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ILEANA BORTOLOMAI
Degree in Biology

IDENTIFICATION AND CHARACTERIZATION OF TUMOUR INITIATING CELLS FROM GYNAECOLOGICAL MALIGNANCIES

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ABSTRACT

In the first part of the study, I developed a model to study the CSC/TIC population of the A431 cell line based on the capability of these cells to form spheres in suspension.

Putative A431 CSC/TIC were characterized for 'stemness' properties such as self-renewal and clone forming capability, presence of a SP and ALDH enzymatic activity, putative stem cell marker expression and *in vivo* tumorigenicity. The results indicate that the growth of A431 cells, as spheres, was not sufficient by itself to define a stem like population, but it was essential for the emergence of a small population of tumour cells with CSC properties.

Then, I investigated CSC/TIC in Epithelial Ovarian Cancer (EOC), with a focus on CD133 and CXCR4 molecules.

In a wide range of primary EOC and ascites the expression of CD133 and CXCR4 was found restricted within two separate subpopulations of tumour cells.

Enrichment in mRNA levels of *OCT4*, *NANOG* and *NESTIN* was observed with good reproducibility in CXCR4 positive cells, whereas it was more variable in CD133 positive cells. Real Time analyses of ABC transporter expression showed there was a slight increase in CXCR4 positive cells while in CD133 positive cells this increase was much more evident, revealing a clear difference between CXCR4 and CD133 subpopulations in term of chemoresistance potential.

Furthermore positive cells isolated from xenograft-derived ascites and sorted for CXCR4 and CD133 were not characterized by higher tumorigenic capacity.

In summary, no correlation between CD133 positivity and the ovarian cancer stemness phenotype was found. However detection of CD133 positive cells may be useful to predict the efficacy of specific cytotoxic therapy.

On the contrary, CXCR4 positive cells may identify those tumour cells which maintain a partially un-differentiated state (high levels of stem cell marker expression) and possibly responsible for tumour invasion and metastasis.

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CHAPTER 1

INTRODUCTION

Normal and cancer stem cells

"The simplest view appears to me undoubtedly to be that in an early stage of embryonic development more cells are produced than are required for building up the part concerned, so that there remains inappropriate a quantity of cells, it may be very few in number, which, owing to their embryonic character, are endowed with a marked capacity for proliferation... the real cause of the subsequent tumour is to be sought in a fault or irregularity of the embryonic rudiment."

JULIUS COHNHEIM, 1889

1.1 Stem cells in the life of an organism.

Embryonal, germinal and somatic stem cells

There are three kinds of stem cells: embryonal, germinal, and somatic or adult stem cells (6). Embryonal stem cells (ES) derive from the first five or six divisions of the fertilized egg. The progeny of embryonal stem cells are the precursors for all of the cells in the adult organs. The unicellular zygote is recognized to be the first stem cell in a human life and is identified as being totipotent, due to its ability to generate an entire organism (7). After about four days these totipotent cells begin to specialize, forming a "structure of cells" called the *morula*. The first recognized differentiation event in development occurs at the late *morula* stage, when the outer cell layer of the embryo adopts epithelial features and initiates the formation of blastocysts that contains two cell types: trophectoderm and inner cell mass (ICM) (8) (FIG. 1.1). The trophectoderm gives rise to the trophoblast, while the ICM undergoes a second differentiative step to form the epiblast and the endoderm, thereby progressing toward the blastocyst stage (9). The epiblast or primitive ectoderm further gives rise to the embryo, while the primitive endoderm develops into the extraembryonic endoderm, which provides nutrient and developmental cues to the embryo and contributes, together with the trophectoderm, to the development of the yolk sack and placenta.

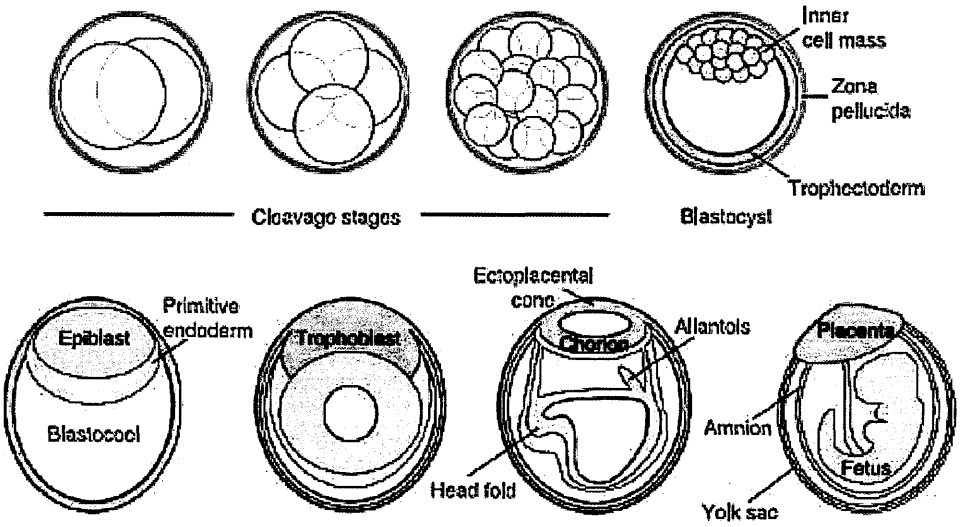


Figure 1.1: Differentiation event during embryo development. Figure from (1)

At the blastocyst stage ICM stem cells are no longer totipotent but are recognized as being pluripotent (10) (1). This partial commitment in combination with the similarities observed between mouse and human ES cells suggest that human ES cells are actually equivalent to the early post-implantation epiblast, rather than its ICM progenitor (11). During further embryonic development, a gradient of decreased regenerative potential is produced and distributed in specific stem cell compartments in a developing embryo. This decrease in regenerative capability leads to a subsequent loss of totipotency but a concurrent retention of pluripotency by stem cell in the embryo, in a poorly understood process called determination. The next recognized landmark during embryonic differentiation is gastrulation, when the epiblast is transformed into the three germ layers of the embryo (ectoderm, mesoderm, and endoderm) and the basic body plan of the animal is established (12). The remaining events of embryogenesis, a major part of

which is organogenesis, rely of the functioning of localized stem cells that together are committed to generate specific organs in response to surrounding microenvironment or niche (13) (14) (15) (16) (FIG. 1.2).

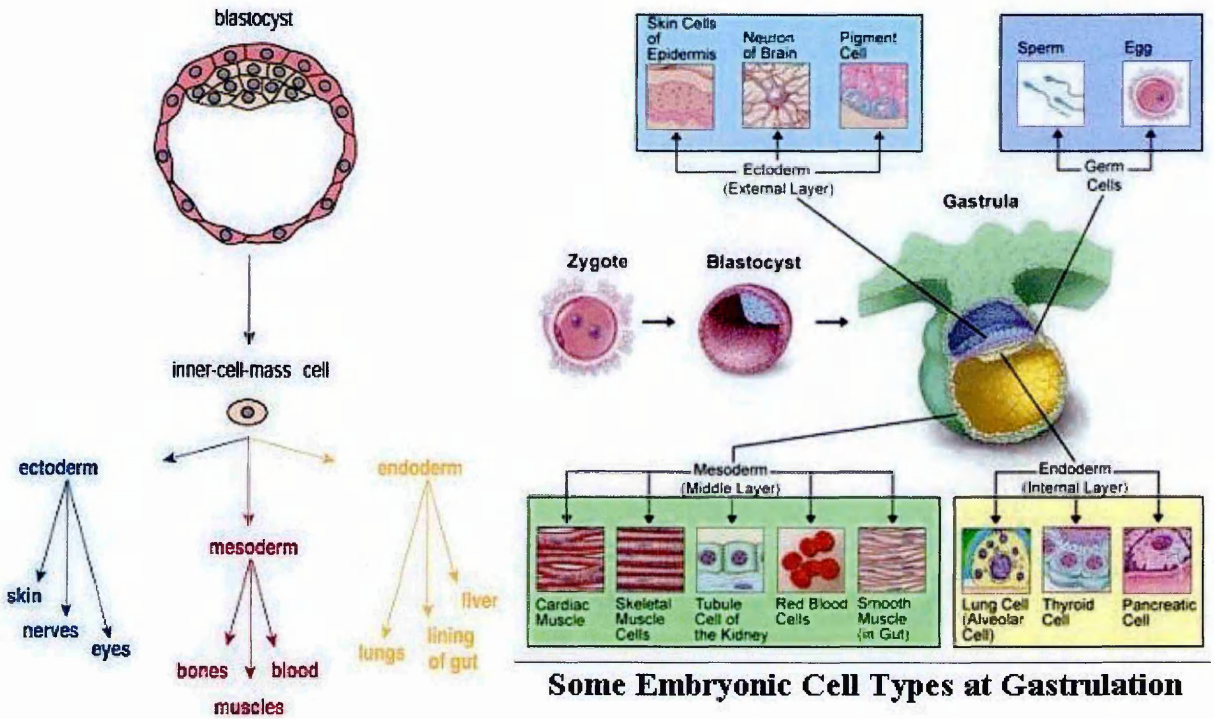


Figure 1.2: Differentiation of human tissues (Image from www.ncbi.nlm.nih.gov)

The formation of germ cells during embryogenesis is of crucial importance for the maintenance of every species. Development of germ cells begins with the specification of the primordial germ cells. Primordial germ cell (PGC) specification or formation marks the initiation of the life cycle of the germ cell lineage in all species. The multipotent germ cells, derived from PGC, have been identified in neonatal gonads, testes, and ovaries in mouse and human (17) (18).

Germ cells in the male enter into mitotic arrest and are reactivated to initiate spermatogenesis after birth (6). Spermatogenesis continues during

adult life in most males, but whether these stem cells self-renew or differentiate is heavily influenced by surrounding somatic cells, in a microenvironment often referred to as a stem cell niche. The continuation of the spermatogenic process throughout life relies on the proper regulation of self-renewal and differentiation of the spermatogonial stem cells.

After birth, male germline stem cells develop into spermatogonial stem cells (SSCs). They can self-renew and generate a large number of differentiated germ cells. These cells could be reprogrammed to embryonic stem cell-like cells and can spontaneously differentiate into derivates of all three germ layers *in vitro* (19). These lines of evidence clearly suggest the pluripotency of germ cells in all stages of development.

Although males retain germline stem cells (GSCs) for spermatogenesis throughout adult life, oocytes production in females of most mammalian species is believed to cease before birth (17) (20). A central dogma of mammalian reproductive biology is that females are born with a finite, non-renewing pool of germ cells, all of which are arrested in meiosis I (oocytes) and are enclosed by somatic cells in structures referred to as follicles (17). Oocyte numbers decline throughout postnatal life (20) through mechanisms involving apoptosis (21), eventually leaving the ovaries devoid of germ cells. In humans, exhaustion of the oocyte reserve occurs around the fifth decade of life, driving the menopause (22). The process that is believed to occur in female mammals with respect to germ-cell development differs from that of several invertebrate organisms, including *Drosophila Melanogaster*, in which GSCs maintain oocyte production in adult ovaries

(23) Interestingly, a new line of research has recently established the existence of proliferative germ cells that sustain oocyte and follicle production in the postnatal mammalian ovary (24) (25).

While diversification of cell types is largely complete at or shortly after birth, many tissues in the adult undergo self renewal and accordingly must establish a life-long population of relatively pliable stem cells. It is generally accepted that adult tissues contain tissue-determined stem cells or somatic stem cells responsible for normal tissue renewal (26) (24) (27). The effective maintenance of a population of healthy stem cells within a particular tissue involves the concurrent operation of multiple genetic and epigenetic factors, along with stringent controls within each niche, that create perfect harmony among the different cells of various organ system (28). It has become apparent that adult stem cells not only reside and function in highly regenerative tissues like the bone marrow, intestine and epidermis, but are also found in tissues of low cell turnover, such as neural, liver, prostate and pancreas.

In adult tissues, somatic stem cells function is the maintenance of tissue homeostasis by replenishing functional tissue cells lost by apoptosis (29) (30). Following injury, normally quiescent adult stem cells undergo cell division producing transit amplifying (TA) cells that rapidly proliferate to repair the lost tissue with sufficient numbers of functional differentiated cells. In some cases, such as liver and pancreas, fully mature cells have the capacity to revert to a proliferative phenotype to effect tissue replacement in a poorly understood process (31). It appears that mature cells may de-

differentiate into stem-like cells with a concomitant change in transcriptional profile or fully mature cells have latent stem cell capacities (32).

1.2 Possible origins of cancer stem cells

1.2.1 Concept of cancer stem cells

The term cancer stem cells (CSCs) is used to indicate a tumour-initiating cell subset that can give rise to a heterogeneous progeny, similar in composition to the tissue from which it was originally isolated.

Like their normal tissue counterparts, tumours are composed of heterogeneous populations of cells that vary in their apparent state of differentiation. This observation suggests that tumours are not a simple monoclonal expansion of cells, but might be similar to abnormal organs sustained by a mutated "cancer stem cell" population, which is endowed with the ability to self-renew and undergo aberrant differentiation (33). This hypothesis is further emphasized by the fact that cancer is known to result from the accumulation of multiple genetic mutations in a single target cell, sometimes over a period of many years (34). Because stem cells are the only long-lived cells in many organs, they are the natural candidates in which early transforming mutations may accumulate.

How CSCs arise in tissue and progress to give rise to a new organ / tumour is a hot field of investigation. Research over the last decade has tried to associate cellular mechanisms with mutagenic effects leading to the emergence of CSCs. The possibilities that emerge include: transformation of normal stem cells, or transformation of a local pool of early progenitors that re-acquire self renewal properties or a series of effective mutations that render committed transient-amplifying (TA) progenitor immortal (de-

differentiation) (35) and, finally, the possible fusion of circulating bone marrow derived stem cells with tissue residing cells (36).

The relative abundance of TA progenitor cells makes them likely candidates for initial transforming events. Several lines of evidence support the concept that a committed progenitor can be the cancer-initiating cell as a result of oncogenic transformation (37).

Similarly the preservation of the expression of cell surface markers between normal and cancer stem cells suggests that normal tissue stem cells may be the targets of oncogenic transformation (37).

Although no direct experimental evidence is currently available for the cell fusion origin of CSCs, cell fusion has been shown to be one of the mechanisms for the apparent cellular plasticity associated with tissue stem cells (38). Conceptually, cell fusion between stem cells and mutated cells might lead to regaining of self-renewal capacity to allow further accumulation of transforming mutations (39).

Future studies are necessary to provide definitive evidence for identifying the origin of CSCs. It is also important to remember that the possible origins for CSCs are not mutually exclusive; demonstrating one model for the formation of CSCs in a given system does not necessarily exclude other mechanisms (FIG. 1.3).

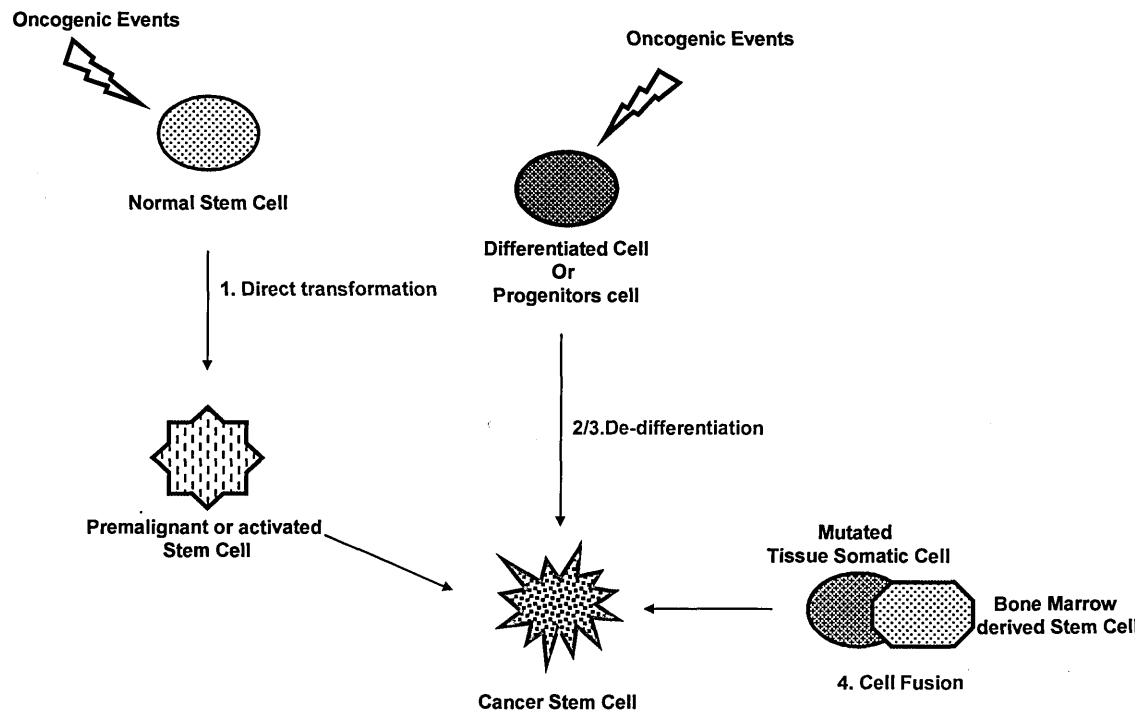


Figure 1.3: Possible origin of CSCs

The observations that classically define the existence of a CSC population are that: 1) only a minority of cancer cells within each tumour are usually equipped with tumorigenic potential when transplanted into immunodeficient mice; 2) although tumours originate from a single transformed cell, the cancer cells within tumours may also display different phenotypes, somewhat reminiscent of the normal tissue from which they originate (40) and, finally, 3) tumours derived from tumorigenic cells contain mixed populations of tumorigenic and non-tumorigenic cancer cells, recreating the phenotypic heterogeneity of the parental tumour.

In conclusion, cancer can be thought of as a disease resulting from abnormal growth and from chronic activation of stem cells, leading to the long term proliferation of these cells. The abnormally dividing stem cell

could be subject to additional genetic events (repression of tumour suppressor genes and activation of oncogenes), leading to autonomous growth, the loss of cell cycle regulation and resistance to apoptosis, all well understood properties of cancer cells (33) (41) (FIG. 1.3).

1.2.2 Cancer stem cell theory

Although monoclonal in origin, most tumours appear to contain a heterogeneous population of cancer cells. This observation is traditionally explained by postulating variations in tumour microenvironment and coexistence of multiple genetic subclones, created by progressive and divergent accumulation of independent somatic mutations.

Two general models of heterogeneity in solid cancer cells have been assumed (FIG. 1.4). The stochastic model supposes that each population of cells within a heterogeneous tumour has an equal but extremely low tumorigenic potential (42) (FIG. 1.4a). In this regard, tumour progression is a constant process based on the positive selection of genetically unstable clones that guarantees a survival advantage on a tumour within its surrounding microenvironment. The stochastic model also accounts for the emergence of drug resistance during chemotherapy through selection of cells with genotypes that allow survival from the drug insult (43).

The fact that from organ specific stem cells derived all the differentiated cell of a given organ has led the proposal of a stem cell hierarchical model for tissue development, maintenance and repair. Deriving from this, is the hierarchical cancer stem cell model (FIG. 1.4b), which proposes that the tumorigenic potential of tumours is limited to a very small clonogenic population of cells, the CSCs (1), whereas the large population of cancer cells, descendants of CSCs, do not have self renewal capacity and are organized in the form of hierarchy. This model postulates that not all cells

in a tumour are equal and the tumorigenic cells are a rare subset with a distinct phenotype.

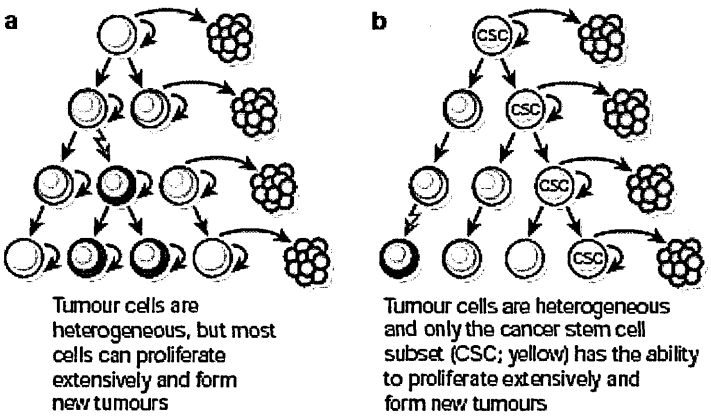


Figure 1.4: image from (2)

It was first fully documented for leukaemia and multiple myeloma that only a small subset of cancer cells is capable of extensive proliferation (44) (45). It has also been shown for solid cancers that the cancer cells are phenotypically heterogeneous and that only a small proportion of cells are clonogenic in culture and *in vivo* (46) (47) (48).

