, resulting in virtually complete elimination of CSCs, significantly reduced tumourigenic and metastatic activity, and long-term event-free survival.

8. Cancer stem cells-targeted treatment strategies – ready for clinical translation?

While CRG triple therapy has been demonstrated to be highly effective against pancreatic CCS, combining conventional chemotherapy with these two inhibitors may bear many potential risks. Those organ systems that do not rely constantly on their tissue-resident stem cells pool (e.g. the liver) will probably not be affected by this treatment. Some other organs, however, may be affected in a much stronger fashion. This is rather likely in organs with a high cellular turnover rate, which depend on regeneration from a pre-existing stem cell pool (e.g. skin, intestine, bone marrow). Apparently, the most relevant and dangerous of these undesired side-effects is bone-marrow depression, with subsequent leucopoenia and a highly increased risk for infections. Potentially toxic effects on the bone marrow may require autologous bone-marrow transplantation, a procedure used in the treatment of leukaemia after chemotherapy-induced aplasia. Interestingly, Mueller et al. show that application of this triple therapy in tumour-bearing nude mice seems not to have a significant effect on white blood cell counts, suggesting preservation of normal bone-marrow function (Mueller et al., 2009). While this is an important result, indicating that the therapy is well tolerated by mice, further safety studies are mandatory before clinical application of CRG as a novel therapy against pancreatic cancer.

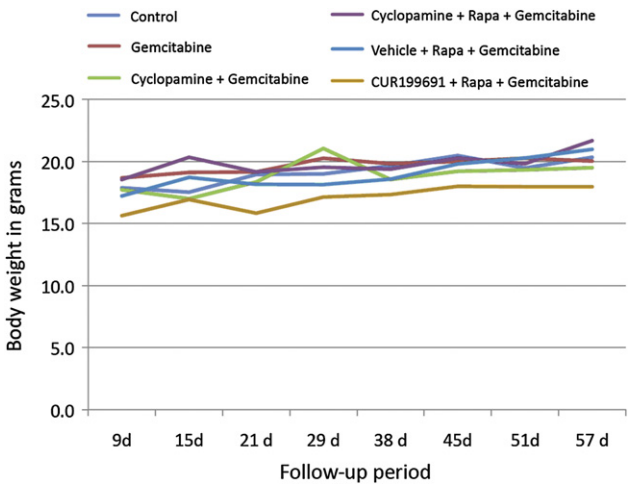

Figure 5 – Weekly measurements of mouse body weights provide no evidence for treatment-induced cachexia in CRG-treated animals.

To follow-up on this issue, an additional safety study using wild type C57/Bl6 mice was performed. Mice were treated with cyclopamine, rapamycin, and gemcitabine for 3 weeks, and then with gemcitabine only for another 4 weeks. In order to elucidate the effects of the different inhibitors in more detail, groups treated with virtually every possible combination. In the context of this study, the body weights and white blood cell counts (WBC) of these mice were monitored weekly, starting with baseline data before the application of the different agents (Figs. 5 and 6). It has to be kept in mind that WBCs are subject to considerable inter-individual differences, as well as short-term changes. Whereas an initial decrease in WBC numbers occurs following application of gemcitabine, no long-term decrease indicative of a manifesting bone-marrow depression was recorded.

Importantly, the proposed novel combination therapy for targeting pancreatic CSC bears several advantages, making it available for clinical application soon: Firstly, all drugs involved are already in clinical use, making long and costly approval studies unnecessary. This significantly reduces the effort that will have to be made until the therapy can be used. Secondly, since these drugs have already been used in patients for quite some time, physicians are already familiar with (un-)expected side-effects. This further reduces the potential danger of this novel approach.