Existing therapeutic approaches have been mainly based on the stochastic model, but the failure of these therapies to cure most solid cancers suggests that the hierarchical CSCs model may be more accurate.

The large number of tumour types with a subpopulation of exclusively tumorigenic and therapy resistant cells suggests that, despite the unanswered questions, the CSC hypothesis has a rightful role to play in tumour biology. At the same time, experimental evidence supporting the established alternative theory of clonal evolution can be found as well.

Therefore, a model that describes cancer initiation and progression should combine elements of clonal evolution and CSC theory.

1.2.3 Rethinking the concept of CSCs

The theories proposed by Campbell and Polyak (49) and Adams and Strasser (50) have tried to combine the competing models of clonal evolution and CSCs. According to these, evidence can be found for both models and their prevalence is probably unique to every tumour and may essentially change as the tumour progresses. Although CSCs seem to be a special subset of cancer cells, recent studies show that CSCs themselves are still a heterogeneous population with different biological properties and that multiple populations with CSC characteristics can coexist in the same tumour. Ma et al. investigated hepatocellular carcinoma (HCC) cell lines and were able to separate subpopulations with different tumorigenic potential based on CD133 and ALDH expression (51), which however contradicts the original CSC hypothesis of just one population with tumour forming capabilities. Herman et al found that CD133+ CXCR4− and CD133+ CXCR4+ pancreatic cancer cells do not differ in tumorigenicity, but only the CD133+ CXCR4+ population migrates and metastasizes (52). The CSC hypothesis explains this heterogeneity with the existence of cancer progenitor cells, which still possess some residual stem cell traits. Whether the heterogeneity of the CSC population is caused by clonal evolution or partial differentiation of the cancer initiating cell is difficult to prove.

It is also becoming clear that several properties, which we thought were intrinsic to CSCs, are modulated by the microenvironment of the cancer cells (53) (54) and such key traits as metastasizing and growth (39) may depend on the normal stromal cells that interact with the cancer cells (55) (Table 1.A).

Table 1.A: Evidences supporting and contradicting the stem Cells and Cancer Stem hypothesis (adapted (5))

Experimental evidence	
Supporting the CSC hypothesis	Contracdicting the CSC hypothesis
Large number of cells are needed for xenotransplantation of tumours	In congenic transplantations substantially fewer cells are needed
The required number of putative CSC for xenotransplantation of tumours is relatively small Xenograft tumours can be serially transplanted, but only with the CSC subpopulation NON-CSC populations do not initiate tumour growth in vivo, or require more cells than the CSC population to do so	Transplantability of malignancies is not restricted to one subpopulation in congenic transplantations
A small fraction of tumour cells are capable of sustained growth under stem cell culturing conditions	CSC markers do not identify a pure CSC population
CSCs have higher clonogenicity in vitro	The CSC population is heterogenous in itself, with differences in metastatic and tumorigenic potential
Cultured CSCs can give rise to progeny with non-CSC phenotypes	
CSCs have intrinsic in vitro and in vivo therapt resistance	

1.3 Stem cells and cancer stem cell properties: similarities and variations

To understand the biology of cancer stem cells, we need to define the unique properties of normal stem cells. Adult stem cells play pivotal roles in mammalian tissue and are identified through three distinctive properties:

- SELF-RENEWAL: the ability to undergo division and form new cells with a potential identical to the mother cell (56).

- DIFFERENTIATION: the ability to give rise to a heterogeneous population of cells, arranged in a hierarchical manner, includes various tissue-specific lineages, thereby building up the requisite critical mass toward replenishing the tissue of short-lived, differentiated cells (57).

- HOMEOSTASIS: the ability to regulate and balance differentiation and self-renewal in the tissue or organ (58).

This unique combination of properties imparts to stem cells a continuing role during the entire life of an organism and ensures that all developed tissues harbour stem cells. The regulation of stem cells in an adult is tightly controlled, to allow for the growth replenishment of tissue and to permit their repair after damage occurred. The critical balance of self renewal and differentiation is achieved by specific gene expression programs of stem cells, which regulate activation and/or inactivation of mechanism allowing the maintenance of the pluripotent state or permitting differentiation into

more specialized states. A disruption in such homeostatic plan can lead to the abnormal state of cancer (59).

In normal tissue three different compartments can be described: 1) a self renewal compartment with quiescent stem cells. These "reserve" stem cells are very few in number as most of the cellular renewal is accomplished by tissue determined transit-amplifying cells. 2) A proliferative compartment with proliferating TA cells with a limited self-renewal potential and 3) a terminal compartment with differentiated cells or apoptotic cells (30).

Diverse poorly-differentiated adult stem/progenitor cell types, which have generally a small size relative to the terminally differentiated cells and express specific stemness markers have recently been identified in the most mammalian tissues/organs (14) (60) (61) (62). Among the tissues and organs harboring a very small number of specific multipotent and undifferentiated adult stem/progenitor cells, there are BM, vascular walls, adipose tissues, skeletal muscles, heart and brain as well as epithelium of lung, liver, pancreas, digestive tract, skin, retina, breast, ovaries, prostate and testis (14) (60) (61) (62). All of the multipotent or bipotent adult stem/progenitor cell types display a long-term self-renewing capacity and can give rise to all of mature and specialized cell types of distinct lineages in the tissues/organs from which they originate. Despite certain adult stem/progenitor cells found in BM, skin and gastrointestinal tract usually show a rapid turnover to replenish the cell loss along lifespan, other adult stem/progenitor cell types remain under a quiescent state and rarely divide

in normal conditions, and undergo only a sustained proliferation after intense tissue injuries. Prevailing models assume the existence of a single quiescent population of stem cells residing in a specialized niche of a given tissue. Emerging evidence indicates that both quiescent (out of cell cycle and in a lower metabolic state) and active (in cell cycle and not able to retain DNA labels) stem cell subpopulations may coexist in several tissues, in separate yet adjoining locations (63) (64) (65). In the zoned stem cell model, active stem cells are the primed subpopulation that account for most of the replenishment of corresponding tissues, whereas quiescent stem cells function as a backup or reserve subpopulation (65) (66). This reserve population can be activated either by a stochastic mechanism (65) or by feedback upon loss of active stem cells or extensive tissue damage (66). The combination of these reciprocal backup systems would provide a robust mechanism to ensure a high rate of physiological self-renewal as well as flexible damage repair, after which the original hierarchy could be re-established.

1.3.1 Self Renewal

The key properties of stem cells , indefinite self-renewal and multilineage potential, were first discovered in the bone marrow and described experimentally in the 1961 (67).

Self-renewal is the process by which a stem cell divides asymmetrically or symmetrically to generate one or two daughter stem cells that have a developmental potential similar to the mother cell (68). The ability to self-

renew is essential for stem cells to expand their numbers during development, to be maintained within adult tissues, and to restore the stem cell pool after injury (69) (70) (71). Self-renewal is not the same as proliferation, although both processes depend on cell division. Proliferation is a more general term that incorporates all types of stem and progenitor cell divisions, self-renewing and otherwise. Most stem cells can divide by either asymmetric or symmetric modes of division, and the balance between these two processes is controlled by developmental and environmental signals in order to produce appropriate numbers of stem cells and differentiated daughters (72) (FIG. 1.5).

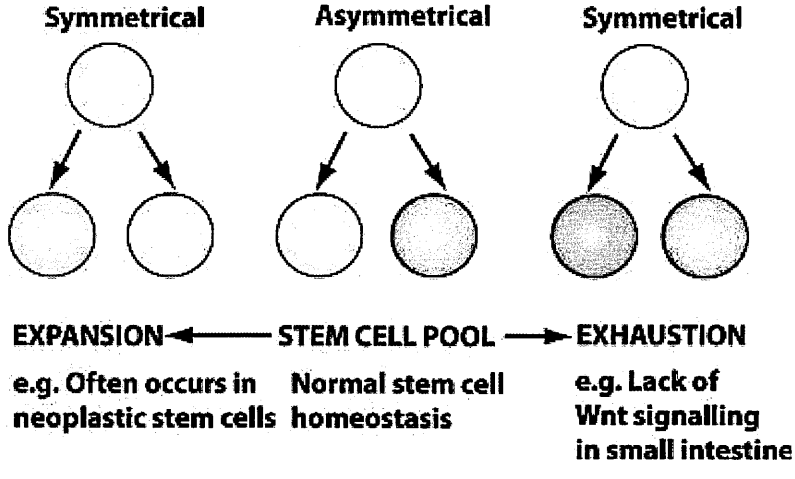


Figure 1.5: image from (3)

Symmetric stem cell division offers a mechanism to increase the stem cell population mostly during development and body formation, and it was secondarily important during tissue regeneration, where asymmetric division is generally preferred. Symmetric stem-cell divisions are common in developing tissues, but they can also be observed in adults, as

exemplified by the adult *Drosophila* ovary. Adult *Drosophila* germline stem cells normally divide asymmetrically (73); however, it seems that adult *Drosophila* germline stem cells are regulated to divide asymmetrically or symmetrically, depending on their specific location within the niche (74) (75).

As in *Drosophila*, also some mammalian stem cells seem to switch between symmetric and asymmetric cell divisions. For example, both neural and epidermal progenitors change from primarily symmetric divisions that expand stem-cell pools during embryonic development to primarily asymmetric divisions that increases differentiated cell numbers in mid to late gestation (76) (77) (78) (79). Experimental evidence indicates that at least some adult stem cells divide asymmetrically under steady-state conditions to maintain population size. Nevertheless they also retain the capacity to divide symmetrically to restore stem-cell pools depleted by injury or disease, as has been observed in the nervous and haematopoietic systems (80) (81) (82) (83).

The role of asymmetric cell division in stem-cell control, coupled with the mechanisms that regulate this process, is fundamental for generating diversity in multicellular organisms (84) (85). Two main types of mechanism govern asymmetric cell divisions. The first, called "intrinsic", relies on the asymmetric partitioning of cell components that determine cell fate; the second, indicated as "extrinsic", involves the asymmetric placement of daughter cells relative to external cues (3) (FIG. 1.6). Intrinsic

mechanisms include regulated assembly of cell polarity factors and regulated segregation of cell fate determinants.

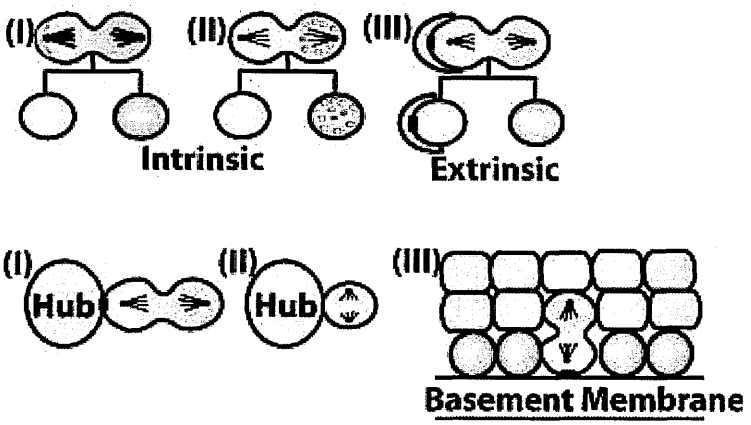


Figure 1.6: image from (3)

A classic example of an asymmetric division that is controlled by an extrinsic mechanism is provided by the *Drosophila* germline stem cell, which divides with a reproducible orientation to generate one daughter that remains in the stem-cell niche and retains stem-cell identity, and one daughter that is placed away from the niche and begins to differentiate (85) (3) (86) (87) (FIG. 1.4). In conclusion it is important to note that asymmetric divisions can be governed by both intrinsic partitioning of fate regulators and asymmetric exposure to extrinsic cues (88) (89) (90) (62) (91).

1.3.2 Multilineage potential and control of stem cell differentiation

The differentiation potential of a stem cell is defined by all the types of differentiated progeny it can finally generate. There are different possibilities explaining how multipotent stem cells can give rise to a repertoire of progenies; one possibility is that multipotent stem cells might express a set of transcriptional factors which separately specify different lineages or combinations of lineages, such as Ikaros for lymphoid lineages (92). Theoretically, the entire developmental potential of a given multipotent stem cell could also be determined by a single specific factor, which generates a regulatory and hierarchical mechanism of differentiation. An example is the forkhead transcriptional regulator FOXL2, which is required to prevent trans-differentiation of an adult ovary to a testis (93). Competence and multilineage potential may also be specified by expression of signal transduction molecules. In several cases the expression of specific receptors seems necessary to respond to fate-determining signal, leading to activation of specific differentiation pathways (94).

The differentiation of stem cells involves both the exit from the uncommitted state and entry into a particular developmental pathway. It is not yet discovered whether exit from the stem cell state and initiation of differentiation are independently controlled in mammals. From one point of view, differentiation might be an obligatory option executed by a stem cell after its removal from the niche. On the other extreme, internal and external signals might promote differentiation and, as consequence, the exit

from the stem cell state. In the Central Nervous System (CNS) both mechanisms act separately, depending on the presence or the absence of basic Fibroblast Growth Factor (bFGF). *In vitro*, bFGF stimulates self renewal of CNS stem cells, but its withdrawal rapidly promotes their differentiation (95).

In mammalian system, there is considerable evidence that growth factors and cell-cell interactions can influence the outcome of fate decision by multipotent progenitors. A single stem cell could be influenced by growth factors in a selective or instructive manner. In a selective mechanism, the stem cells differentiate into a particular lineage independently of the growth factors, which subsequently control the survival and the proliferation of the progenitors. For example, forced expression of bcl-2 in immortalized hematopoietic progenitor cells leads to differentiation in the absence of cytokines, underling that these growth factors are dispensable for their differentiation. Conversely in the neural crest, the expression of BMP2, bFGF and TGFß promotes the differentiation toward the three cell types of the CNS (95).

Understanding the interplay between extracellular and intracellular regulatory factors in controlling lineage determination remains an important challenge for the future.

1.3.3 Relationship between normal and cancer stem cells: CSC plasticity as a stem cell dysfunction

CSCs share many characteristics in common with normal stem cells, including self-renewal and differentiation. With the growing evidence that cancer stem cells exist in a wide array of tumours, it is becoming increasingly important to understand the molecular mechanisms that regulate self-renewal and differentiation because corruption of genes involved in these pathways likely participates in tumour growth. Studies of normal and cancer stem cells from the same tissue have shed light on the ontogeny of tumours. Understanding the biology of cancer stem cells will contribute to the identification of molecular targets important for future therapies.

Many observations suggest that analogies between normal stem cells and cancer cells may be appropriate. Both normal stem cells and tumorigenic cells have extensive proliferative potential and the ability to give rise to new normal or abnormal tissues. Both tumours and normal tissues are composed of heterogeneous combinations of cells, with different phenotypic characteristics and different proliferative potentials (96). Because most tumours have a clonal origin (97), tumorigenic cancer cells must give rise to phenotypically diverse progeny, including cancer cells with indefinite proliferative potential. Although some of the heterogeneity in tumours arises as a result of continuing mutagenesis, it is likely that heterogeneity also arises through the aberrant differentiation of cancer stem cells. In other words, both normal and tumorigenic stem cells give rise

to phenotypically heterogeneous cells that exhibit various degrees of differentiation (62).

In contrast to normal stem cells, it has been proved that CSCs undergo genomic alterations that allow them to escape cell cycle regulation and achieve growth factor and anchorage independence proliferation and resistance to apoptosis, besides contributing to dysregulation of self renewal and expansion (2). The acquisition of each of these characteristics is complementary to the others and requires a suitable microenvironment in which the transformed stem cells are believed to proliferate and differentiate into an entire tumour (41). The plasticity gained by CSCs is regulated by a cooperative effect of cell intrinsic (autocrine) factors, which may either involve changes in DNA sequences of genes or gene silencing through methylation or altered chromatin architecture (genetic and epigenetic effects), together with cell extrinsic (paracrine or derived from tumour microenvironment) factors (98). Plasticity makes also possible for differentiated cells to acquire cancer stem cell properties in the presence of the appropriate oncogenic insults. Thereby, proliferating proto-oncogenic stem cells appear to require at least one additional permanent genetic mutation to drive them along a trajectory toward transformation (99) (100). This could be achieved either through oncogene activation or by silencing of tumour suppressor genes, which effectively supplements the perturbed shift toward self renewal; continuing mutagenesis would further ensure clone amplification and disease progression (91).

Another common feature of tumour and tissue stem cells is the utilization of similar signal pathways that normally control cell fate (101) (102). Such regulatory signal molecules, including components of the Notch, Wnt and Hedgehog pathways, have been shown to play roles in controlling stem cell self renewal and in regulating lineage fate in different systems. In numerous tumours, however, the signalling cascades initiated by these molecules have recently been demonstrated to be dysregulated. In the skin, liver, colorectal and pancreatic cancers, for example, Wnt signalling has been demonstrated to be aberrantly activated (103) (104). In ovarian cancer, the Wnt signal transducer ß-catenin is overexpressed at an advanced stage of tumour progression (105). The Hedgehog cascade, a regulator of patterning during embryonic development, has been shown to be associated with breast (106), ovarian (107) and prostatic cancers (107), whereas Notch over-stimulation has been strongly implicated in T cell malignancies (108). An important difference in the signal between normal and transformed states is that those in normal tissue are transiently expressed as stem cell activating signals, whereas in cancer these signals dominate and lead to a state of long term or permanent activation (106) (107) (Table 1.B).

Table 1.B: Summary of the common properties between normal and cancer stem cells

CHARACTERISTICS SHARED BY NORMAL AND CANCER STEM CELLS
• Capacity for asymmetric division (self renewal), which gives rise to a quiescent stem cells and a committed progenitor and contributes toward developing a critical mass of cells
• Regulation of self renewal by similar signalling pathways (Wnt, Sonic Hedgehog and Notch) and at the epigenetic level by Polycomb genes (BMI-1)
• Expression of factors such as Oct4, Nanog and Sox2, which maintain a functional plasticity by promoting pluripotency and immortality
• Extend telomeres and telomerase activity that increases the cellular life span
• Expression of ABC transporters, contributing to cellular resistance against specific growth-inhibitory drugs
• Expression of similar surface receptors (e.g. CXCR4, CD133 CD117, CD44) that are either identified as stem cell markers or associated with homing and metastasis.
• Predisposition for growth factors independence and stimulation of angiogenesis through secretion of growth factors, cytokines and angiopoietic factors.

1.4 Therapeutic implication of cancer stem cells: chemoresistance and radioresistance

1.4.1 Genome integrity and cytoprotective strategies

As the longest-lived, mitotically active cells in the body, stem cells are particularly sensitive to the accumulation of genetic lesions. Preserving genomic integrity is important for maintaining normal function as well as preventing carcinogenesis. This is more important in stem cells than in other cells because stem cells pass mutations on to large numbers of progeny, amplifying the risk of cancer. Many mechanisms protect stem cells from endogenous and exogenous mutagens or enhance their capacity to repair the damage that occurs. In cases where the damage is too extensive to be repaired, stem cells may undergo apoptosis or senescence. The long-term self-renewal potential of tissue stem cells depends on mechanisms that maintain their genomic integrity, such as those involved in ROS detoxification, DNA damage repair and telomere maintenance.

Reactive oxygen species (ROS) are toxic products of oxidative metabolism. Although important for certain physiological processes such as intracellular signal transduction and combating pathogens, excessive levels of ROS within cells can damage lipids, proteins, RNA, and DNA, thus impairing cellular function.

Stem cells reside in their niches where the oxygen tension is thought to be extremely low (1-4%) (109) (110). These hypoxic environments favourably

support the undifferentiated stem cells to perform anaerobic metabolism. A metabolic shift from anaerobic glycolysis to mitochondrial respiration, for a more efficient production of ATP, is required for the higher energy demand of cells undergoing differentiation. At the same time, the coordinated up-regulation of antioxidant enzymes ensures a proper redox environment for differentiating cells to prevent excess ROS and oxidative stress resulting from the exuberant oxidative phosphorylation.

FoxO family transcription factors are tumour suppressors that protect stem cells and other cells from oxidative damage, reducing mutagenesis and extending cellular lifespan (111). FoxO transcription factors increase the expression of genes required for the detoxification of ROS including superoxide dismutase and catalase as well as genes that promote quiescence (112). Orciani and collaborators determined the susceptibility to oxidative stress of isolated mesenchymal stem cells from human skin (S-MSCs) in comparison with keratinocytes (113). Human keratinocytes seem to have much greater antioxidant defence to counteract the oxidative injury to which they are continuously exposed in the skin. The S-MSCs are surrounded by a complex microenvironment that protects them from external insults, and so they do not have a particularly efficient defence system, and they were generally less responsive to enhanced pro-oxidant challenge. As a matter of fact, S-MSCs seem particularly prone to apoptotic events, which might thus represent their primary defence mechanism (113).

The genomic DNA of normal cells is under continuous assault from intrinsic insults, such as oxidative stress, and extrinsic insults, such as ultraviolet (UV) light and ionizing radiation (IR). In dividing cells, the DNA may also suffer from the introduction of errors during the replication required for mitosis. Cells have therefore had to evolve mechanisms to maintain genomic stability. In response to DNA damage, a cell may trigger a checkpoint response that induces cell-cycle arrest and allows the cell time to repair the DNA damage before cell-cycle progression is resumed. Alternatively, if the DNA damage is too severe, the cell becomes senescent or undergoes apoptosis. A defect in a DNA damage checkpoint response can result in unchecked mutation and genomic instability.

The consequences of these processes for stem cells can be profound: diminution in stem cell pools, or an increased chance for stem cell differentiation or malignant transformation (114) (115). In normal stem cells, genomic stability is maintained through enhanced DNA damage recognition and repair, which provide a robust defence for the cell. Studies of gene expression in stem cell populations have established that a small number of genes in stem cell populations are consistently over-expressed. A surprising number of these are DNA repair genes, including those involved in mismatch repair (MMR) and non-homologous end joining (NHEJ) repair systems (116) (117).

Little is known about molecular mechanisms implicated in DNA damage response in somatic stem cells; however, a peculiar response to DNA damage could be observed in stem cells during the transition from

pluripotency to lineage commitment. It is likely that the DNA damage response is different depending on the specific stages along the transition from multipotent stem cells to terminally differentiated progenies. A more radical solution, such as elimination of repair defective cells by apoptotic mechanisms, appears to be adopted by stem cells at earlier stages to ensure the genomic stability of their progenies (118) (119) (120). Moreover essential players of the DNA damage-activated responses typical of proliferating cells, such as ATM, p53, ATR and Chk1, appear involved in the regulation of DNA damage response in stem cells during renewal and homeostasis (118).

Mechanisms involved in telomere maintenance are also crucial for genomic integrity and self renewal potential of stem cells. Mammalian telomeres are repetitive DNA sequences (thousands of TTAGGG repeats) along with specialized protein complexes that are located at the ends of chromosomes (121). Telomeres are required to protect chromosomes from fusing to each other, from exonuclease activity, and from the loss of coding sequences due to the end replication problem (121). In most somatic cells telomere length shortens with each cell division. In cells with a high capacity of self renewal, such as stem cells and cancer cells, routine telomere shortening is prevented by expression of the telomerase complex. In stem cells telomerase expression is necessary for maintaining self renewal potential, reducing the rate at which telomeres erode and increasing their replicative capacity (109) (122).

A general conclusion is that differentiation and apoptosis represent the major mechanisms adopted by different stem cell types to protect their genome integrity. If the damage does not impair the stem cell pool and the consequent mutations do not alter progenitor functions, stem cells lead to differentiation. In the case of extensive damage, apoptosis and senescence represent the only mechanisms avoiding the risk of mutated progeny, leading to cancer. In both cases a direct and extreme consequence of this is that unrepaired DNA lesions may cause stem cell exhaustion.

Many stem cells have acquired the ability to withstand cytotoxic insults through either efficient enzyme-based detoxification systems or by the ability to rapidly export potentially harmful xenobiotics. The ABC superfamily of membrane transporters is one of the largest protein classes known, and is characterized by expression of an ATP-binding cassette region functioning to hydrolyse ATP, supporting energy-dependent substrate exportation across membranes, principally from the intracellular cytoplasm to the extracellular space (123). The large superfamily of ABC transporters is portioned in smaller sub-families with functional differences. The ABCA sub-family members have been mostly related with lipid trafficking in a wide range of body locations, whereas ABCD, ABCE and ABCF have roles in very long chain fatty acid transport, initiation of gene transcription and protein translation, respectively (124) (125). In humans, the three major types of multidrug resistance (MDR) proteins include members of the ABCB (ABCB1/MDR1/P-glycoprotein), the ABCC

(ABCC1/MRP1, ABCC2/MRP2, probably also ABCC3-6, and ABCC10-11), and the ABCG (ABCG2/MXR/BCRP) subfamily. Although recognized substrates are mostly hydrophobic compounds, MDR pumps are also capable of extruding a variety of amphipathic anions and cations. ABCB1 preferentially extrudes large hydrophobic molecules, while ABCC1 and ABCG2 can transport both hydrophobic drugs and large anionic compounds (123) (126).

The expression of transport proteins and multidrug resistant proteins protect stem cells against toxins and are associated with the efflux of xenobiotic toxins, a low rate of cell division and active DNA repair. ABC transporters have emerged as an important field of investigation in the regulation of stem cell biology (69) and, significantly, ABCG2, an ABC transporter found in many stem cells, is up-regulated in hypoxic environments, such as the stem cell niches, mediated by HIF1-α (123) (69). ABCG2 has a unique function in stem cells and its role is to maintain progenitor cells in an undifferentiated state. This hypothesis was supported by the results of bone marrow transplantation studies (127). Transplantation of ABCG2-overexpressing bone marrow cells into lethally irradiated mice resulted in a lower number of mature hematopoietic cells. This finding was interpreted as an indication that ABCG2 causes the efflux of a substance important for differentiation. In any case, high ABCG2 expression in stem cells suggested that it has a functional role in stem cell survival and that it can be used as a tool for stem cell enrichment (128) (129).

ABC transporters are not the only cytoprotective molecules present in adult stem cells; the aldehyde dehydrogenase (ALDH) gene superfamily encodes detoxifying enzymes for many pharmaceuticals and environmental pollutants (123) (130) (131). Indeed, Aldehyde dehydrogenases are a group of NAD (P) +/- dependent enzymes involved in the metabolism of a wide variety of aliphatic and aromatic aldehydes. They serve to detoxify xenobiotic aldehydes (e.g. cyclophosphamide) and many other intracellular aldehydes, e.g. ethanol and vitamin A (132) (133) (134). In conjunction with other markers, high levels of ALDH activity have been shown to characterize highly clonogenic, undifferentiated multipotential stem/progenitor cells in both human bone marrow (135) and blood (136). High ALDH activity has also been used to detect putative stem/progenitor cells in a variety of solid organs (137) (138).

In normal human mammary epithelia, the ALDH+ population entirely contains the clonogenic cells that can generate both myoepithelial and luminal cells (137). This role of ALDH as stem cell marker has also been investigated in cancer tissue. A growing body of evidence indicates that ALDH activity represents a promising CSC marker (139) (140). Among ovarian epithelial tumours the role of ALDH1 as CSC marker is debated. Sun and collaborators showed that the isolation of ALDH positive cells had greatly enriched for clonogenic, tumorigenic, and metastatic cells in adenoid cystic carcinoma (141). Contrarily, in another paper ALDH1 immunohistochemical staining of a large cohort of ovarian carcinomas suggested that high expression of this transporter was a good prognostic

factor (142), the opposite of what is found for many tumour types, particularly the breast.

A further mechanism that stem cells appear to employ to reduce susceptibility to potential toxins is through low expression of certain cytochrome P450 enzymes, a superfamily of haemoproteins involved in oxidative metabolism, which catalyze largely oxidative reactions, including those of pharmacological and toxicological importance (143).

1.4.2 Drug resistance in cancer stem cells and new therapeutic strategies

Several mechanisms have been hypothesized as a basis for drug resistance in solid tumours, including drug inactivation, quiescence, and increased DNA repair in chemoresistant tumour cells (144). The classical drug resistant model sustains drug selection of clones possessing mutations that confer a survival advantage from various drug insults. From this point of view, each cell within the tumour would have an equal probability of gaining such mutations (144). However the above mentioned phenotypes also characterized normal stem cells, as described in the previous paragraph. CSCs are expected to share many of the typical properties of normal stem cells, which may underlie their capacity to survive to common therapeutic protocols (145).

As a consequence of the CSC theory, it is believed that malignancies, that initially undergo complete remission but subsequently relapse to a refractory state (146) (147), are expected to possess tumour stem cells

that have acquired the property to escape clinical treatments. From the CSC model point of view, chemotherapeutics preferentially target TA cells, causing tumour regression, but fail to eradicate drug-resistance CSCs (FIG. 1.7).

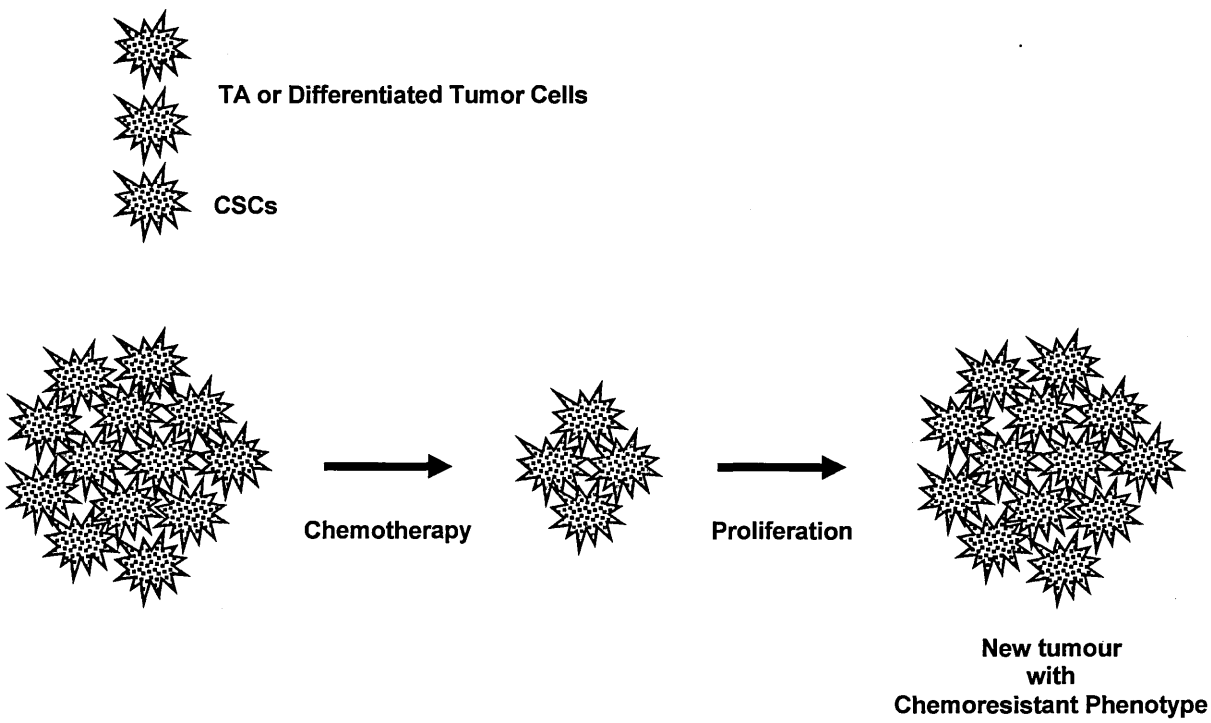


Figure 1.7: Model proposed for drug-resistant CSCs

Following false complete remission of the disease, CSCs proliferate and most probably confer the drug resistant phenotype to their progeny, resulting in a tumour that is fully refractory to further treatment. Therapies are greatly needed that exclusively target the small percentage of tumour progenitors in addition to the non-tumorigenic progeny that comprise the bulk of the tumour mass.

In thyroid cancer, resistance to therapy-induced cell death results from the high expression of anti-apoptotic proteins and has been connected with the autocrine production of interleukin-4 (IL-4) (148). More recently, an IL-4 antagonist was shown to strongly enhance the anti-tumour efficacy of conventional chemotherapeutic drugs through selective sensitisation of colon cancer stem cells identified by CD133+ expression (149). It seems that the efficacy of conventional chemotherapy regimens may be significantly improved when combined with adjuvant treatment, such as anti-IL4 (150). Thus, targeted therapies that suppress or destroy tumour stem cells directly may synergize with traditional therapies to provide increased efficacy in tumour eradication.

Resistance to radiotherapy has been associated with reactive oxygen species (ROS), critical mediators of radiation-induced cell killing. Lower ROS levels in CSCs results from increased expression of free radical scavenging systems; therefore pharmacological depletion of ROS detoxifying enzymes could significantly increase radio-sensitization of CSCs (151).

Moreover, as tumour stem cells maintain the ability to differentiate, agents that induce differentiation may play a therapeutic role against CSCs eradication. Both DNA methyltransferases (DNMTs) and Histone deacetylase (HDACs) may have a role in suppressing genes related to differentiation. As consequence, HDACs and DNMTs inhibitors, alone or in combination with conventional chemotherapies, are strongly anti-proliferative agents to drug resistant human cancer cells, whereas no

effects were observed on normal epithelial cells. These date suggest that these therapies may hold the potential to directly target tumour stem cells (152) (153).

Recently, high-throughput screening for selective CSC inhibitors was successfully carried out on EMT-induced breast epithelial cells as an *in vitro* model of mammary CSCs (154). This screen led to the identification of a compound, salinomycin that specifically inhibits proliferation of mammary CSCs and significantly reduces their relative proportion by more than 100-fold relative to paclitaxel, the commonly employed chemotherapeutic drug for breast cancer.

The ABCG2 and ABCB1/MDR1 genes are expressed in the majority of stem cells and in most of CSCs (145). The combined use of conventional chemotherapeutics drugs and ABC transporters inhibitors, such as Apatinib, a small-molecule multi-targeted tyrosine kinase inhibitor (155), could be used to specifically target CSCs, but this type of therapy may have toxic effects on a patient's normal stem cells. Therefore, this approach needs to be adjusted carefully to avoid excessive toxicity.

Another approach to inhibiting CSCs is to target the proteins essential for the growth and maintenance of stem cells. For example, Patched (PTCH) is the receptor for Hedgehog (HH) molecules, an important pathway containing several genes either tumour suppressor genes or oncogenes and is mutated or over expressed in several type of cancers (156) (157) (158). Constitutive HH expression could be an important component in stem cell activation in many tumours and therefore an attractive target for cancer

therapies. Other pathways critical to embryonic development and potentially important in cancer have also been described and include the WNT and NOTCH pathways (104) (159). A number of experimental inhibitors of these pathways have been developed (160). Inhibition of the Notch signalling pathway through pharmacologic and genomic approaches prevented sphere formation, proliferation, and/or colony formation in soft agar of breast CSCs and could be also useful tool for a combined therapy against CSCs in solid tumours.

These targeting strategies still require refinement but the discovery of an effective therapy specifically targeting the CSC subpopulation could help eradicate the cancer cell subpopulation capable of evading traditional therapy and so increase disease free survival. Essential in the optimal development of CSC targeted therapies will be the ability to disrupt the CSC subpopulation without affecting normal somatic stem cells. Identifying regulators of tumour initiating cells that are less critical in normal stem cell biology will be important in achieving greater success in control CSC growth and tumour relapse.

1.5 The stem cell niche in health and malignancies

Stem cells are regulated and supported by the surrounding microenvironment, referred to as the stem cell niche, which provides stem cells with physical anchorage as well as membrane-bound and secreted factors that regulate survival, polarity, quiescence and differentiation. The concept of the stem cell niche was first introduced in 1978 by Schofield, who hypothesized that the stem cell in the multicellular organism is regulated by its environment, which imparts to it properties that may be altered by removal and isolation of the stem cell from this niche (161). Pertinent signals to the stem cell may be direct interactions with resident niche cells or extracellular matrix molecules or diffusible autocrine, paracrine and even endocrine signals (162), which are all integrated to provide the proper regulation and maintenance of the stem cell. The niche must be highly specialized and requires specific organization to balance and control the self-renewal and differentiation capabilities of the stem cell. The niche could be a single cell, as is the case in the *Caenorhabditis Elegans*, or a group of cells, as in the case in the *Drosophila Melanogaster* germline.

The microenvironments regulating the stem cells of higher organisms are more complicated. In mammals the stem cell niche is composed of a group of cells in a specific tissue location for the maintenance of stem cells. The niche's overall structure is variable, and different cell types can provide the niche environment. In adults, the niche prevents tumorigenesis by

controlling stem cells in the arrested state and maintaining the balance between self-renewal and differentiation. In this context, any mutation that leads stem cells to escape from the niche control may result in tumorigenesis (163). It is therefore reasonable to hypothesize that one of the differences between normal stem cells and cancer stem cells is that cancer stem cells may no longer be dependent on niche signalling (53). Some common features, structures, and functions of the stem cell niche in different tissues could be summarized as follow:

- The niche functions as a physical anchor for stem cells. E-cadherin-mediated cell adhesion is required for anchoring GSCs and SSCs in *Drosophila*, and N-cadherin may be important for anchoring HSCs in the bone marrow niche. Other adhesion molecules, such as integrins, may help anchor stem cells to extracellular matrices (164) (90).
- The niche generates extrinsic factors that control stem cell fate and number. Many signal molecules have been shown to be involved in regulation of stem cell behaviour, including Sonic Hedgehog (Shh), Wnt, Bone Morphogenetic Proteins (BMPs), Fibroblast Growth Factors (FGFs), Notch, Stem Cell Factor (SCF), Angiopoietin-1 (Ang- 1). The BMP and Wnt signal pathways have emerged as common pathways for controlling stem cell self-renewal and lineage fate from *Drosophila* to mammals (86) (165).
- In invertebrates and mammals, the stem cell niche exhibits an asymmetric structure. Upon division, one daughter cell is maintained in the niche as a stem cell (self-renewal), whereas the other

daughter cell leaves the niche to proliferate and differentiate, eventually becoming a functionally mature cell (166).

Locating and further identifying stem cell niches in mammals have been difficult, owing to their extremely complicated anatomic structures. Studies regarding stem cells and their location/niche in other genetic model systems, including those of *Drosophila* and *Caenorhabditis Elegans*, have been fruitful. In *Drosophila*, GSCs were located in the anterior region of ovary germarium. The germarial tip adjacent to GSCs was defined as the niche supporting GSCs in the *Drosophila* ovary (164), whereas the hub, located at the tip of *Drosophila* testis, served this function (167). In *C. Elegans,* a distal tip cell (DTC) located at the tip of the germ line organization region was found to function as the niche in supporting GSCs (168).

Adult mammalian stem cell niches are more complex due to the presence of multiple potential sites for stem cells as well as a number of candidate niche cells and extracellular components that are involved in the regulation and maintenance of the tissue specific stem cells. In mammals, a stem cell niche was successfully identified in the bulge area of hair follicles, in the intestinal crypt base, in the mammary gland, in the Subventricular Zone (SVZ) of the Nervous Central System (NCS) and in the haematopoietic system (110) (169) (170).

Two stem cell niches have been recognized in the mammalian brain, one in the SVZ and a second in the subgranular zone (SGZ). NSCs isolated from the SVZ, the best characterized, differentiate into both neurons and glia *in*

vitro (171) and are regulated by both contact-dependent factors and diffusible signals. In both the SVZ and SGZ structures, endothelial cells that form blood vessels and the specialized basal lamina are an essential component of the NSC niche. Endothelial cells provide attachment for SVZ and SGZ astrocytes and generate a variety of signals that control stem cell self-renewal and lineage commitment (172). Brain tumors appear to occupy similar niches to normal NSCs. Recent work demonstrate that Nestin+ CD133+ brain CSCs associated directly with vascular endothelial tubes, and a mouse xenograft model, where co-injection of endothelial cells with malignant cells led to more rapid tumor growth (173). Recently, cells expressing stem cell markers were found to localize not only to the perivascular niche but also to areas of necrosis (174). This raises the possibility of a hypoxic niche for brain CSCs, located surrounding vascular tubes in strict association with endothelial cells.

From a general point of view, a majority of mutagenic agents described to confer a risk for cancer also perturb normal stem cell homeostasis and their niche, besides inducing changes in the DNA of some stem cells and impairing or enhancing some of their characteristics properties (175).

These mutated stem cells may remain in a state of perpetual activation and may be controlled by intercellular communications or by autocrine/paracrine factors produced by the niche. The niche could play a pivotal role in cancer stem cell self renewal and tumour progression, accentuating CSC activation and further differentiation. Another function of the tumour niche is the active recruitment of new endothelial and stromal

cells into tumours that is essential for developing a pro-angiogenic environment that enhances tumour survival under adverse conditions (176) (177).