9. Summary and perspectives

Cancer stem cells represent a new and very intriguing target for therapy but the CSC concept still bears many open questions (Fig. 7). For the first time, emerging evidence shows successful CSC-targeted therapy in a preclinical setting with a marked survival benefit in treated mice. Whereas further studies are needed to strengthen these results, a depletion of CSC may well become a powerful tool in clinical cancer therapy. Since normal stem cells often share common pathways with CSCs, the use of drugs/molecules that specifically target the CSC population is a promising strategy, yet a great challenge. While several markers have been described to

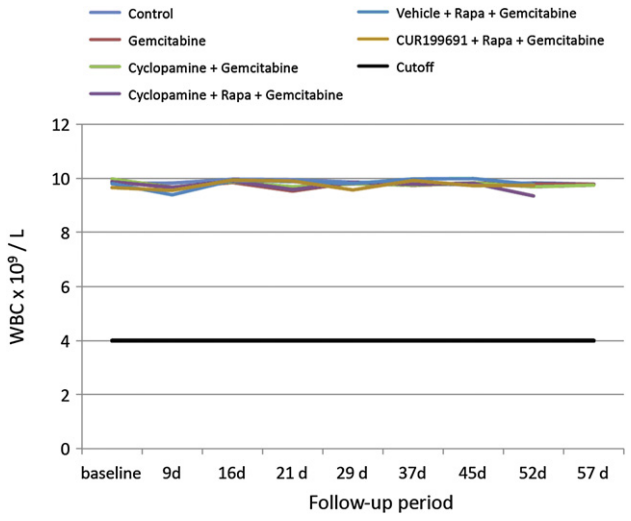

Figure 6 – Weekly measurements of white blood cell counts demonstrate no evidence for long-term decrease of WBC below cut-off.