In conclusion, it could be possible that stemness is a function of microenvironment. The properties of CSCs in different tumours may vary significantly, depending on their location within the tumour mass and the different cell types or soluble factors characterizing the tumour environment. Up to now, several models for the existence and composition of CSCs niche have been postulated (178). Firstly, CSCs may not require a specific tumour niche and may be capable of surviving in the normal stem cell niche. Alternatively CSCs may depend on a pre-activated CSC niche for their expansion and survival. Another possibility is that CSCs may influence the local microenvironment by secreting different signalling molecules, creating their own niche. Finally, CSCs may acquire the ability to autonomously provide the necessary factors for expansion and self renewal, becoming niche independent.

1.6 Cancer stem cells and metastasis

In the vast majority of cancers, primary malignancies are responsible for a mere 10% of deaths whereas the main cause of mortality is metastases. Metastasis is a complex, multi-step process where specific cellular and molecular mechanisms are involved. In the case of carcinoma this process includes basement membrane destruction and local invasion into adjacent tissue, intravasation and survival in the bloodstream, extravasation into distant organs and, finally, cancer cell colonization, which implies the cell capacity to survive and proliferate at the metastatic site (39). During all these steps, tumour cells undergo profound changes in cell adhesion properties, involving cell-to-cell contacts and cell-to-extracellular matrix adhesion, in addition to the expression of extracellular proteases. Those changes lead to the remodelling of cell-cell contacts, degradation of the extracellular matrix and migration of the cancer cells into adjacent tissues (179).

Epithelial to mesenchymal transition (EMT) is the biological process through which epithelial cells acquire a mesenchymal identity (180). EMT is a highly conserved process that governs meaningful events during embryo development and in the adult life. In the adult organism, EMT is engaged during wound healing, tissue regeneration and organ fibrosis (181). In cancer, EMT is thought to confer on cancer cells the ability to detach from the primary mass and to acquire more motile features. At the metastatic

site the recapitulation of the primary mass is achieved by the reverse process of mesenchymal to epithelial transition (MET) (180).

In general terms, the EMT process involves a complex genetic program which is characterised by the repression of epithelial markers and the induction of mesenchymal and migration-related markers. Numerous pathways, such as those triggered by EGF and TGFß growth factors, activate the EMT program, inducing loss of cell polarity and down-regulation of the epithelial marker E-cadherin, whose functional repression is considered the hallmark of EMT (180) (182). E-cadherin is a central component of cell-cell adherent junctions and is required for the formation of epithelia in the embryo and to maintain epithelial homeostasis in the adult (183). During tumour progression E-cadherin down regulation is a frequent event and its inactivation or silencing can be achieved by different mechanisms (184) (185). Most, if not all, well characterised E-cadherin repressors are known as EMT inducing developmental regulators. Two of the major members of the Snail zinc-finger transcription factors family, known as Snail (or Snail1) and Slug (or Snail2), play an important role during EMT, blocking E-cadherin expression. Other important regulators of E-cadherin transcription, involved in the EMT process, are SIP1 (or ZEB-2), ZEB-1 and Twist (185) (186).

Snail and Slug play crucial roles during EMT, not only repressing E-cadherin expression, but also conferring additional properties. Both Snail and Slug seem to confer protection against cell death induced by external

stimuli and resistance to genotoxic agents, including γ-irradiation and chemotherapeutic compounds (186). Thus, it is possible that the expression of Snail and Slug in cancer cells stimulates not only the EMT process but also confer to a tumour cell cancer stem cell properties, such as metastatic and chemo/radio resistance attributes. To confirm this hypothesis Bapat and co-workers has recently published a brilliant paper (186) where Snail and Slug were demonstrated to regulate not only EMT in ovarian cancer cells, but also to be involved in the acquisition of stem cell properties toward resisting radiotherapy or chemotherapy mediated cellular stress. In this study they showed that Snail and Slug directly participate in p53 mediated pro-survival signalling through active repression of pro-apoptotic genes. Furthermore Snail and Slug regulate indirect activation of a self renewal program and the expression of stemness genes, such as *OCT4*, *NESTIN* and *BMI1* (186).

Another character frequently associated with EMT is cadherin switching, from the epithelial (E-cadherin) to mesenchymal type (N-cadherin). By expressing N-Cadherin on the surface of carcinoma cells, their affinity to stromal cells, naturally expressing N-Cadherin, is increased. Malignant cells acquire a fibroblastic morphology and the ability to degrade the basal lamina, invade through the surrounding tissues and metastasize (FIG. 1.8).

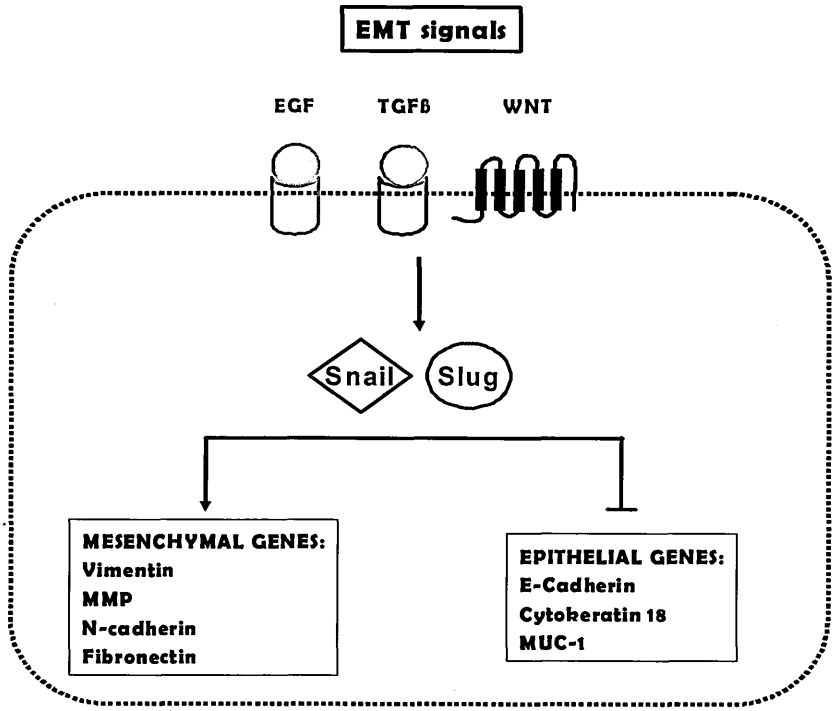


Figure 1.8: EMT and CSCs

Recent studies support the notion that metastasis capacity is pre-determined by genetic changes acquired at the initial stages of tumour development (187) (188). The biology of CSCs may have important implications when applied to the study of cancer metastasis. In many solid tumours, both circulating tumours cells (189) and cells at established sites of metastasis (190) contain a higher proportions of cells expressing stem cell markers, suggesting that CSCs are more capable of metastasizing than differentiated tumour cells, and/or that CSCs survive better in metastatic sites than the other malignant cells.

Several characteristics of CSCs make them the major candidates able to occupy unfamiliar sites and sustain metastatic growth. The inherent plasticity of stem cells makes them more adept to survive in a diverse environment where growth factors and other signalling molecules are

different than in the primary tumour site. Moreover, CSC tumour-initiating capacity is necessary at any metastasis site to give rise to a new tumour mass. Therefore, even if non-CSCs migrate, only the CSC subpopulation is able to expand into heterogeneous metastatic lesions.

Numerous molecules and pathways have recently been identified to regulate both stem cell migration and cancer metastasis (191). These molecules comprise a complex network of cellular interactions that makes possible the formation of an organ microenvironment for migrating CSCs.

Cell surface receptors and their ligands required for their activation, such as SDF1 and CXCR4, are expressed during normal stem cell homing and mobilization as well as cancer cell metastasis. It is notable that the SDF-1 chemokine receptor CXCR4 is expressed on immunophenotypically identified CSCs in many primary solid tumours, including breast and glioblastoma (192). CXCR4 expression may mediate homing of CSCs to metastatic sites such as the liver and BM that express high levels of SDF-1 at baseline.

Also soluble factors participate in directing tumour metastasis and in creating a favourable environment for tumour spreading. Cytokines produced by the primary tumour may actually create the metastatic microenvironment in distant organs (193) (187). For example, VEGFR1+ haematopoietic progenitor cells are stimulated by cytokines released from the primary tumours and travel to sites of future metastasis. It is thought that the presence of these cells changes local microenvironments to make them more favourable for metastases (194). Whether this process might

induce a niche that is particularly helpful for CSCs represents an intriguing pathway for investigation. It is believed that these microenvironmental changes not only promote tumour progression but also enhance CSC survival.

In light of significant advances in metastasis and stem cell research, a CSC-based model for both tumorigenesis and metastasis could be proposed. During the establishment of the CSC pool, CSCs inherit a unique set of genetic and/or epigenetic changes that determine the cancer malignancy, metastatic potential and the tissue tropism. Molecular crosstalk between the primary tumour and the pre-metastasis niche through secreted stimulatory signals helps manage the homing of metastatic CSCs. Trafficking towards favourite tissues and organs of metastatic CSCs is guided by cues such as oxygen gradients or other chemo-attractants derived from niche sites (120) (195) (171) (172). CSCs and metastatic CSCs at primary and secondary sites can either takeover the niches of normal stem cells or recruit new components to form a permissive niche. Metastatic CSCs may then either proliferate at the new site or stay dormant, similar to the quiescent state of normal stem cells (196). Stimulatory factors from the niche can lead to reactivation of the CSCs and formation of a metastatic lesion, which may partly explain temporal patterns of primary tumours versus secondary tumours (197). Metastatic CSCs at metastatic sites maintain most of the genetic programs acquired at the primary tumour site through self-renewal, which explains the phenotypic similarities between primary and metastatic cancers (198).

However, metastatic CSCs in secondary sites are able to evolve independently by accumulating additional genetic alterations that render them resistant to treatments that are effective against primary tumours.

1.7 Chemokines in cancer stem cells and metastasis

1.7.1 Roles of chemokines and their receptors in cancer

Numerous clinical studies have clarified that selected chemokine receptors are often up-regulated in a large number of common human cancers, including those of the breast, lung, prostate, colon, ovary and melanoma (199) (40) (200). Chemokine receptors and their corresponding chemokine ligands have been shown to play a number of non-redundant roles in cancer metastasis to vital organs as well as regional lymph nodes, the most frequent site of cancer metastasis. Indeed, chemokine receptors may potentially facilitate tumour dissemination at several key steps of metastasis, including adherence of tumour cells to endothelium, extravasation, metastatic colonization, angiogenesis, proliferation and protection from the host immune response by activation of well known survival pathways (201) (202).

The function of chemokines was firstly demonstrated for leukocytes, which use these proteins to arrest at inflamed blood vessels and as guides to get to specific sites of inflammation. The specific roles of chemokines for leukocyte site-specific homing at inflammatory sites also suggest their possible contribution in several of the key steps of metastasis (39) (199).

Chemokines are small chemotactic cytokines which bind to G-protein linked receptors (203) and are divided into the following four subgroups: CXCL, CCL, CX3CL and CL (204).

Homeostatic chemokines are involved in the development and the homeostasis of the immune system, whereas inflammatory chemokines are released in consequence of special stimuli (injuries, inflammation) and induce leukocyte homing to the affected tissue site. They are released during inflammation and engage lymphocytes in the course of the immune response (203).

In the context of cancer, chemokines regulate tumorigenesis in at least four different ways:

- By acting as homing factors, as well as by autocrine growth stimulation (205) (206).

  Chemokines secreted by tumours not only draw infiltrating cells into tumour sites but they may also contribute to tumour cell growth.

- By modulation of angiogenesis.

  In this regard, a specific set of chemokines act as a direct chemotactic factor for endothelial cells and can stimulate the creation of new blood vessels within the tumour. CXCL12, the ligand of CXCR4, is produced by both stromal cells and cancer cells and increases the expression of Vascular Endothelial Growth Factor (VEGF) in endothelial cells, thus stimulating neo-angiogenesis within the tumour mass (205) (207).

- By activating the tumour-specific immune response.

Tumour cells, stromal cells, as well as inflammatory cells contribute to the chemokine milieu at the tumour site (208).

Inflammatory cells play positive and negative roles in tumorigenesis (209) (210). Tumour associated chemokines may control inflammation within tumours and as a consequence, inhibit or increase the capability of the immune system to eliminate cancer cells.

Ovarian cancer cells, for example, produce inflammatory chemokines such as CCL2 and CCL5 (211). High levels of CCL2 stimulate the homing of macrophages in the same area that release a variety of factors, including matrix metalloproteinases (MMPs); MMPs may increase the invasiveness of cancers and are clinically associated with poor outcomes in cancer patients (212). In contrast to CCL2, the presence of CCL5 was correlated with infiltration of CD8+ T cells and may favour immune responses against tumour cells (211).

Tolerance to tumour cells may also be mediated by a subpopulation of naturally occurring T regulatory cells (Tregs). Through contact-dependent mechanisms, Tregs suppress the cytotoxic ability of CD8+ cytolytic T cells (CTL) (213). Ovarian cancer cells and tumour-infiltrating macrophages are both able to secrete the CCR4 ligand, CCL22, and appear to be responsible for the recruitment of Tregs (CD4+ CD25+ CD3+ CCR4+) into tumour tissue in a CCL22-

dependent manner. Thus, CCR4+ Tregs apparently infiltrate into tumours in advanced disease and inhibit the activity of tumour specific CTL, possibly leading to the poor outcome observed in this subset of patients (214).

Another mechanism by which certain tumours escape the immune system is through the chemorepulsive activity of high levels of CXCL12 (SDF-1). At low concentration, CXCL12 acts as a T cell chemoattractant (215), otherwise can repel T cells both *in vitro* and *in vivo via* a CXCR4 dependent mechanism (209).

In conclusion, according to which chemokines are released within the tumour mass, opposite events may occur: copious production of pro-inflammatory chemokines can pilot a strong inflammatory response and can potentiate angiogenesis, thus favouring a rapid neoplastic growth. Alternatively, high levels of monocytes and/or neutrophil infiltration, for example, in response to lower levels of pro-inflammatory chemokines, can be associated with angiostasis, cytotoxicity, and possible tumour regression (210).

1.7.2 CXCL12/CXCR4 axis plays a pivotal role in CSC metastasis

CXCR4 is one of the most common chemokine receptors that has been demonstrated to be over-expressed in certain human cancers (203) (216). The role of the SDF-1-CXCR4 axis was initially studied in haematopoietic cells (204), where it regulates the trafficking and homing of CXCR4 positive

HSCs, pre-B lymphocytes, and T lymphocytes (217) (218). However, besides this role, CXCR4 is also expressed on the surface of various kinds of tissue stem cells (200), supporting their trafficking during development, tissue injury, and regeneration.

The binding of CXCL12 to CXCR4 leads to the activation of divergent signalling pathways, which can stimulate multiple responses such as chemotaxis, cell survival and proliferation and increase intracellular calcium concentration or activate transcription of different genes (FIG. 1.9) (4). The following figure summarizes some of the key signalling pathways thought to be involved in CXCR4 signal transduction. The exact nature of these pathways may be tissue-dependent and thus may diverge between cell types.

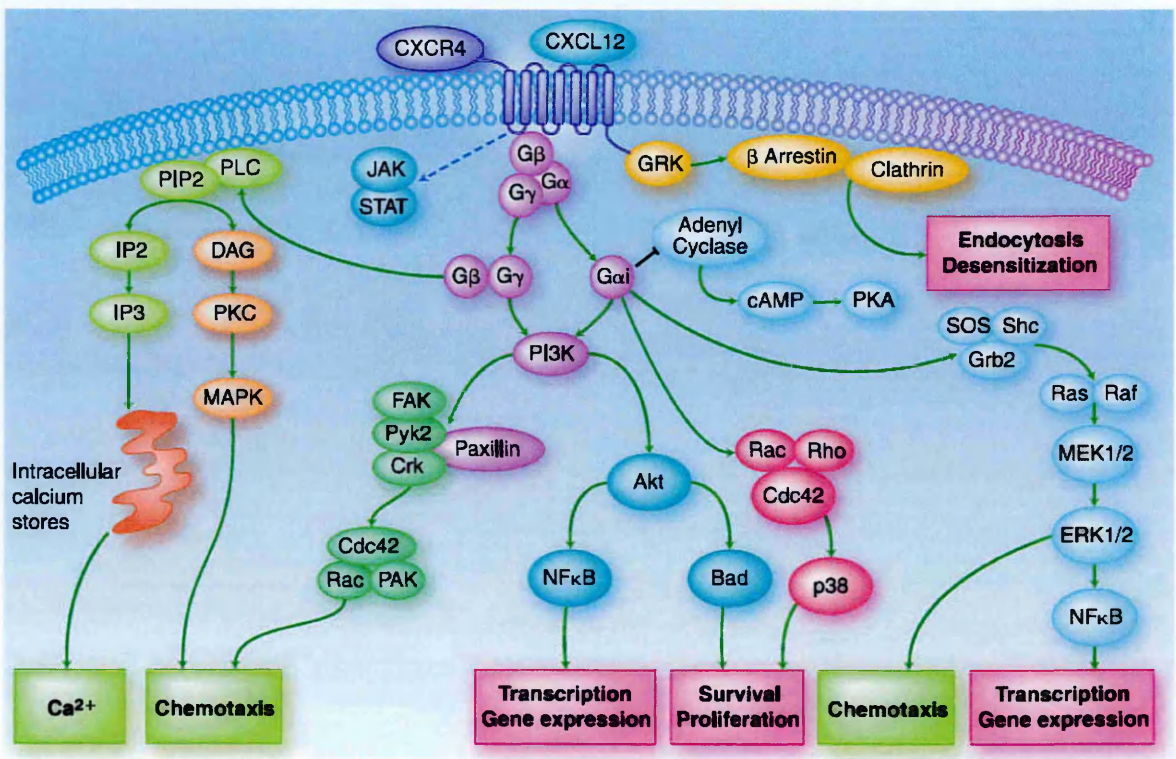


Figure 1.9: A schematic picture of the CXCL12/CXCR4 intracellular signal transduction pathways (4)

The expression of CXCR4 is regulated at the molecular level by several transcription factors associated with organ development as well as those related to stress and tissue damage. *PAX* genes are over-expressed in different type of cancers and are responsible for high levels of CXCR4 expression observed in tumours (219) (220). Beside *PAX* genes, CXCR4 expression may also be positively regulated by transcription factors related to stress, hypoxia and tissue damage, such as NF-κB (221), hypoxia-inducible factor (HIF-1) (222), lysophosphatidylcholine (223), TGF-β1 (224), VEGF (225), and several interleukins (IL-2, IL-4, and IL-7) (226). Thus, it is likely that stress-related conditions, often present in the tumour mass or after tissue damage, may up-regulate CXCR4 expression on both normal and malignant stem cells. Moreover, the sensitization of normal and cancer stem cell chemotaxis to a CXCL12 gradient is reliant on cholesterol content in the cell membrane and on the incorporation of the CXCR4 and the small GTPase Rac-1 into membrane lipid rafts. This co-localization of CXCR4 and Rac-1 in lipid rafts helps the activation of CXCR4 pathway and allows normal or cancer stem cells to better sense a CXCL12 gradient (227). In this regard, drugs that perturb lipid raft formation by depleting cholesterol from cells could negatively influence the metastatic properties of cancer stem cells (227).

Preliminary data, together with the reported role of various chemokines in metastasis, strongly support the idea that chemokine-mediated metastasis could play a key function in migrating CSCs.

This implies that the CXCL12-CXCR4 axis may influence the nature of tumours and direct the metastasis of CXCR4 positive CSCs by chemoattracting them to organs that highly express its ligand. CXCL12 can be found on the surface of liver, lung or bone cells, therefore, an increased number of metastases in these organs can be observed. Sustaining this notion, it has been recently reported that several CXCR4 positive cancers, such as breast, ovarian, prostate cancers and neuroblastoma, metastasize to the bones in a CXCL12-dependent manner (228) (229) (230) (207).