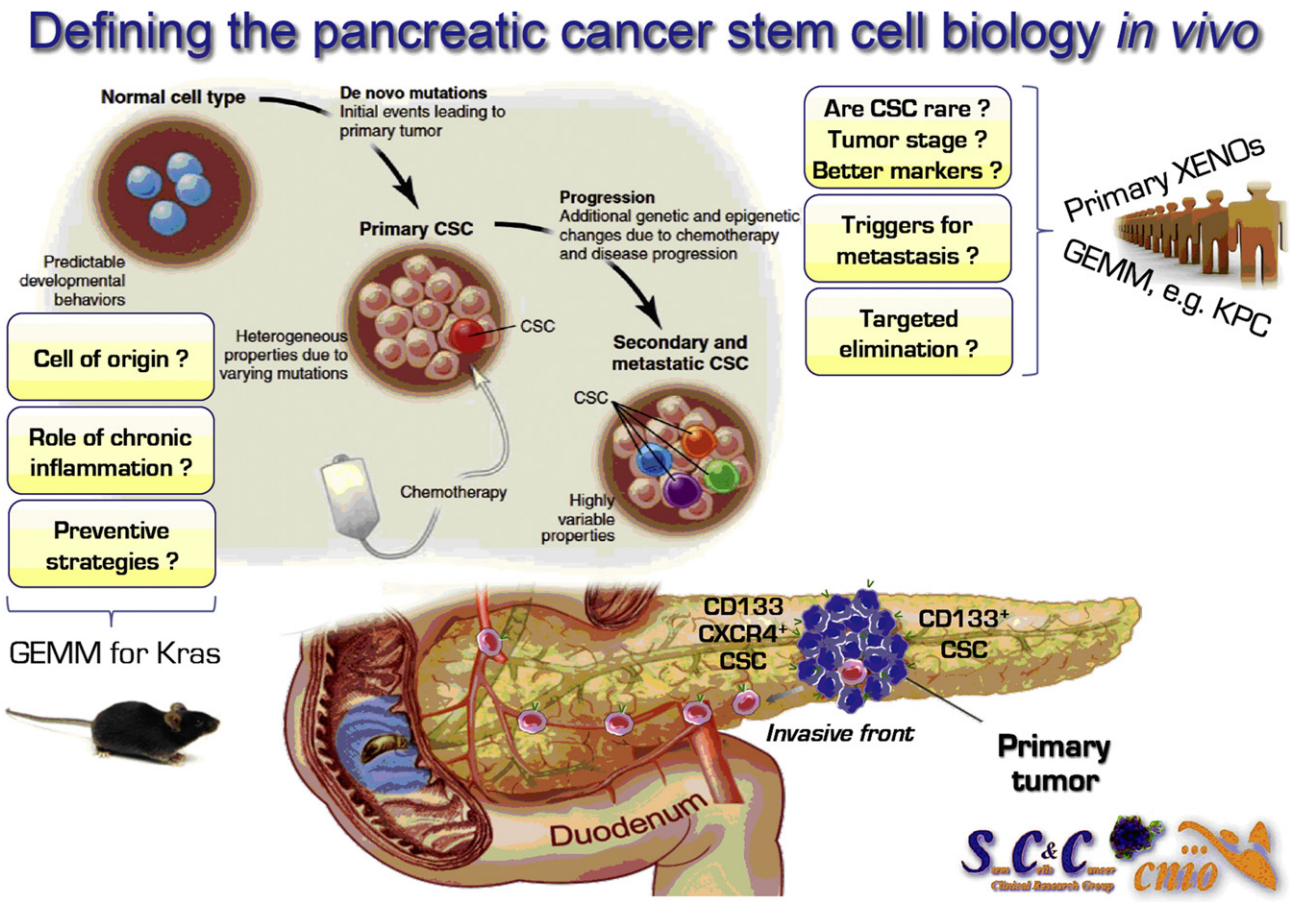

Figure 7 – Future studies need to address essential aspects of cancer stem cell biology.

characterize CSCs, there is relatively little overlap among the CSC markers reported in different tumours or species (mouse, human).

Furthermore, as these markers are often expressed by normal (stem) cells, so it is crucial to find markers that exclusively identify CSCs. It is also very important to define if and how a single CSC can give rise to a tumour, from which cell it originates, and how it determines both the 'stemness' degree and the heterogeneity of its progeny. A major goal in order to discover the best therapies against tumour progression and to address the question of clonal evolution could be the analysis of patient samples at different stages of disease and in particular a follow-up of CSC numbers during and after treatment. While CSCs may be an important target for therapy, it still remains to be determined if they are the unique target, and what is the best way to neutralize their capacities of progression, expansion and resistance to the treatments in the host environment.

REFERENCES

Ahlgren, J.D., 1996. Chemotherapy for pancreatic carcinoma. Cancer 78, 654–663.

Al-Hajj, M., Wicha, M.S., Benito-Hernandez, A., Morrison, S., Clarke, M.F., 2003. Prospective identification of tumorigenic breast cancer cells. Proc. Natl. Acad. Sci. U.S.A. 100, 3983–3988.

Bao, S., Wu, Q., Mclendon, R., Hao, Y., Shi, Q., Hjelmeland, A., Dewhirst, M., Bigner, D., Rich, J., 2006. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. Nature 444, 756–760.

Bar, E.E., Chaudhry, A., Lin, A., Fan, X., Schreck, K., Matsui, W., Piccirillo, S., Vescovi, A.L., DiMeco, F., Olivi, A., et al., 2007. Cyclopamine-mediated hedgehog pathway inhibition depletes stem-like cancer cells in glioblastoma. Stem Cells 25, 2524–2533.

Barabe, F., Kennedy, J.A., Hope, K.J., Dick, J.E., 2007. Modeling the initiation and progression of human acute leukemia in mice. Science 316, 600–604.

Bonnet, D., Dick, J.E., 1997. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat. Med. 3, 730–737.

Bruce, W.R., Van Der Gaag, H., 1963. A quantitative assay for the number of murine lymphoma cells capable of proliferation in vivo. Nature 199, 79–80.

Burkert, J., Otto, W., Wright, N., 2008. Side populations of gastrointestinal cancers are not enriched in stem cells. J. Pathol. 214, 564–573.

Burris 3rd, H.A., Moore, M.J., Andersen, J., Green, M.R., Rothenberg, M.L., Modiano, M.R., Cripps, M.C., Portenoy, R.K., Storniolo, A.M., Tarassoff, P., et al., 1997. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. J. Clin. Oncol. 15, 2403–2413.