It has been experimentally proved, using breast cancer cell lines, that the abrogation of CXCL12/CXCR4 signalling impaired breast cancer metastasis formation to regional lymph nodes and lung (231). Interestingly, inhibition of CXCR4 does not prolong the overall survival in mice with experimental lung metastases, but is able to delay or at least impair the proliferation of both the primary tumour and metastases (232). Furthermore, the formation of the EGFR/Neu heterodimer enhances both the expression of CXCR4, required for c-erbB-2 breast cancer cell invasion *in vitro* and *in vivo*, and inhibits ligand-induced CXCR4 degradation (233) (234). In addition to c-erbB-2 (HER2/neu), recent data point out that also CD24 affects CXCR4 function in breast cancer cells (235). Indeed, CD24 expression reduces CXCL12-mediated cell migration and signalling *via* CXCR4. By contrast, the loss of CD24 was correlated with an enhanced CXCR4 lipid raft association concomitant with an increased cell migration (235). These results suit well to the phenotype of breast cancer stem cells: $CD44^+$ $CD24^{-/low}$ ESA^+ (236). Thus, if CD24 is truly linked to CXCR4 expression, breast cancer stem cells

negative for CD24 should express elevated CXCR4 levels, which may be responsible for metastatic CSC spreading. However this hypothesis has not yet been proven.

The progression and organ-specific spreading of different tumours, such as breast, colon, and melanoma has been associated with $\alpha_v\beta_3$-integrin expression. A lot of data support the evidence of a critical relationship between $\alpha_v\beta_3$-integrin expression and the CXCL12/CXCR4 axis in prostate cancer. Recent findings show that not only CXCR4, but also the expression and activation of $\alpha_v\beta_3$-integrins is regulated by CXCL12 (237), which suggests a positive feedback loop for the adhesion, and subsequent prostate cancer metastasis formation in CXCL12 expressing tissues (238) (239). Based on these results prostate cancer stem cells, which are identified by the phenotype $CD44^+/\alpha_2\beta1$ integrin $^{high}/CD133^+$, may also express CXCR4 and may use this receptor as cellular adhesion components and/or as extracellular matrix components for establishing tissue-specific metastasis.

Human pancreatic cancer tissue contains CSCs expressing CD133 that are the most tumorigenic and highly chemotherapy resistant cells. Moreover, in the invasive front of pancreatic tumours, a distinct subpopulation of CD133+/CXCR4+ CSCs was identified as the population responsible for the metastatic phenotype of this tumour (240).

1.7.3 Role of CXCL12/CXCR4 in metastasis of human ovarian cancer

A central role for the CXCL12/CXCR4 axis in ovarian tumour metastasis was also identified and a correlation between the activity of this chemokine system and an enhanced intraperitoneal dissemination of Epithelial Ovarian Cancer (EOC) was described (241). Recent analysis showed the expression of CXCR4 and CXCL12 also in normal ovary, but their localization was confined to the follicular cells and it was not detected in normal epithelium (242).

Clinical studies also found that the prognosis of patients with a high level of CXCR4 is drastically worse than that of the patients with a low CXCR4 level, indicating that CXCL12-CXCR4 axis plays an important role in the biology of ovarian tumours (243) (207) (244).

In vitro studies confirmed that, in the presence of CXCL12, CXCR4 controls both ovarian cancer cell proliferation and migration, through the activation of the ERK1/2 and Akt pathway (245). In addition it was also reported that CXCL12 effects on ovarian cancer cell lines are mediated by EGFR trans-activation through a mechanism involving the activity of cytosolic tyrosine kinases belonging to the c-Src family (246). A possible important "cross-talk" between CXCR4 and EGFR intracellular pathways might connect signals of tumour progression and proliferation and provided a plausible explanation for the poor overall survival of patients whose cancer tissue co-expressed both CXCR4 and EGFR (246) (207).

Human peritoneal mesothelial cells (HPMCs) coating the peritoneal cavity bind to EOC cells in the preliminary step of peritoneal metastasis. CXCL12

is principally expressed in HPMCs rather than in EOC cells, while CXCR4 was found in both EOC and HPMCs, thus creating an extracellular chemotactic milieu for ovarian epithelial cancer cell migration (241) (247).

The over-expression of HER2/neu in breast cancer enhances the metastatic potential through the up-modulation of CXCR4 (234). In a similar study performed on a group of ovarian tumour samples (248), HER2/neu positive patients did not show a higher CXCR4 expression. Intraperitoneal treatment with AMD3100 (CXCR4 inhibitor) resulted in reduced dissemination in nude mice inoculated with ES-2 ovarian cancer cell line, suggesting that CXCR4 inhibition could suppress the formation of peritoneal metastasis (241). Due to the particular way of ovarian cancer spreading and metastasis, it is possible that a complex cohort of chemokines, produced by different types of cells within the peritoneum, is responsible for the invasiveness and the metastatic potential of EOC.

1.8 CD133 and its potential role as CSC marker

Prominin/CD133 is a 115/120-kDa integral membrane glycoprotein specifically associated with plasma membrane protrusions in epithelial and non-epithelial cells including neuroepithelial and haematopoietic stem cells (249). It displays a unique membrane topology with five membrane-spanning domains and eight potential N-glycosylated extracellular sites (FIG. 1.10, red circle) (250). CD133 (AC133) was the first identified member of the prominin family of pentaspan membrane proteins. The specific functions and ligands of the prominins are still relatively unclear, but they are distinct in their restricted expression within plasma membrane protrusions, such as epithelial microvilli and epididymal ductal epithelial sterocilia (FIG. 1.10 from (250) and (251)).

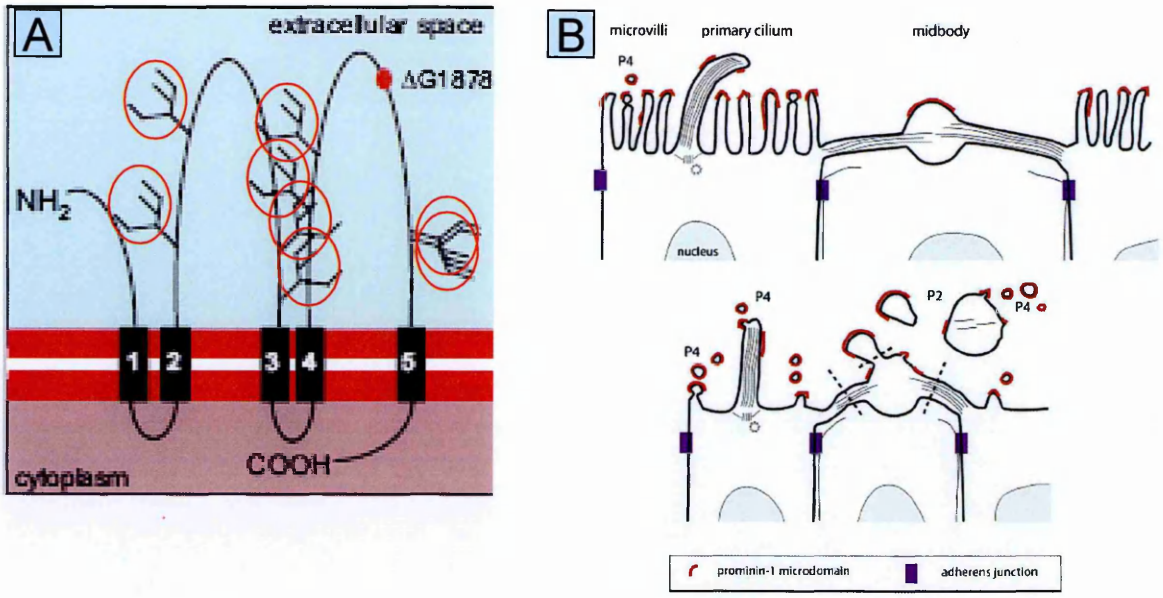


Figure 1.10: CD133 structure and cellular localization

A) CD 133 is a pentaspan membrane protein with eight potential N-glycosylated extracellular sites. B) CD133 is specifically associated with plasma membrane protrusions in epithelial and non-epithelial cells.

Due to its location in membranous protrusions of the plasma membrane such as the microvilli of epithelial cells, CD133 was attributed a functional role as an 'organizer' of plasma membrane architecture (250). Interactions between CD133 and cholesterol within membrane micro-domains (250) suggested that CD133 might also be essential in maintaining an appropriate lipid content within the plasma membrane (252).

Since its expression was found in haematopoietic progenitor cells (253), much attention has been focused on the potential role of CD133 as a cell surface marker of adult stem cells. Indeed, in human haematopoietic lineages, CD133 antigen expression is restricted to CD34+ cells, although CD133 transcripts have been found in many human cell lines and differentiated cells (254).

Human AC133+ cells, isolated from human peripheral blood and manipulated *in vitro* to undergo myogenesis, were shown to improve disease through a direct contribution to muscular regeneration when transplanted into a lineage of dystrophic mice, a model of Duchenne's muscular dystrophy (255). Bussolati *et al* showed that a population of CD133+ cells isolated from the adult human kidney were capable of both self-renewal and multi-lineage differentiation *in vitro* and *in vivo*, and could contribute to renal tissue regeneration by differentiation to form both epithelial and endothelial lineages (256).

Expanding evidence highlights the role of CD133 as a marker of CSCs in various human tumours (257) (258). This assertion was supported not only by the fact that CSCs isolated from numerous tumours expressed this marker (259) (260) (261) (262) (263), but also by the fact that CD133 expression is regulated by several pathways generally mutated in different types of cancer (264) (265) (266).

CD133 was expressed in combination with CD44+ and $\alpha_2\beta_1^{high}$ in approximately 0.1% of cells within a large series of prostate tumours, irrespective of their grade or metastatic state (262). These cells were able to self-renew and to proliferate and showed multi-lineage differentiation capabilities *in vitro* in order to recapitulate the original tumour phenotype (267) (262).

CSCs are significantly enriched in CD133+ subpopulations derived from human colon cancer and hepatocellular carcinomas, as shown by their

potential to both self-renew and differentiate, to form colonies and proliferate *in vitro*, and by their ability to reform the original tumour phenotype when transplanted either subcutaneously or into the renal capsule of immunodeficient mice (263).

Ovarian cancer CD133 expressing cells were able to self renew, giving rise to both CD133 positive and negative cells, and were associated with enhanced resistance to platinum-based therapy (260). CD133 positive cells in ovarian cancer were also shown to be involved in the establishment of tumour vasculature that is critical for tumour cell survival during disease progression (268). A controversy exists regarding the potential tumorigenic capability of ovarian CD133 positive cells. Only one paper has demonstrated that CD133 + ovarian tumour cells have increased tumorigenic capability over CD133-cells and have the ability to regenerate a heterogeneous tumour similar to that found in the original patient (261).

A recent study assessed the changes in human cord blood-derived CD133+ cells following culture in medium conditioned by cells that stably expressed specific Wnt signalling molecules (264). Regulation of the differentiation and proliferation status of CD133+ cells by Wnt signalling matches with the role of CD133 as a CSC marker. Indeed, Wnt signalling dysregulation through genetic alterations of its downstream components, such as APC or β-catenin, is a common event in the pathogenesis of several cancer types (265).

A recent paper asserted that the stemness of medulloblastoma-derived CD133+ cells was related with the Notch signalling pathway (269). Notch

blockade resulted in a 5-fold loss in the CD133+ sub-population and in elimination of the CD133+ side-population featuring high Hoechst dye exclusion (270). Moreover, in the highly aggressive brain tumour, glioblastoma multiforme, the HEDGEHOG (HH)-GLI pathway has been shown to regulate the self-renewal of the CD133+ cells and, importantly, expression of the 'stemness- associated gene signature' (266).

A major problem with CSCs is their resistance to current therapies such as chemo- or radiotherapy (271) (272) (273). CD133+ TICs are resistant to therapy (273), consistent with their stem-like nature as reported both for chemotherapy (271) and radiation treatment (272). However, since the function of CD133 is unknown, it is not obvious if CD133 is just a marker of resistant cells or whether high expression of CD133 in TICs could contribute to the resistance to therapy. Frank et al. (272) recently showed that CD133+/ABCG5+ melanoma cells were resistant to doxorubicin treatment and that melanoma tissue isolated from patients expressed high levels of both CD133 and ABCG5. Moreover, CD133+ glioma cells are also resistant to radiation therapy (271). Following radiation, glioma cells acquired a higher level of resistance, associated with increased CD133 expression.

A lot of research strongly links high expression of the surface marker CD133 with tumour-initiating capacity and greater resistance to chemo- and radio-therapy, suggesting potential methods for selectively eradicating the TICs by targeting associated signaling pathways (265) (269) or the cell cycle machinery (271).

1.9 Squamous cell carcinoma of the cervix: an overview

1.9.1 Epidemiology and aetiology

Cervical cancer is the second most common malignancy in women worldwide. Squamous cell carcinoma (SCC) remains the most frequent form of cervical cancer. However, the incidence of cervical adenocarcinoma has increased significantly over recent years (274).

Cervical cancer offers a unique opportunity for prevention because it is preceded by a well recognized pre-malignant form: cervical intra-epithelial neoplasia (CIN) in the case of squamous cell carcinoma and cervical glandular epithelial neoplasia in the case of adenocarcinoma. The introduction of cervical screening programs, based on the Papanicolaou smear, has significantly decreased the incidence of SCC of the cervix in developed countries. In contrast, the incidence of adenocarcinoma has continued to rise, even in developing countries, despite the introduction of screening programs (275). The reason of this discrepancy is partially due to difficulties in detecting the precursor lesions with conventional screening (276).

Human papilloma virus (HPV) has been demonstrated to be the main aetiological agent in both squamous and adenocarcinoma of the cervix, with high-risk HPV types 16 and 18, detected in more than 95% of cervical cancers (277). Other cofactors may influence the progression from

infection to cancer. These cofactors can either be specific to the host, to the virus itself or to the environment. Tobacco, Chlamydia infection in HPV-positive patients, multiparity, family history of cervical cancer, immunosuppression and HIV infection are statistically associated with cervical cancer, whereas the effects of oral contraceptives are controversial (278).

The human papillomavirus is organised into two groups: the low-risk types that include HPV 6, 11, 31, 33, 35, 42, 43 and 44, and high-risk forms that include HPV 16, 18, 45 and 46. The high-risk viruses cause lesions which more frequently progress to malignancy, and are present in 70% of invasive squamous cell carcinomas. 90% of squamous cell dysplastic lesions are caused by HPV 16, and more than 50% of the invasive squamous intraepithelial tumours contain HPV 16. In contrast, HPV 18 is implicated in small cell undifferentiated tumours and adenocarcinomas (277).

Of the three causative molecular mechanisms of cervical cancer, two are associated with HPV: firstly, the effect of the viral oncogenes, E6 and E7 and secondly, integration of the viral DNA into chromosomal regions of tumour DNA. The third process involved is the repetitive loss of heterozygosity in some chromosomal regions.

E6 and E7 influence pathways involved in cell cycle control by interacting with two important tumour suppressor proteins, p53 and RB (277). Binding of E6 protein with p53 results in a loss of p53 activity within the cells. The infected cells, expressing a mutant form of p53, fail to exhibit cell cycle

arrest in G1 phase after DNA damage and acquire the ability to escape apoptosis induced cell death (277). The E7 viral protein binds to the retinoblastoma protein (RB), which negatively regulates cell growth and proliferation, and inactivates RB function within the cells. E7 expression inactivates not only RB but also its related proteins, such as p21 and PCNA, therefore avoiding cell cycle arrest after DNA damage. However, evidence suggests that HPV alone is not sufficient to induce malignant transformation and other host genetic and epigenetic variations are equally important in the development of cervical cancer (279) (280).

1.9.2 Evidence for the existence of CSCs in squamous cell carcinoma

The vertebrate skin is composed of an epidermis and a dermis which derive from embryonic ectoderm and mesoderm, respectively. The epidermis is a stratified squamous epithelium made up of multiple layers of keratinocytes resting on a basement membrane that separates it from the underlying dermis. The basal layer of epidermis is a heterogeneous population of proliferative and differentiating cells. A subpopulation in the basal layer, known as epidermal stem (EPS) cells, give rise to transit amplifying (TA) cells by symmetric or asymmetric division (281). The interfollicular epidermis, hair follicle, and sebaceous gland all arise from TA cells (282). The EPS cells are characterized by unlimited capacity for self-renewal, high expression of β1-integrins, and rapid adhesion to extracellular matrix (ECM) proteins (283). The TA cells are programmed to undergo terminal differentiation after a few cell divisions. They also

express lower levels of β1-integrins and adhere more slowly on ECM proteins than the stem cells (283) (284). Like stem cells of other tissues, EPS cells are important because they not only play a central role in homeostasis and wound repair but also represent a major target of tumor initiation.

The mechanism by which HPV alters cell cycle regulation and may drive cervical EPS into carcinogenesis, remain the main questions to be answered. The self renewal of normal stem cells is tightly regulated and responds to feedback mechanisms which regulate cell division and differentiation. HPV infection could involve normal cervical stem cells and *via* E6 and E7 viral proteins effects could be responsible for their malignant transformation. In this scenario infected cervical stem cells may directly acquire CSC properties, such as abnormal proliferation, cell cycle de-regulation and evasion from apoptosis and senescence mechanisms (277).

These considerations, combined with the existing evidence obtained from analysis of human epidermal tumours and genetically modified mice, suggest that the stem cell populations in different epidermal compartments may be the targets of tumorigenesis. Tumours, including squamous cell carcinomas, sebaceous adenomas and basal cell carcinomas display characteristics of multilineage differentiation, an aspect of the normal parental stem cell. This evidence supports the theory that these types of tumours are propagated and maintained by a population of cancer stem cells (285).

In work published in 2008 it was demonstrated that markers that are co-expressed in normal stem cells were not co-expressed in the tumour mass of poorly differentiated human SCCs and immortalized SCC cell lines (oral cavity and cervix). Down-regulation of two markers, Lrig1 and MAP4, and up-regulation of a third, MCSP, were correlated with poor differentiation status and increased proliferation in primary tumours (286). However, this does not correlate solely to an extension of the epidermal stem cell compartment, but it is probably due to TA cell amplification. This suggests that SCC cells assume certain properties of the normal epidermal stem cells, but also down-regulate the pathways involved in stem cell quiescence (286).

Particular attention in the epidermal cancer stem cell field has been given to Head and Neck Squamous Cell Carcinoma (HNSCC), which are often invasive, recurrent and chemotherapy-resistant (287). The cancer stem cell subpopulation of HNSCC was characterized using methods previously adopted to typify breast cancer stem cells (236).

Prince et al. demonstrated that both SCC primary tumours and secondary xenografted tumours expressed the CSC marker CD44 to a variable degree. Only cancer cells positive for CD44 were tumorigenic and showed an undifferentiated basal-cell like phenotype. The tumours formed by purified CD44+ cells reproduced the original tumour heterogeneity and could be serially propagated, demonstrating two crucial properties of stem cells: the ability to self-renew and to differentiate. Furthermore, the tumorigenic

CD44+ cells differentially expressed the *BMI1* gene, at both the RNA and protein levels (288).

In another work, cultured cell lines derived from HNSCC were also shown to contain a cell subpopulation with stem cell properties (287). All cell lines had a fraction of cells able to export Hoechst dye but the rate of exclusion varied distinctly from one cell line to the other. Similarly, all cell lines formed cancer spheres when grown in suspension but there was difference in the size of spheres and in their patterns of formation. High expression levels of stemness markers, such as β-catenin, CD44, β-integrin and CD133, were limited to the surface region of holoclone cells, which characterize the undifferentiated fraction of tumour cells. Interestingly SCC cells sorted for CD44/CD133 are clonogenic, but the double positive population was very small and not yet functionally characterized (287).

Another interesting paper published in 2009 identified a subpopulation of CD44+/CK17+ cells with stem-like properties in patient primary carcinoma of the cervix uteri. From the 50% of tumour samples analyzed they were able to obtained primary cultures of spheres, which self renew and proliferate extensively both *in vitro* and *in vivo*. CD44+/CK7+ sphere cells were partially inhibited by chemotherapeutic drugs and expressed stemness-related genes, such as Oct-4, Stat3, C-myc and Sox2 (289).

These *in vivo* and *in vitro* studies support the hypothesis that SCC cancer stem cells are enriched in a subpopulation of CD44 positive cells. The heterogeneity of CD44 expression is not sufficient alone to identify the

tumour initiating cells subpopulation within this type of cancer and therefore other markers need to be discovered.

Recently, podoplanin was identified as another putative marker for tumour-initiating cells in one SCC cell line, A431 (290). Podoplanin positive cells also expressed CD44, had higher colony-forming potential *in vitro* and induced tumours more rapidly than unsorted or podoplanin negative cells (290). In addition podoplanin has been previously shown to promote EMT (291), suggesting its possible role in the SCC stem cell phenotype. However, like CD44, podoplanin appears to only enrich for the population of SCC stem cells, because the dose of cells required for tumorigenesis *in vivo* was still too high (290). In light of these studies, it appears that there is still a need to identify more SCC cancer stem cell/tumour initiating cell markers to more precisely identify the apparent hierarchy of cancer cell populations within the tumours.