Clarke, M.F., Dick, J.E., Dirks, P.B., Eaves, C.J., Jamieson, C.H., Jones, D.L., Visvader, J., Weissman, I.L., Wahl, G.M., 2006. Cancer stem cells – perspectives on current status and future

directions: AACR workshop on cancer stem cells. Cancer Res. 66, 9339–9344.
Deng, S., Yang, X., Lassus, H., Liang, S., Kaur, S., Ye, Q., Li, C., Wang, L.P., Roby, K.F., Orsulic, S., et al., 2010. Distinct expression levels and patterns of stem cell marker, aldehyde dehydrogenase isoform 1 (ALDH1), in human epithelial cancers. PLoS One 5, e10277.
Ding, L., Ellis, M.J., Li, S., Larson, D.E., Chen, K., Wallis, J.W., Harris, C.C., McLellan, M.D., Fulton, R.S., Fulton, L.L., et al., 2010. Genome remodelling in a basal-like breast cancer metastasis and xenograft. Nature 464, 999–1005.
Feldmann, G., Dhara, S., Fendrich, V., Bedja, D., Beaty, R., Mullendore, M., Karikari, C., Alvarez, H., Iacobuzio-Donahue, C., Jimeno, A., et al., 2007. Blockade of hedgehog signaling inhibits pancreatic cancer invasion and metastases: a new paradigm for combination therapy in solid cancers. Cancer Res 67, 2187–2196.
Goodell, M.A., Brose, K., Paradis, G., Conner, A.S., Mulligan, R.C., 1996. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J. Exp. Med. 183, 1797–1806.
Hermann, P., Huber, S., Herrler, T., Aicher, A., Ellwart, J., Guba, M., Bruns, C., Heeschen, C., 2007. Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. Cell Stem Cell 1, 313–323.
Hermann, P.C., Huber, S.L., Heeschen, C., 2008. Metastatic cancer stem cells: a new target for anti-cancer therapy? Cell Cycle 7, 188–193.
Hermann, P.C., Mueller, M.T., Heeschen, C., 2009. Pancreatic cancer stem cells – insights and perspectives. Expert Opin. Biol. Ther. 9, 1271–1278.
Hingorani, S.R., Petricoin, E.F., Maitra, A., Rajapakse, V., King, C., Jacobetz, M.A., Ross, S., Conrads, T.P., Veenstra, T.D., Hitt, B.A., et al., 2003. Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. Cancer Cell 4, 437–450.
Hirschmann-Jax, C., Foster, A.E., Wulf, G.G., Nuchtern, J.G., Jax, T.W., Gobel, U., Goodell, M.A., Brenner, M.K., 2004. A distinct "side population" of cells with high drug efflux capacity in human tumor cells. Proc. Natl. Acad. Sci. U.S.A. 101, 14228–14233.
Hong, S.P., Wen, J., Bang, S., Park, S., Song, S.Y., 2009. CD44-positive cells are responsible for gemcitabine resistance in pancreatic cancer cells. Int. J. Cancer 125, 2323–2331.
Immervoll, H., Hoem, D., Sakariassen, P., Steffensen, O., Molven, A., 2008. Expression of the "stem cell marker" CD133 in pancreas and pancreatic ductal adenocarcinomas. BMC Cancer 8, 48.
Inoki, K., Corradetti, M.N., Guan, K.L., 2005. Dysregulation of the TSC–mTOR pathway in human disease. Nat. Genet. 37, 19–24.
Jemal, A., Tiwari, R.C., Murray, T., Ghafoor, A., Samuels, A., Ward, E., Feuer, E.J., Thun, M.J., 2004. Cancer statistics, 2004. CA Cancer J. Clin. 54, 8–29.
Ji, Q., Hao, X., Zhang, M., Tang, W., Yang, M., Li, L., Xiang, D., Desano, J.T., Bommer, G.T., Fan, D., et al., 2009. MicroRNA miR-34 inhibits human pancreatic cancer tumor-initiating cells. PLoS One 4, e6816.
Jimeno, A., Feldmann, G., Suarez-Gauthier, A., Rasheed, Z., Solomon, A., Zou, G.M., Rubio-Viqueira, B., Garcia-Garcia, E., Lopez-Rios, F., Matsui, W., et al., 2009. A direct pancreatic cancer xenograft model as a platform for cancer stem cell therapeutic development. Mol. Cancer Ther. 8, 310–314.
Kemper, K., Sprick, M.R., de Bree, M., Scopelliti, A., Vermeulen, L., Hoek, M., Zeilstra, J., Pals, S.T., Mehmet, H., Stassi, G., et al., 2010. The AC133 epitope, but not the CD133 protein, is lost upon cancer stem cell differentiation. Cancer Res. 70, 719–729.
Li, C., Heidt, D.G., Dalerba, P., Burant, C.F., Zhang, L., Adsay, V., Wicha, M., Clarke, M.F., Simeone, D.M., 2007. Identification of pancreatic cancer stem cells. Cancer Res. 67, 1030–1037.