An alternative approach to identify SCC cancer stem cells has been to discover and characterize the SP cells by their ability to efficiently pump out Hoechst 33342 dye. Because SP cells of normal epidermis show characteristics of epidermal stem/progenitor cells (31), SP cells may be also potential candidates for the identification of cancer stem cells in SCC. In one study, three different oral SCC cell lines were shown to contain a SP (287) and a subsequent study using a different set of SCC cell lines revealed that the SP has a higher clonogenic ability and proliferation rate than their parental cell lines (292). After serial *in vitro* propagation, these cells maintain elevated expression levels of the epidermal stem cell marker

CSPG4/MCSP. Significantly, purified SP cells also showed increased tumorigenicity, even though the injected tumour cell dose was comparatively high (292). Thus, SCC SP cells display characteristics of cancer stem cells and could be combined with CD44 and podoplanin expression for further analysis of SCC tumour initiating cells.

We have also contributed in this field of research, investigating the tumour fraction with cancer stem/tumour initiating cell characteristics in three human cervical carcinoma cell lines A431, Caski and SiHa (293). Considering the already published data, our primary approaches for the isolation of SCC stem cells were the growth as non-adherent spheres in specific media and aldehyde dehydrogenase (ALDH) enzymatic activity. A good correlation between the two parameters was observed and the highest levels were observed in the A431 cell line that was selected for characterization of the CSC/TIC fraction (see Results chapter for more details). Together our results indicated that the growth of A431 cells as a non-adherent sphere was not sufficient by itself to define a stem-like population, but it was essential for the emergence of a small population of tumour cells with CSC properties.

In conclusion, a lot of data have highlighted the possible phenotypes associated with SCC cancer stem cells both *in vitro* and *in vivo*, but it remains to be established the specific marker profile of this subpopulation in SCC and how to translate this knowledge for patient benefit.

1.10 Human ovarian cancer

1.10.1 Epidemiology and aetiology

Ovarian cancer is the major cause of mortality among gynecological malignancies, and represents the fifth most common cause of cancer death in women in the Western World. The age at diagnosis, degree of disease, success of primary surgery, and the histopathological features of the tumour are important prognostic markers (294). Over the last decades, ovarian cancer incidence has remained stable and, despite recent advancements in treatment, the overall five-year survival rate continues to be low, probably because over 70% of ovarian tumours are diagnosed when regional or distant involvement has already occurred (295).

The aetiology of ovarian cancer is poorly understood. Factors associated with an increased risk for invasive epithelial ovarian cancer include age, race, nulliparity and family history of ovarian cancer, endometrial or breast cancer. Factors associated with a reduced risk are history of one or more full-term pregnancies, use of oral contraceptives, history of breast feeding, tubal ligation, and hysterectomy. Other factors such as infertility drugs, hormone replacement therapy, age at menarche, age at menopause, dietary factors, lactose intolerance, talc use, coffee and alcohol consumption have been suggested, but their role is still inconclusive (296).

Depending on the cell and anatomic structures from which the tumour presumably originates, most of the ovarian cancers can be placed into one of three major categories (297):

1. Epithelial ovarian carcinoma: ovarian surface epithelial cells (OSE) are organized as a single cell layer on a sheet of basement membrane. In ovarian carcinoma (EOC), genetic and epigenetic changes enable OSE cells to escape positional control and proliferate in a disorganized fashion. Two type of ovarian surface epithelial lesion have been described as a possible precursor of carcinoma (297).

2. Stromal tumours: are ovarian tumours that probably originate from theca cells and/or granulosa cells. These two types of cells are located within the stroma of the organ, surrounding the germinal cells that form the follicles. Some of these tumours have a fibrous appearance and therefore are named fibromas or thecomas. Most of the stromal tumours are benign and surgical excision is curative (297).

3. Germ cell tumours: are ovarian tumours originating from cells believed to derive from primordial germ cells. More than half of the ovarian neoplasms that develop in children and adolescents are of germ cell origin, with one-third of these being malignant. Conversely, in adults, germ cell tumours are relatively infrequent, and the great majority of them are benign (297).

1.10.2 Epithelial ovarian cancer

It is broadly accepted that the bulk of ovarian cancer arises from ovarian epithelial cells of the surface or inclusion cyst epithelium, which represent some 90% of all ovarian malignancies (298). Recently it has been suggested

that the fallopian tube epithelium may play a more important role in the development of Epithelial Ovarian Cancer (EOC) (299) (300).

Ovarian epithelium is derived from the coelomic epithelium and this developmental heritage of pluripotency might have conferred to OSE cells the potential to differentiate into a variety of structures seen in their respective forms of cancer.

EOC are classified as benign if they lack exuberant cellular proliferation and invasive behaviour, as borderline if there is exuberant cellular proliferation but no invasive behaviour and as malignant if there is invasive behaviour (297).

The high mortality of this tumour is largely explained by the fact that the majority (75%) of patients present at an advanced stage, with widely disseminated metastatic disease within the peritoneal cavity.

Ovarian carcinoma metastasizes either by direct extension from the ovarian/fallopian tumour to neighbouring organs (bladder/colon) or when cancer cells detach from the primary tumour. Exfoliated tumour cells are transported throughout the peritoneum by physiological peritoneal fluid and disseminate within the abdominal cavity. Extensive seeding of the peritoneal cavity by tumour cells is often associated with ascites, particularly in advanced, high-grade serous carcinomas. These cancers grow rapidly, metastasize early, and have a very aggressive disease course. Unlike most other cancers, ovarian carcinoma rarely disseminates through the vasculature. However, pelvic and/or para-aortic lymph nodes can be involved.

Current treatment strategies for advanced EOC consist of aggressive surgery ("cytoreduction" or "tumour debulking"). Postoperatively, all women, except those with very well-differentiated early stage cancer, receive chemotherapy with platinum (carboplatin, rarely cisplatin) and a taxane (Taxol, rarely taxotere). Although patients respond initially to the standard combination of surgery and chemotherapy, a large majority succumb later through development of recurrent, therapy-resistant disease (301).

EOC are classified into five major subtypes: serous, mucinous, endometrioid and clear cell (299).

Highly malignant epithelial ovarian tumours lacking any specific differentiation are classified as undifferentiated. Serous or mucinous tumours identical to those occurring in the ovary may arise in multiple locations within the pelvic and abdominal cavities. By convention, when the ovaries appear to be incidentally involved and do not appear to be the primary origin of the tumour, the tumour is recorded as an extra-ovarian peritoneal carcinoma (297).

Serous Epithelial Ovarian Cancer

This type of EOC is formed by cells that resemble those of the internal lining of the fallopian tube. They represent the majority of EOC and two-thirds are bilateral. Serous carcinoma is widely disseminated at the time of diagnosis, often invades through the ovarian capsule and grows on the surface of the ovary (297) (298).

Mucinous Epithelial Ovarian Cancer

Mucinous tumours are epithelial ovarian cancers formed by cells that resemble either those of the endocervical epithelium (endocervical or Mullerian type) or, more frequently, those of the intestinal epithelium (intestinal type). Malignant mucinous tumours represent 5-10% of all malignant ovarian neoplasms and one-third are bilateral. Late extra-peritoneal recurrences, particularly in the lungs, are characteristic of malignant mucinous tumours (297) (298).

Endometrioid Ovarian Cancer

Endometrioid tumours are epithelial ovarian tumours formed by cells that resemble those of the endometrium. They may be associated with the aberrant presence of endometrium outside the uterus (endometriosis) and with overgrowth (hyperplasia) or cancer of the endometrium. These tumours make up the second most common malignant ovarian surface epithelial tumour type and are rarely bilateral. Most malignant endometrioid tumours are confined to the ovaries and adjacent pelvic structures. Malignant ovarian endometrioid carcinomas are considered to have a better prognosis than either mucinous or serous carcinomas (302).

Clear Cell Ovarian Cancer

Little is known about the development and progression of this type of EOC. Most of them are malignant and studies have shown that 5-10% of cases are associated with endometriod lesions. Two-thirds of all women with malignant clear cell tumours have never given birth, and 50-70% have

endometriosis. They can be predominantly solid or cystic with one or more polypoid masses protruding into the lumen. Survival rates for clear cell carcinomas are poorer than for other surface epithelial carcinomas (303).

Studies of ovarian carcinoma have successfully connected genetics to tumour behaviour, giving a good explanation to such variety within the same subtype of ovarian cancer. Indeed, the four major histopathologic types of ovarian carcinoma are characterized by rather distinctive, though not necessarily unique, genetic abnormalities (299) (304). Low grade serous carcinoma carry activating mutation in *KRAS* and *BRAF* genes, whereas deregulation of the tumour suppressing pathway p53 and BRCA1/2 is more common in high grade serous tumours, even if they may be present in their low grade counterparts. Moreover KRAS mutation is supposed to be an early event also in ovarian mucinous carcinogenesis.

Silencing mutations of *PTEN* tumour suppressor and concomitant activation of the PI3/Akt cascade, as well as deregulation of the Wnt/β-catenin/Tcf pathway, are highly specific to endometrioid carcinoma. The profiles of clear cell carcinomas showed that they were similar to those of renal and endometrioid carcinomas, implying that certain molecular events are common to clear cell tumours, regardless of the organ of origin. In this type of EOC, p53 mutation is rare, but Her2/Neu expression is two times higher than that observed for the other subtypes. (304)

More recently, a comparison of the gene expression profiles of serous, endometrioid, and clear cell types of ovarian cancer with that of normal ovarian surface epithelium revealed 43 genes that were common to all

types of EOC (305), suggesting that the process of ovarian tumorigenesis involves a common pathway (306).

1.10.3 Human ovarian cancer stem cells

A clear correlation with surface markers that leads to the identification of specific stem and progenitor subsets have been adequately achieved in the haematopoietic system, or in organs such as breast and brain. Using the same markers, CSCs were thereby isolated and studied in the corresponding tumours. Over the last few years, several studies have focused on the isolation, identification, and characterization of stem cells from normal ovarian tissue as well as from ovarian tumour samples.

Differentiation potential in the ovary has been connected with the ovarian surface epithelium. OSE has retained properties of relatively uncommitted pluripotential cells as reflected by its growth potential, its capacity to reverse their phenotype in response to environmental changes, and its ability to differentiate along numerous pathways. These properties make OSE cells responsible of wound repair after post ovulatory rupture. This immature state may be responsible, in part, for the propensity of OSE to undergo neoplastic transformation, and may be the principle cause that makes OSE cells the possible source of ovarian cancer stem cells (299).

Bukovsky et al. maintained that mesenchymal cells in human ovarian tunica albuginea are capable of differentiating into OSE cells by a mesenchymal-epithelial transition *in vivo*. Through this transition, mesenchymal cells in the tunica albuginea give rise to primitive granulosa cells in the ovarian

cortex and to primitive germ cells which differentiate into oocytes and assemble with granulosa cell nests to form new primary follicles (16).

Another study showed that granulosa cells may undergo a de-differentiation process which revert these cells to a stem cell state, supported by the expression of *OCT4* and by their ability to differentiate into neuronal, chondrocytic and osteoblastic lineages (307) (FIG. 1.11). Up to now, a unique source of normal and cancer ovarian stem cells is not yet defined and future studies need to be undertaken to clarify this issue.

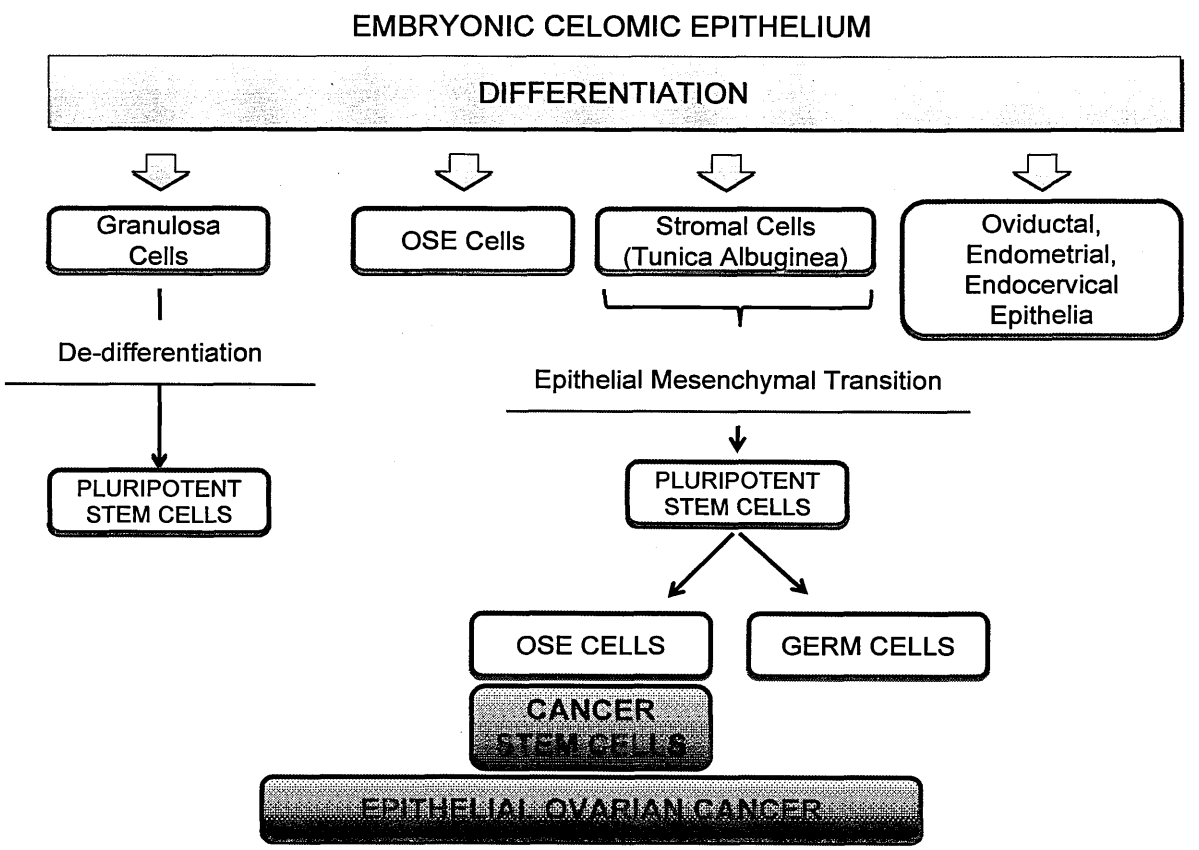


Figure 1.11: Possible origin of normal and cancer ovarian stem cells

Lack of accurate information regarding the origin and biology of normal ovarian stem cells, has been a restrictive factor in the identification of ovarian CSCs. For the isolation of putative ovarian CSCs from the bulk of an ovarian malignancy a number of different approaches have been used.

In the first study, the authors used tumour cells isolated from ascitic fluid from patients with advanced stage ovarian carcinomas (308). Characterization led to the identification of two spontaneously immortalized clones, among nineteen, that showed characteristics of CSCs. All the clones were positive for the presence of cell surface markers such as c-kit, stem cell factor (SCF), CD44, EGFR and E-cadherin, but only the two CSC clones had the ability to form spheroids in suspension, multicellular colonies in soft agar and were associated with *in vivo* tumorigenicity. This study was the first to support the association of cancer stem cells with serous ovarian cancer.

Another work performed in the same laboratory showed, through mitochondrial genome profiling, that the ovarian CSCs are probably derived from a single mutant stem cell clone (309).

Beside the ability to grow *in vitro* as spheres, in an anchorage-independent manner, another common approach used to isolate and identify the CSC subpopulation is based on the cell surface phenotype. A lot of efforts were spent on the identification of a specific surface-marker profile for ovarian CSCs. In this context, CD133 antigen has proved to be a marker of tumour cells with stemness features in several human malignancies (310).

Upon comparing the percentage of CD133 positive cells between normal ovaries, benign ovarian tumours, primary ovarian carcinoma and omental metastasis, Ferrandina et al. found that the primary tumours expressed the highest levels of the two CD133 epitopes (AC133-1 and AC133-2) (259). The CD133+ cells represented less than 1% of the total population, which is one of the defining characteristics of CSCs. The CD133+ cells were able to form colonies and had a higher proliferation potential than CD133- cells *in vitro*. A limitation in this work is that the authors did not identify a strict connection between CD133 and the functional criteria for defining the CSC subpopulation, such as *in vivo* self renewal and chemoresistance.

Subsequently, another study supported the putative role of CD133 positive cells in ovarian tumours and additionally identified that epigenetic dysregulation of CD133 may be connected with neoplastic transformation (260). This research demonstrated that ovarian cancer CD133 expressing cells were able to self renewal, giving rise to both CD133 positive and negative cells, and were associated with enhanced resistance to platinum-based therapy. However, the fact that ovarian CD133 + cells showed a gene expression signature similar to that of other cancer-initiating cells seemed to be an indirect support of the hypothesis. Another limitation to the idea that CD133 + cells represent ovarian CSCs arises from the fact that CD133 negative cells could also generate tumours in mouse models.

This anomaly was more recently clarified by Curley et al. They demonstrated that CD133 + ovarian tumour cells have more tumorigenic

capability than CD133-cells and have the ability to regenerate a heterogeneous tumour similar to that found in the original patient (261).

CD133 positive cells in ovarian cancer were also shown to be involved in the establishment of tumour vasculature that is critical for tumour cell survival during disease progression (268). Using mouse models, the authors demonstrated that endothelial cells are actively recruited by functional CSCs for tumour neovasculogenesis, but, conversely to Curley's reports but similar to Baba et al., they did not observe any difference in terms of tumorigenic potential between CD133 + and CD133- cells.

In conclusion, although CD133 initially has appeared to be a weak candidate for the identification of ovarian CSCs now there is increasing evidence supporting its role as an ovarian CSC marker.

Another putative ovarian CSC marker that has emerged in the last few years is CD44, the hyaluronic acid receptor. This molecule is expressed in numerous tumours and was also described as CSC marker in breast, colon and squamous head and neck cancers. Two major papers have been published supporting this role for CD44 in EOC (311) (312). In the first report an initial enrichment of cancer stem cells from ovarian tumour samples was obtained through their ability to grow in suspension as spheres. Sphere cells expressed stem cell markers, such as Bmi-1, Nanog, Oct4, ABCG2, and were characterized by the surface phenotype CD44 and CD117 (c-Kit). Similar to ovarian cancer spheroids, injection of 100 CD44+/CD117+ cells were able to propagate the original tumour, whereas the double negative subpopulation remained non- tumorigenic even when

injected at a much higher cell dose (311). Alvero et al. added new insight into the role of CD44 in ovarian CSCs. CD44 positive ovarian cancer cells had distinct genetic and miRNA profiles, are responsible for the ability to recapitulate the original tumour and are able to proliferate in presence of chemotherapeutic compounds. Moreover CD44+/MyD88+ cells showed constitutive NF-κB activity and cytokine and chemokine production, high capacity for repair, resistance to TNFα-mediated apoptosis, capacity to form sphere *in vitro* and self renewal/tumorigenicity *in vivo* (312).

The involvement of MyD88 may connect ovarian CSCs to tissue repair process dependent on TLR4 (Toll Like Receptor-4)/MyD88 pathway (313) (314). Thus, CD44+/MyD88+/TLR4+ EOC cells could respond to TLR4 ligand by activating the NF-κB pathway, sustaining the process of repair/differentiation triggered by the cancer stem cells.

The same group also demonstrated that CD44 + EOC cells are able to form vessel-like structures *in vitro* and are able to acquire an endothelial-specific phenotype (CD44+/VE-Cadherin+/CD34+) dependent on IKKβ/NF-κB pathway and not on the Vascular Endothelial Growth Factor (VEGF) (315). Finally, Bourguignon et al. demonstrated the involvement of CD44 positive cells in the drug resistance mechanism, using an ovarian cancer cell line SKOV3.ipl, established from ascites developed in nude mice after an intraperitoneal injection of SKOV3 cells. The binding of hyaluronan (HA) to its receptor CD44 promotes Nanog association with CD44, Nanog nuclear translocation and subsequent expression of Nanog target genes, such as *Rex1* and Sox2, pluripotent stem cell regulators. Therefore, it is

likely that the HA-CD44 interaction serves as an upstream activator of Nanog signaling. Moreover hyaluronan-CD44 interaction recruits Nanog and favours its functional association with Stat3. This complex enhanced the transcription of Stat3 specific target genes, leading to MDR1 gene expression and tumour cell growth, as well as multidrug resistance (316).

To complete the spectrum of ovarian CSCs markers, two other papers have to be mentioned. The first published by Gao et al. identified CD24 positive EOC cells as those that possessed stemness properties. Conversely to what has been observed in breast CSCs, ovarian CD24 positive cells seem to possess stem cell-like characteristics, such as quiescence and chemoresistance, as well as a specific capacity for self-renewal and differentiation. In addition, CD24 + cells were more tumorigenic than an equal number of CD24 - cells and expressed higher mRNA levels of some 'stemness' genes, including *NESTIN* , *β-CATENIN*, *BMI-1*, *OCT3/4*, *NOTCH1* and *NOTCH4*. Furthermore CD24 + cells possessed a mesenchymal phenotype, as suggested by their lower E-Cadherin mRNA level than the CD24- cells (317).

In another work, Lin28 and Oct4 human embryonic stem cell markers were suggested as putative ovarian CSC markers. In this report, Lin28 and Oct4 co-expression was found both in ovarian cancer cell lines and patient tumour samples and was correlated with advanced tumour grade. When the expression of these two proteins was repressed in the same cells, there was a significant reduction in cell growth and survival. Consequently the authors proposed that Lin28 and Oct4 may have an important role in the

initiation and progression of EOC, and may serve as an important molecular and therapeutic target for future treatment strategies (318).

The capacity to extrude Hoechst dye has also been used to identify ovarian cancer stem-like cells (319) (320). This dye-excluding side-population (SP) ability in used in various normal and cancer tissues to select for presumptive stem-like cells. It is reasonable that the SP phenotype coupled with specific marker expression may be the most functional assay to isolate and characterize the CSC subpopulations from tumour samples. In this regard Szotek et al. identified and characterized SP cells from genetically engineered mouse ovarian cancer cell lines, human ovarian cancer cell lines and human ovarian patient tumours. SP cells from both mouse and human ovarian cancer showed the ability to regenerate tumours with lower latency and higher frequency than the non-SP cells that remain responsive to doxorubicin and to Mullerian Inhibiting Substance (MIS) (321). The finding that SP-derived CSCs were highly resistant to doxorubicin, through ABCG2 expression, accounts for evasion mechanisms under chemotherapy; it further supports the candidature of these cells as being CSCs.

Recently, another exhaustive study addressed the optimization parameters essential to identify and isolate the SP in ovarian cancer, using the human ovarian cancer cell line OVCAR3 as an *in vitro* model (322). In this study only 0.9% of the whole population was isolated as SP and these cells had the capacity to form holoclones and to express the self-renewal marker Oct4.

Similarly Hu L et al. showed that SP of ovarian cancer cells, collected from primary ovarian cancer patient ascites and from different types of epithelial ovarian cancer cell lines, expressed the stem cell markers Nanog, Stellar, Oct4 and ABCG2. SP cells were more tumorigenic than non-SP cells showed an extensive cell proliferation capacity and were more resistant to chemotherapy *in vitro* and *in vivo,* compared with non-SP (323).

The tumorigenic and chemoresistant phenotype of ovarian SP were confirmed by another study. In collaboration with our group, Moserle et al. investigated the presence of SP in epithelial ovarian cancer and found it to be present in 33% of primary tumour samples analyzed, as well as in 4 of 6 cultures from xenotransplants (324). They found that SP cells had higher proliferation rates, were much less apoptotic compared with non-SP cells, and gave rise to tumours faster than non-SP cells. Moreover, IFN-alpha treatment was followed by a marked reduction in SP size in tumour cell lines of different origin. Isolated SP cells treated with IFN-alpha were associated with a peculiar change in their transcriptional profile. These findings could have important clinical implications because they imply that tumours bearing large SP numbers, albeit rare, could be sensitive to IFN-alpha treatment.

Together these reports have emphasized the role of SP in CSC enrichment and have tried to optimize the technical procedures for identifying the SP within heterogeneous cancer cell populations.

Drug resistance is a hallmark of CSC subpopulations and a lot of effort and experimental approaches have been developed for the identification of this

property. Besides the isolation of the SP, the analysis of ALDH1 activity has also been used to identify the tumour initiating and drug resistant cancer cell population within different type of cancer (134). In the field of ovarian CSCs, three different and partially opposite examples were reported. In 2009, Chang et al. analyzed a large cohort of ovarian carcinomas using tissue microarrays to identify a possible correlation between ALDH1 expression and clinical factors, such as diagnosis, tumour grade and stage, and clinical response to chemotherapy. The results showed that ALDH1 was a favourable prognostic factor in ovarian cancer patients, contrary to what was observed in breast cancer where it is associated with poor patient prognosis. Probably ALDH1 may have a different function in ovarian cancer and its functional role in normal and neoplastic ovarian tissue needs to be determined with more efficiency (142).

On the other hand, Deng et al. showed that the percentage of ALDH1 positive cells in different types of tumour is significantly correlated with the level of ALDH1 expression in the corresponding normal tissue. In normal ovary the expression of this marker is limited compared to that observed in ovarian cancer samples and is significantly associated with poor clinical outcome in serous ovarian cancer (325).

A recent paper, published in October 2010 by Landen and co-workers (326), confirmed that ALDH positivity correlated negatively with progression-free survival of patients with EOC. Additionally, ALDH positive ovarian cancer cells, isolated from two pairs of ovarian parental and

chemoresistant cell lines (SKOV3ip1/SKOV3TRip2 and A2780ip2/A2780cp20), had increased but not absolute tumorigenicity. ALDH1A1 *in vitro* down-regulation sensitized ovarian cancer cells to chemotherapy whereas *in vivo* its down-modulation had an effect on tumour growth only if it was combined with chemotherapeutic treatments. These results suggest that the ALDH1 positive population had properties of CSCs and it is associated with ovarian cancer cell chemoresistance. Ovarian cancer cell lines can be re-sensitized to chemotherapy by down-regulation of ALDH1 both *in vivo* and *in vitro*.

In summary, these data indicate that the prognostic value of ALDH1 in ovarian cancer, although potent, may be histotype-specific. Indeed, based on clinic-pathological and molecular studies, different histotypes of EOC have remarkably individual molecular backgrounds and biological behaviours. They may be considered as different diseases, even though they are located in the same anatomic location. Consequently, the prognostic value of ALDH1 in different types of ovarian epithelial cancers still needs to be further investigated in large-scale independent sample sets.

A number of studies have contributed to the knowledge about the various phenotypes associated with ovarian cancer-initiating cells both *in vitro* and *in vivo*, but there remains a paucity of information about how to translate this knowledge for patient benefit. The ongoing characterization of these cells, combined with new technologies in sequencing, microarrays and imaging, will soon allow us to highlight the pathways and mechanisms

driving ovarian cancer-initiating cell function as well as the potential vulnerabilities that can be exploited for targeting these cells.

CHAPTER 2

MATERIALS and METHODS

2.1 Experimental approaches commonly used for cancer stem cell identification and isolation

2.1.1 Isolation assays

Marker expression: Fluorescence Activated Cell Sorting (FACS) or magnetic beads selection

There are several concerns regarding the isolation of stem cells based on marker expression, such as the enzyme treatment, loss of cell viability during the sorting procedure and the purity of the isolated fraction. Treatment of the tissue sample with enzymes, such as trypsin, would destroy the surface antigens and not only affects the ability to sort the suspension into specific populations, but might also influence the ability of these molecules to play a role in the early stages of repopulation *in vivo* or *in vitro*. Thus, techniques for dissociating cells must be carefully developed.

In addition, phenotypes, based on marker selection, often use terms, such as high, middle, low, and non-expression, to describe the properties of the

sorted cells for each marker; these terms are subjective and can vary depending on the method used for cell preparation. Additionally gate setting and the antibody preparation used could affect the efficiency of the approach. Although flow cytometry offers a sensitive, specific, and robust method of cell isolation and purification, some of its technical limitations make its application to stem cell purification a high risk. An alternative and common approach for the isolation of CSC is the immuno-magnetic beads selection/depletion. This system is based on super-paramagnetic particles of approximately 50 nanometers in diameter directly conjugated with a specific antibody or able to detect the fluorochrome of the primary antibody (anti-fluorochrome micro bead). After the staining, the magnetically labeled cells are separated over a specific column placed in a magnetic field-separator. The cells are retained on the column, while unlabeled cells pass through. The retained cells can then be eluted from the column and analysed by FACS.

Sphere assay

Several *in vitro* assays have been used to identify stem cells and CSC, including sphere assays and serial colony-forming unit (CFU) assays (replating assays).

Forced growth in suspension in the presence of specific growth factors was found a useful tool for isolation of undifferentiated multipotent neural and breast stem cells (327). Under these conditions, most of the cells die and surviving cells give rise to floating spherical colonies (spheres). One of the

most important characteristics of stem/progenitor cells is the ability to self-renew; only cells with self-renewal capability are able to sustain the growth in suspension, giving rise to non-adherent colonies. The growth in suspension has also been applied to the isolation and propagation of putative tumour stem cells from tumour cell lines and from patient tumour specimens (308) (328). However the rapidity of sphere growth in many systems makes it unlikely that they arose from single cells solely through clonal expansion. The complexity of distinguishing the relative contributions of aggregation and proliferation in sphere formation poses a major impediment to their use in isolating this subpopulation of cells.

Side Population and Aldefluor assays

Another phenotype used to distinguish cancer stem cells within a tumour is their presence within the side population (SP) fraction defined by Hoechst dye efflux properties. SP is a distinct, small cell population composed of unstained cells in the left lower quadrant of a Flow Activated Cell Sorter (FACS) profile. SP cells are identified and electronically gated on a cell sorter station after excitation of the Hoechst dye with 150 mW of 350 nm UV light. SP fluorescence emissions are directed toward a 610-nm dichroic filter and captured simultaneously through both a blue (450-nm) band-pass and a red (675-nm) long-pass filter on a linearly amplified fluorescence scale. Goodell et al. (329) have demonstrated that the exclusion of Hoechst 33342 by SP cells is an active process involving multidrug resistance transporters, such as MDR1 and ABCG2. SP has been used to isolate,

characterize and study malignant stem cells, because their ability to exclude nuclear dyes correlates with multidrug resistance, such as the expulsion of cytotoxic drugs (330).

SP has been identified in many normal tissues, including mammary glands (331), lung (332), brain (333) and skin (334) in both human and animal models. In addition to the identification of SP in normal cells, SP cells have been characterized in various tumours and cancer cell lines (293) (335) (336). However, as with cell surface markers, the existence of a SP phenotype is not a universal property of stem cells, and in some tissues, the SP fraction may not contain the stem cells. Experiments that identified normal breast stem cells by their ability to generate mammary glands in cleared fat pads showed that the majority of these cells are not included within the SP fraction. Possible toxicity of the dye should also be considered when interpreting functional assays based on SP cells.

The aldehyde dehydrogenase (ALDH) family is a group of cytosolic isoenzymes responsible for oxidizing intracellular aldehydes, thus contributing to the oxidation of retinol to retinoic acid in early stem cell differentiation (337). Specific ALDH activity is evaluated using the ALDEFLUOR assay based on the different staining in the presence or in the absence of the Aldefluor inhibitor diethylaminobenzaldehyde (DEAB). Aldefluor assay followed by FACS analysis assesses the presence and size of the cell population with ALDH enzymatic activity.

Isolated cancer cells from several types of tumour with relatively high aldehyde dehydrogenase 1 (ALDH1) activity display *in vitro* features of

CSCs, including capacities for proliferation, self-renewal and differentiation, resistance to chemotherapy and CSC marker expression (338) (339) (340). As for SP approach or for specific marker expression commonly used to isolate cancer stem cells, the Aldefluor assay may give misleading results, due to the fact that a high percentage of heterogeneous cancer cells show an elevated level of ALDH activity. For these reasons both SP and Aldefluor assays must be followed by a functional evaluation. Because of these complicating factors, it is safer to first isolate stem cells using other methods and then ask whether that particular stem cell population is indeed included within the SP and or ALDH positive population.

2.1.2 Functional assays

Self-renewal, tumorigenic and differentiation potential

Self-renewal and lineage capacity are the hallmarks of any stem cell. Therefore, as with normal stem cells, assays for cancer stem cell activity should include self-renewal and tumour propagation abilities.

The main approach used to examine the self renewal ability and, in particular, to monitor the type of division, as symmetric or asymmetric, is based on the staining with specific dyes. PKH or FCSE fluorescent cell linkers are lipophilic dyes that enable fluorescent labelling of live cells over an extended period of time, with no apparent toxic effects. During symmetric cell divisions the dye is portioned among the daughter cells, so that, after a number of mitotic divisions it is diluted and all the cells are no longer stained. On the contrary, as consequence of asymmetric division, the dye is retained by the daughter cell that remains a stem cell because it is mainly quiescent. Thus, label retention may be used as a marker of stem cells if they are relatively slow cycling. Since asymmetric division correlates with asymmetric partitioning of cell fate determinants, the analysis of the intracellular localization of a specific protein such as Numb after the first division, is another method to discriminate between symmetric or asymmetric cell division (91).

Up to now, the gold standard assay that is commonly accepted to discriminate between bulk cancer cells and CSCs is the serial

transplantation in animal models that, although imperfect, is considered as the best functional assay, because it demonstrates both self renewal and differentiation potential of the selected CSCs.

Recipients are immune-deficient mice with different grades of immunological defects: nude mice (B cell deficient), severe combined immunodeficient (SCID) and non-obese diabetic (NOD) mice (B cells and T cell deficient). More recently many investigators have turned to NOD/SCID/Il-2rγ^{-} (341) and Rag2$^{-/-\gamma c-/-}$ mice (342) as these mice lack the entire immune system.

In transplantation assays, cells are xenografted in immune-compromised mice that are monitored at various time points for tumour formation. To show self-renewal, cells isolated from the tumours are grafted into a second animal for more passages.

Issues complicating transplantation assays include potential effects of the grafting site. It is well known that normal stem cells are greatly dependent on signals from the surrounding stromal cells but it is not still clear what the effect may be on separating cancer stem cells from any supporting cells during the course of the assay.

Moreover the extraordinarily high level of genetic and epigenetic changes that take place within most cancer cells, may allow some cells to generate diverse cell types not because they are stem cells *per se* but because of their genetic/epigenetic instability.

Although serial transplantation assays remain the best developed method for identifying cells with properties of cancer stem cells, more precise and

simpler assays are likely to emerge as the favourite technique for their identification.

Another useful approach for the characterization of normal and therefore cancer stem cells is the capacity to undergo differentiation processes both *in vivo* and *in vitro*. After asymmetric division, one of two stem daughter cells gives rise to a proliferating transit-amplifying cell destined to terminal differentiation. Differentiation potential *in vivo* is usually assessed by the ability of isolated cancer stem cells to give rise to a heterogeneous tumour that resembles the original one. On the other hand, differentiation potential of cancer stem cells could be also evaluated by culturing cells under conditions favourable for tissue-specific differentiation. After culture in lineage specific conditions, the expanded cancer stem cells may be highly differentiated in to their tissue-specific lineages, as assessed on the basis of phenotypic characterization and histological analysis.

When analyzed *in vitro,* normal human skin keratinocytes give rise to three subpopulations based on their patterns of clonal expansion (343). Colonies with a round compact form, termed holoclones, are considered to be derived from stem cells, while clones with initial or advanced differentiation are called meroclones and paraclones respectively, being representative of early and late TA progenitor cells (344). The presence of these stem cell derived hierarchies is a functional and simple method to characterize cancer stem cell multilineage potential (293), but it is also an indication of the heterogeneous composition of the CSC subpopulation.

All the considerations listed above indicate that the cancer cell population is heterogeneous with respect to one of the key criteria that define stem cells and cancer stem cell isolation. As a result, it remains to be elucidated whether the term CSCs defines a specific state or, alternatively, indicates a gradient of phenotypic features along a spectrum of stemness and commitment that is permissive to self renewal.

2.2 General procedures and reagents

All the solutions were made using double-distilled H_2O stored in sterile containers. Details of all the general reagents used for experimental work of this thesis are tabulated below. Chemicals were supplied by Sigma-Aldrich (UK) or Merck (Germany).

Table 2.A: List of reagents

PBS	5.84g NaCl, 4.72g Na_2HPO_4, 2.64g NaH_2PO_4, 2 H_2O (pH=7.2) in 1 L
PBS $CaCl_2$ (10x)	79,4g NaCl, 2g KCl, 2g KH_2PO_4, 14,2g $Na_2HPO_4 \cdot 2$ H_2O, 1g $MgCl_2 \cdot 6H_2O$ in 1L +1, 32g $CaCl_2 \cdot 2H_2O$ in 1L
TAE (Tris Acetate EDTA)-50X	2M Tris, 2M glacial acetic acid, 50mM EDTA, pH=8.0
TBE (Tris Borate EDTA) 10X	0.5M Tris, 0.5M boric acid, 13mM EDTA, pH= 8.0
Gel loading dye buffer (6x):	25mg bromophenol blue, 1,25ml SDS10%, 12,5ml glycerol
MPBS	dried skimmed milk powder Marvel in PBS (Marvel is dried skimmed milk powder) at different %
PBST 0.1%	PBS - 0.1%Tween 20

Table 2.B: Western Blot reagents

Lysis buffer (RIPA buffer)	50mM Tris-HCl (pH=7.4), 150mM NaCl, 1% NP40, 0.1% SDS, 0.5% NaDOC, protease inhibitor (complete mini, Roche)
Running buffer (MOPS)	50mM sodium acetate, 10mM EDTA, and 10mM EGTA, treated with DEPC (INVITROGEN)
Blotting buffer	39mM glycine, 48mM Tris , 0.037% SDS and 20% methanol

Table 2.C: List of antibodies and isotype controls

ANTIBODY SPECIFICITY	CONJUGATE	HOST SPECIES	SUPPLIER
Mouse anti-human CD44	APC	Mouse IgG2b	Becton Dickinson (Franklin Lakes, NJ)
Mouse anti-human CD45	FITC	Mouse IgG2a	MACS Miltenyi (Germany)
Mouse anti-human CD133	PE	Mouse IgG1	MACS Miltenyi (Germany)
Mouse anti-human CD117	PerCP-Cy5.5	Mouse IgG1	Becton Dickinson (Franklin Lakes, NJ)
Mouse anti-human CD184 (CXCR4)	APC	Mouse IgG2a	Becton Dickinson (Franklin Lakes, NJ)
Mouse anti-human EpCAM	PE	Mouse IgG1	eBioscience (San Diego, CA)
Mouse anti-human CD133	/	Mouse IgG1	MACS Miltenyi (Germany)
Mouse anti-human Folate Receptor	/	Mouse IgG	Home made
Mouse anti-human CD44	/	Mouse IgG1	DAKO (Denmark)
Mouse anti-human Vimentin	/	Mouse IgG1	Novocastra/Leyca Microsystem (Germany)

Rabbit anti-human N-Cadherin	/	Rabbit IgG polyclonal	Santa Cruz (Santa Cruz, CA)
Mouse anti-human Ki67	/	Mouse IgG1	Chemicon (Temecula, CA)
Mouse anti-human E-Cadherin	/	Mouse IgG1	Zymed Laboratories (Carlsbad, CA)
Mouse anti-human Cytokeratin 8/18	/	Mouse IgG1	Novocastra/Leyca Microsystem (Germany)
Mouse anti human SSEA1	/	Mouse IgM (ES cell marker sample kit)	Chemicon (Temecula, CA)
Mouse anti human SSEA1	/	Mouse IgM	Zymed Laboratories (Carlsbad, CA)
Mouse anti-human OCT4	/	Mouse IgG (ES cell marker sample kit)	Chemicon (Temecula, CA)
Rabbit anti-human OCT4		Rabbit IgG (polyclonal)	ABCAM (Cambridge, UK)
Mouse anti-Human Podoplanin	/	Mouse IgG1	ABCAM (Cambridge, UK)
Mouse anti-human CXCR4	/	Mouse IgG2	R&D System (Minneapolis, MN)
Rabbit anti-human CXCR4	/	Rabbit IgG	Biotrend (Germany)
Mouse anti-human CD117	/	Mouse IgG1	Chemicon (Temecula, CA)
Mouse anti-human Nanog	/	Mouse IgG2	Sigma-Aldrich (St. Louis, MO)
Rabbit anti-acetyl-Histone H3	/	Rabbit IgG	Upstate/Millipore (Billerica, MA)
Rabbit anti-acetyl-Histone H4	/	Rabbit IgG	Upstate/Millipore (Billerica, MA)
Isotype control	FITC	Mouse IgG2	MACS Miltenyi (Germany)
Isotype control	PE	Mouse IgG1	MACS Miltenyi (Germany)
Isotype control	PerCP-Cy5.5	Mouse IgG1	Becton Dickinson

			(Franklin Lakes, NJ)
Isotype control	APC	Mouse IgG2	Becton Dickinson (Franklin Lakes, NJ)
Alexa Fluor 546	/	goat anti- rabbit IgG	INVITROGEN (Paisley , UK)
Alexa Fluor 488	/	Goat anti-mouse IgG	INVITROGEN (Paisley , UK)

Table 2.D: list of probes for REAL TIME assay (APPLIED BIOSYSTEM)

GENES	PROBES
OCT4 (POU5F1)	Hs01895061_v1
NANOG	Hs02387400_g1
NESTIN	Hs00707120_s1
CD133	Hs01009261_m1
CXCR4	Hs00237052_m1
EGFR	Hs00193306_m1
GAPDH (VICMGB)	4325792

Cell lines used in this research and their culture conditions

The following cell lines were purchased from the American Type Culture Collection ATCC (Rockville, MD).