Ma, S., Chan, K., Hu, L., Lee, T., Wo, J., Ng, I., Zheng, B., Guan, X., 2007. Identification and characterization of tumorigenic liver cancer stem/progenitor cells. Gastroenterology 132, 2542–2556.
Maeda, S., Shinchi, H., Kurahara, H., Mataki, Y., Maemura, K., Sato, M., Natsugoe, S., Aikou, T., Takao, S., 2008. CD133 expression is correlated with lymph node metastasis and vascular endothelial growth factor-C expression in pancreatic cancer. Br. J. Cancer 98, 1389–1397.
Moore, M.J., Goldstein, D., Hamm, J., Figer, A., Hecht, J.R., Gallinger, S., Au, H.J., Murawa, P., Walde, D., Wolff, R.A., et al., 2007. Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. J. Clin. Oncol. 25, 1960–1966.
Morrison, S.J., Kimble, J., 2006. Asymmetric and symmetric stem-cell divisions in development and cancer. Nature 441, 1068–1074.
Mueller, M.T., Hermann, P.C., Witthauer, J., Rubio-Viqueira, B., Leicht, S.F., Huber, S., Ellwart, J.W., Mustafa, M., Bartenstein, P., D'Haese, J.G., et al., 2009. Combined targeted treatment to eliminate tumorigenic cancer stem cells in human pancreatic cancer. Gastroenterology 137, 1102–1113.
Muller, A., Homey, B., Soto, H., Ge, N., Catron, D., Buchanan, J.W., McClanahan, T., Murphy, E., Yuan, W., Wagner, S.N., et al., 2001. Involvement of chemokine receptors in breast cancer metastasis. Nature 410, 50–56.
Nakata, B., Fukunaga, S., Noda, E., Amano, R., Yamada, N., Hirakawa, K., 2008. Chemokine receptor CCR7 expression correlates with lymph node metastasis in pancreatic cancer. Oncology 74, 69–75.
Nowell, P.C., 1976. The clonal evolution of tumor cell populations. Science 194, 23–28.
Park, C.H., Bergsagel, D.E., McCulloch, E.A., 1971. Mouse myeloma tumor stem cells: a primary cell culture assay. J. Natl. Cancer Inst. 46, 411–422.
Park, S.W., Davison, J.M., Rhee, J., Hruban, R.H., Maitra, A., Leach, S.D., 2008. Oncogenic KRAS induces progenitor cell expansion and malignant transformation in zebrafish exocrine pancreas. Gastroenterology 134, 2080–2090.
Pece, S., Tosoni, D., Confalonieri, S., Mazzarol, G., Vecchi, M., Ronzoni, S., Bernard, L., Viale, G., Pelicci, P.G., Di Fiore, P.P., 2010. Biological and molecular heterogeneity of breast cancers correlates with their cancer stem cell content. Cell 140, 62–73.
Philip, P.A., Mooney, M., Jaffe, D., Eckhardt, G., Moore, M., Meropol, N., Emens, L., O'Reilly, E., Korc, M., Ellis, L., et al., 2009. Consensus report of the national cancer institute clinical trials planning meeting on pancreas cancer treatment. J. Clin. Oncol. 27, 5660–5669.
Quintana, E., Shackleton, M., Sabel, M.S., Fullen, D.R., Johnson, T.M., Morrison, S.J., 2008. Efficient tumour formation by single human melanoma cells. Nature 456, 593–598.
Rasheed, Z.A., Yang, J., Wang, Q., Kowalski, J., Freed, I., Murter, C., Hong, S.M., Koorstra, J.B., Rajeshkumar, N.V., He, X., et al., 2010. Prognostic significance of tumorigenic cells with mesenchymal features in pancreatic adenocarcinoma. J. Natl. Cancer Inst 102, 340–351.
Reya, T., Morrison, S.J., Clarke, M.F., Weissman, I.L., 2001. Stem cells, cancer, and cancer stem cells. Nature 414, 105–111.
Ricci-Vitiani, L., Lombardi, D.G., Pilozzi, E., Biffoni, M., Todaro, M., Peschle, C., De Maria, R., 2007. Identification and expansion of human colon-cancer-initiating cells. Nature 445, 111–115.
Rosenberg, L., 1997. Treatment of pancreatic cancer. Promises and problems of tamoxifen, somatostatin analogs, and gemcitabine. Int. J. Pancreatol. 22, 81–93.
Rothenberg, M.L., Moore, M.J., Cripps, M.C., Andersen, J.S., Portenoy, R.K., Burris 3rd, H.A., Green, M.R., Tarassoff, P.G., Brown, T.D., Casper, E.S., et al., 1996. A phase II trial of

gemcitabine in patients with 5-FU-refractory pancreas cancer. Ann. Oncol. 7, 347–353.
Shimizu, K., Itoh, T., Shimizu, M., Ku, Y., Hori, Y., 2009. CD133 expression pattern distinguishes intraductal papillary mucinous neoplasms from ductal adenocarcinomas of the pancreas. Pancreas 38, e207–214.
Shmelkov, S., Butler, J., Hooper, A., Hormigo, A., Kushner, J., Milde, T., St. Clair, R., Baljevic, M., White, I., Jin, D., et al., 2008. CD133 expression is not restricted to stem cells, and both CD133+and CD133− metastatic colon cancer cells initiate tumors. J. Clin. Invest. 14.
Singh, S.K., Hawkins, C., Clarke, I.D., Squire, J.A., Bayani, J., Hide, T., Henkelman, R.M., Cusimano, M.D., Dirks, P.B., 2004. Identification of human brain tumour initiating cells. Nature 432, 396–401.
Tian, H., Callahan, C.A., DuPree, K.J., Darbonne, W.C., Ahn, C.P., Scales, S.J., de Sauvage, F.J., 2009. Hedgehog signaling is restricted to the stromal compartment during pancreatic carcinogenesis. Proc. Natl. Acad. Sci. U.S.A. 106, 4254–4259.
Visvader, J.E., Lindeman, G.J., 2008. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. Nat. Rev. Cancer 8, 755–768.
Warshaw, A.L., Fernandez-del Castillo, C., 1992. Pancreatic carcinoma. N. Engl. J. Med. 326, 455–465.
Wellner, U., Schubert, J., Burk, U.C., Schmalhofer, O., Zhu, F., Sonntag, A., Waldvogel, B., Vannier, C., Darling, D., zur Hausen, A., et al., 2009. The EMT-activator ZEB1 promotes tumorigenicity by repressing stemness-inhibiting microRNAs. Nat. Cell Biol. 11, 1487–1495.
Wicha, M.S., 2006. Cancer stem cells: an old idea – a paradigm shift. Cancer Res. 66, 1883–1890 (discussion 1895–1886).
Wilson, A., Laurenti, E., Oser, G., van der Wath, R.C., Blanco-Bose, W., Jaworski, M., Offner, S., Dunant, C.F., Eshkind, L., Bockamp, E., et al., 2008. Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. Cell 135, 1118–1129.
Yauch, R.L., Gould, S.E., Scales, S.J., Tang, T., Tian, H., Ahn, C.P., Marshall, D., Fu, L., Januario, T., Kallop, D., et al., 2008. A paracrine requirement for hedgehog signalling in cancer. Nature 455, 406–410.
Yilmaz, Ö., Valdez, R., Theisen, B., Guo, W., Ferguson, D., Wu, H., Morrison, S., 2006. Pten dependence distinguishes haematopoietic stem cells from leukaemia-initiating cells. Nature 441, 475–482.
Zhou, J., Wulfkuhle, J., Zhang, H., Gu, P., Yang, Y., Deng, J., Margolick, J.B., Liotta, L.A., Petricoin 3rd, E., Zhang, Y., 2007. Activation of the PTEN/mTOR/STAT3 pathway in breast cancer stem-like cells is required for viability and maintenance. Proc. Natl. Acad. Sci. U.S.A. 104, 16158–16163.