- A431 (ATCC, cat. No. CRL-1555) a squamous epidermoid carcinoma cell line, established from an epidermoid carcinoma of the vulva of an 85 year old female patient by D.J Giard, et al. (345).
- CaSki (ATCC, cat. No. CRL-1550) a cervical epidermoid carcinoma cell line, established from a metastasis in the small bowel mesentery.
- SiHa (ATCC cat. No. HTB-35) a cervical squamous cell carcinoma cell line, established from fragments of a primary tumour sample.

These cell lines were maintained in adherent conditions in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum (FBS) (Lonza, Walkersville, MD), and 1% glutamine (Lonza) at 37°C in 5% CO2 and sub-cultured by trypsin/EDTA (Lonza) detachment.

General procedures for maintenance of eukaryotic cancer cell lines

All manipulations involving cell culture were performed in a sterile environment provided by a laminar flow sterile hood. All liquid reagents were either purchased sterile or filtered through a 0.22 μm filter. Routine culturing was performed at 37°C in humidified incubators in the presence of 5% CO_2. All cell lines were routinely tested for mycoplasma infection using the MycoAlert Mycoplasma detection Kit (Cambrex) and positive cells were then excluded. Centrifugation of cells was usually achieved at 1200 or 1500 rpm for 5 minutes. Fetal bovine serum (FBS) (Gibco, CA) was heat

inactivated at 56°C for 40 minutes before using as supplement in the growth media.

Generally cell counts were obtained using a Burker's chamber visualized through an inverted microscope and cell viability was checked by Trypan blue exclusion. When a more precise count was needed, cells were counted with Countess ™ automated cell counter (Invitrogen, Italy) that performs cell count and viability measurements using trypan blue stain.

All cell lines were propagated in adherent conditions for different passages, using the following methods. After removing the culture medium, the cell layer was briefly rinsed with 0.25% (w/v) Trypsin- 0.53 mM EDTA solution to remove all traces of serum. Trypsin-EDTA solution was added to the cell culture and incubated for 5 minutes at 37°C in a humidified incubator in the presence of 5% CO_2. After cell detachment, 10 ml of complete medium was added to the flask in order to inactivate the Trypsin solution. The cell suspension was then centrifuged for 5 minute at 1500 rpm, resuspended in an appropriate volume of complete medium, counted when needed, and seeded to new culture vessels.

Spheres from parental cell lines were obtained by plating the cells at low density (1,000 cells/ml) in MEGM serum free medium, supplemented with 0.4% BPE, 0.1% hEGF, 0.1% hydrocortisone, 0.1% GA-1000, 0.1% insulin, 1% glutamine (MEGM BulletKit) (Lonza). The *in vitro* maintenance of spheres was performed in Ultra Low Attachment Plates (Corning, NY, NY) and spheres were trypsinized with TrypLE™ Express (Invitrogen, Carlsbad, CA). Spheres with a mean diameter of 50-150 μm, were monitored and

trypsinized every 4/5 days. Spheres re-seeded in adherent conditions were cultured in the same culture conditions of the relative parental cell line.

Storage and recovery of eukaryotic cells from liquid nitrogen

Harvested cells were pelleted by centrifugation and resuspended at approximately 10^6 cells /ml in FBS containing 10% v/v dimethylsulphoxide (DMSO), which prevents the formation of ice crystals. Aliquots of 1 ml were transferred to 1.5 ml cryotubes and stored overnight at -80°C. Frozen cells were transferred the following day to liquid nitrogen tanks (-196°C). Recovery of cells from storage was performed by rapidly thawing in a 37°C H_2O bath. Thawed cells were washed in 10 ml fresh and pre-warmed medium, pelleted by centrifugation and then transferred to the appropriate culturing conditions for the cell type of interest.

Processing ovarian tumour samples

Many solid tumours contain a heterogeneous population of normal and cancerous cells. Separation of these cell populations is the key to an accurate assessment of the true genotypic and phenotypic characterization of cancer cells. In order to isolate a subpopulation of cancer cells with characteristic of CSCs we developed different methods to obtain a single cell suspension, starting from surgical ovarian tumours (FIG 2.1).

Clinical specimens were obtained from a series of consenting patients according to the Internal Review and the Ethics Boards of the Istituto Nazionale Tumori of Milan.

All manipulations involving tumour samples were performed under a laminar flow sterile hood. All liquid reagents were either purchased sterile or filtered through a 0.22 μm filter.

Cancer samples were firstly mechanically processed using a scalpel and scissors. Blood, floating fat and necrotic fragments were discarded. Recovered tumour fragments were then re-suspended in specific medium and shredded in to smaller pieces. Single cell suspensions were filtered with 70μc or 100 μc cell strainers (Becton Dickinson) and checked for their vitality. At least one purification step, usually by Ficoll gradient, was applied to eliminate lymphocytes and red cells. Frequently, after processing, samples did not contain tumour cells sufficient to be immediately characterized and sorted for specific markers.

Depending on the consistency of the tumour samples, mechanical shredding alone was not sufficient to obtain and isolate a single cancer cell suspension. For this reason we set up and performed different enzymatic approaches. After the mechanical process, tumour fragments were suspended in the enzymatic solution and digested for different periods of time depending of the tissue consistency and on the reagents used.

Initially tumour fragments were suspended in the Triple Express Trypsin (GIBCO). Digestion was performed at 37°C in a humidified incubator in the presence of 5% CO_2 for a period of time ranging from 2h to overnight incubation. The cell suspension was then filtered with 70 μc or 100 μc cell strainers and checked for vitality. A purification step was required for the presence of lymphocytes and red cells. Using this approach the yield of

tumour cells was generally low and again, after processing, the available samples often did not contain sufficient tumour cells to be immediately characterized and sorted by specific marker expression.

In order to achieve a complete digestion of tumour fragments, we subsequently used the Accumax solution (Millipore), which is a proprietary solution that contains collagenolytic, proteolytic, and DNAse enzymes and is provided as a ready-to use solution. As an added benefit, Accumax solution does not contain any mammalian or bacterial components. Digestion was performed at room temperature with rotation for one or two hours and cell viability was generally checked every thirty minutes. Also in this case, after digestion, the tumour cell suspension was filtered and purified but again the yield of tumour cells was commonly insufficient for a phenotypic characterization by FACS analysis.

To limit the difficulties encountered with the procurement of high purity primary tumour cells and the low yield of purified cells, we used the Panomics' Cancer Cell Isolation Kit (Panomics, CA), which combines specific reagent compositions and handling processes to successfully isolate purified tumour cells. Firstly non-tumour and necrotic tumour tissue were picked out with sterile ophthalmological tweezers and tumour fragments were cut in small pieces. The pelleted material was then resuspended in Tumour Cell Digestion Solution and incubated at 37°C for 2-4 hour with agitation. The heterogeneous cell mixture was then purified with the Tumour Cell Purification solution, filtered with a 100 μc filter and analyzed for viability. With this method we obtained a purified tumour cell

suspension, as validated by FACS analysis, but the number of total cancer cells recovered was again not sufficient for a sorting procedure based on expression of a specific marker (FIG. 2.2).

As for the ovarian solid tumours, we set up different methodologies in order to isolate a purified cancer cell suspension from ascitic samples. Using enzymatic dissociation with the ACCUMAX solution we usually obtained a satisfactory yield of a viable single cell suspension. Digestion was carried out at room temperature with rotation for one or two hours depending of the size of tumour cell population, and cell viability was generally checked every thirty minutes. The tumour cell suspension was filtered and purified to eliminate necrotic tumour cells and inflammatory cells such as leukocytes and lymphocytes.

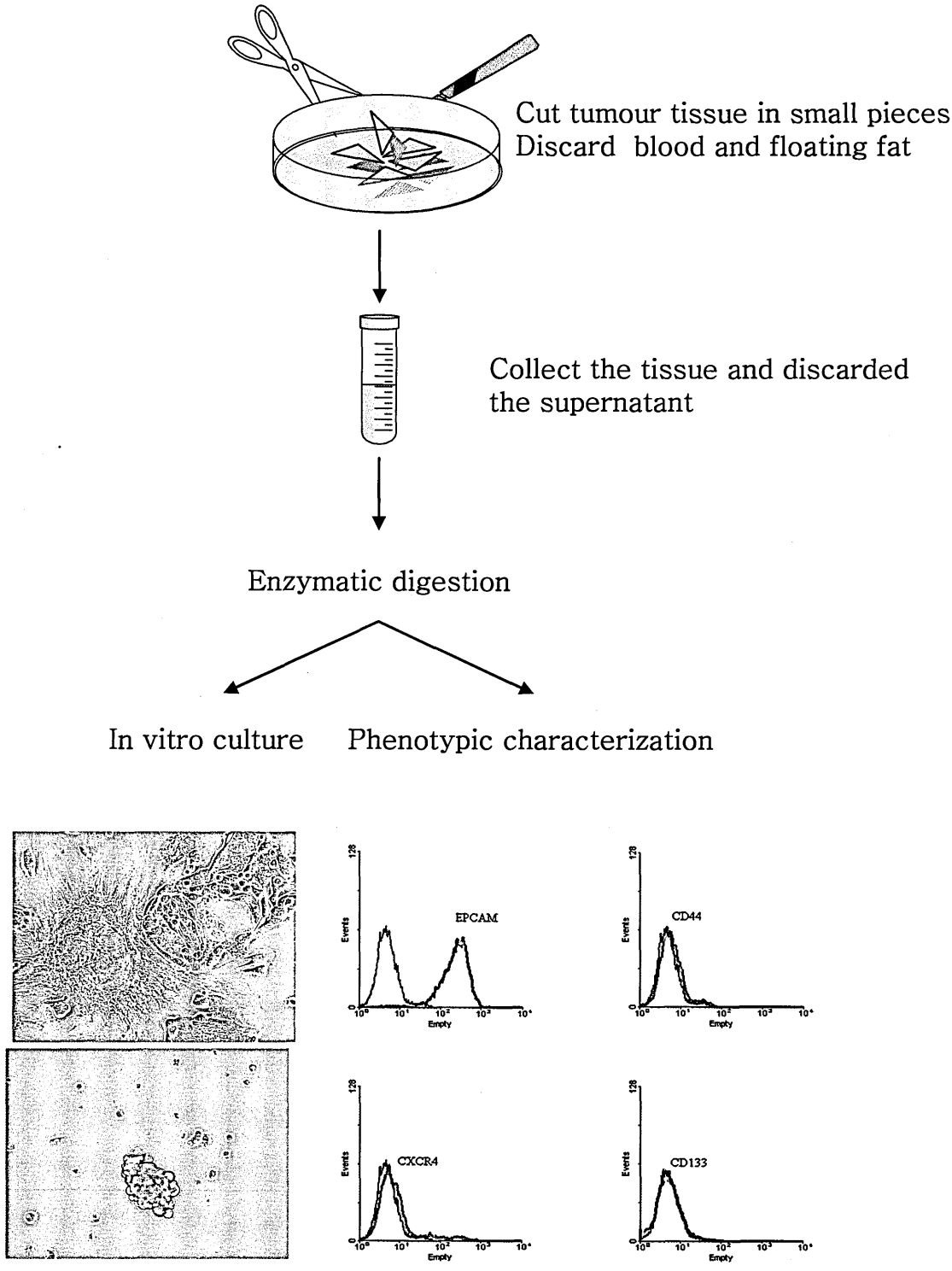


Figure 2.1: Processing ovarian tumour samples

ovarian solid tumours were cut in small pieces and processed with the methods described in the text. Fat and necrotic fragments were discarded. After processing, viable single cells were, when possible, seeded in different culture conditions and phenotypically characterized. Examples of ovarian tumour samples *in vitro* culture and phenotypic characterization are reported.

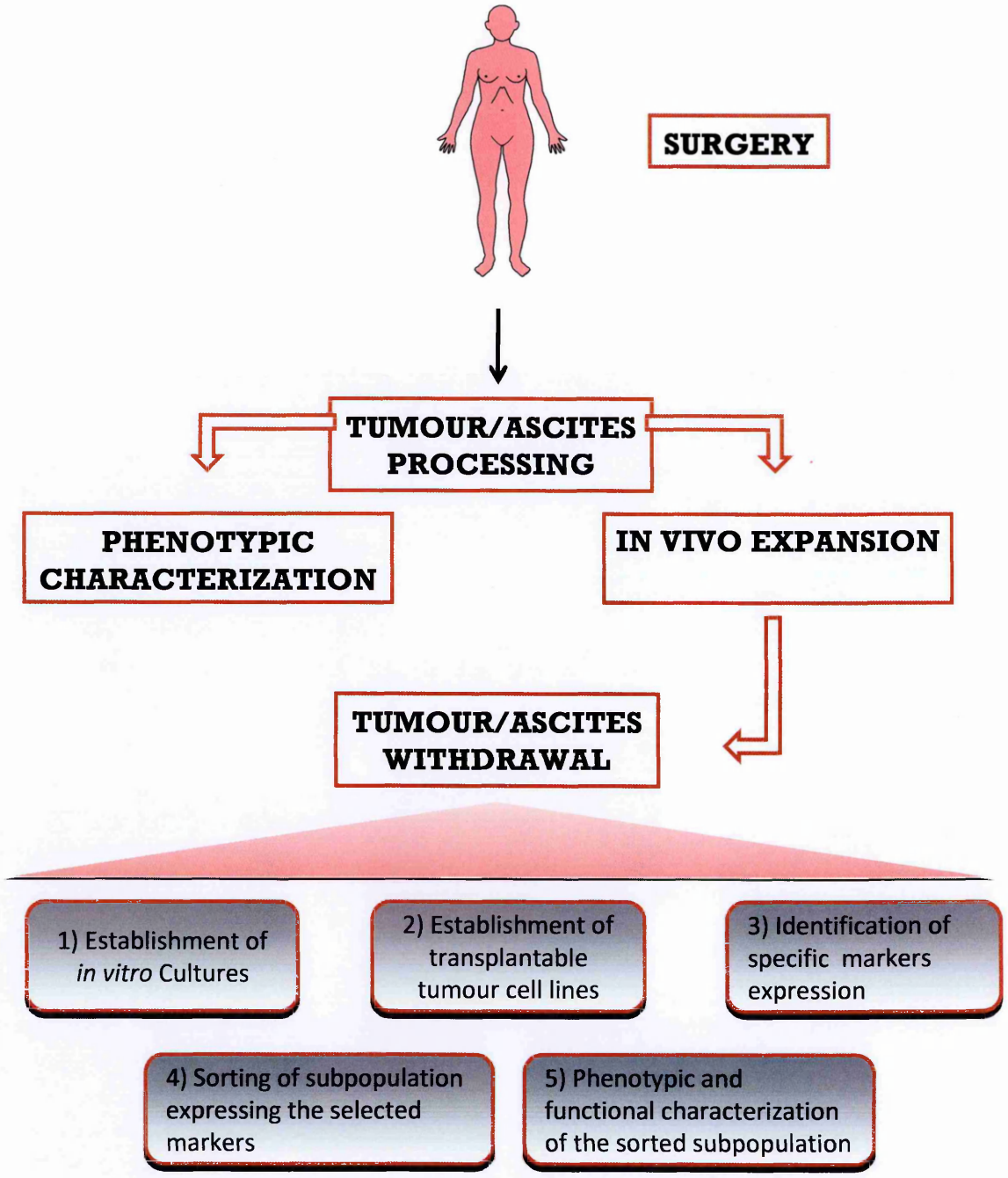


Figure 2.2: Experiment layout

after surgery, ovarian tumour samples, solid masses or ascites, were collected and mechanically and/or enzymatically processed. Tumour cells recovered were phenotypically characterized and expanded *in vivo* in a murine model. *In vivo* expansion could guarantee a continued source of material. After *in vivo* growth, cancer samples were again collected and processed, and the purified tumour cells were characterized for stem cell properties.

Culture conditions of ovarian cancer cells recovered from surgical specimens

All manipulations involving tumour cells were performed under a laminar flow sterile hood. All liquid reagents were either purchased sterile or filtered through a 0.22 μm filter. Routine culturing was performed at 37°C in humidified incubators in the presence of 5% CO_2.

Following solid tumour or ascitic clump digestion the isolated cells were seeded in adhesion for two hours in RMPI 1640 or MCDB 105/MEDIUM 199 1:2 (Sigma-Aldrich, St. Louis, MO), containing 10% fetal bovine serum (FBS) (Lonza, Walkersville, MD), and 1% glutamine (Lonza) to allow inflammatory and/or stromal cells adhesion to the plastic. The supernatant was then collected and cells were seeded in different culture conditions. Aliquots were plated in the presence of different concentrations of serum (10%, 1% and 0.1%) (Lonza, Walkersville, MD) and in different media (RMPI 1640 or MCDB 105/MEDIUM 199 1:2), as described above. Adherent cells were routinely checked and fresh medium added every 5 days. Cell detachment was performed after removing the culture medium; the Trypsin-EDTA solution was then added to the cell culture and incubated for 5 minutes at 37°C in humidified incubators in the presence of 5% CO_2. After complete detachment, 10 ml of complete medium were added to the flask in order to inactivate the Trypsin solution. The cell suspension was then centrifuged for 5 minutes at 1500 rpm, resuspended in an appropriate volume of complete medium, counted when needed, and seeded to new culture vessels.

Following ovarian cancer surgical specimen digestion, cells were seeded in suspension at low cell-density (1,000 cells/well or 5,000 cells/well) in MEGM BulletKit serum free medium, supplemented with 0.4% BPE, 0.1% hEGF, 0.1% hydrocortisone, 0.1% GA-1000, 0.1% insulin, 1% glutamine (Sphere medium) (Lonza). Sphere growth was monitored every two/three days and *in vitro* maintenance was performed in Ultra Low Attachment Plates (Corning, NY, NY). Spheres were trypsinized with TrypLE^{TM} Express (Invitrogen, Carlsbad, CA), as described for the culture of A431 spheroids.

2.3 General methods

RNA extraction

Total RNA was isolated with the RNAspin Mini RNA Isolation Kit (GE Healthcare, Piscataway, NJ). Approximately 10^7 cells were harvested and pelleted by centrifugation at 1200 rpm for 5 minutes. The pellet was lysed in 350 µl of lysis solution RA1 + 3.5µl β-mercaptoethanol. Then the lysates were mixed vigorously and filtered with RNAspin Mini filter units by centrifugation for 1 min at 13400 rpm. The same volume of 70% ethanol was added to the samples and mixed vigorously. The lysates were then filtered through a RNAspin Mini column, which binds and captures the RNA, and centrifuged for 1 min at 12300 rpm. After washing with Membrane Desalting Buffer (MDB), DNaseI solution was loaded on to the column to avoid possible DNA contamination and incubated for 15 min at RT. The RNA was then washed with RA2 buffer that inactivates DNaseI and twice with buffer RA3. Finally the RNA was eluted by applying 40 µl of RNeasy free H_20 to each mini-column

Agarose gel electrophoresis of nucleic acids

Gels were prepared by adding agarose (1 to 1.5% w/v) to 30 ml 0.5x TAE buffer and heated to boiling point in a microwave oven. After cooling, 1 µl of ethidium bromide stock solution (10mg/ml) (Sigma) was added. Gels were poured into a gel tray with a well-comb of the desired number of wells. After setting, the gel was submerged into the electrophoresis tank containing 0.5x TAE buffer. Loading dye (1/6 volume of 6x stock solution) was added to the nucleic acid solutions which were then loaded into the

wells. Electrophoresis was performed at a constant voltage (100-120 volts) for at least 40 minutes or until the desired resolution was obtained. Nucleic acids were visualized with short-wave ultraviolet light. In the case of electrophoresis of DNA fragments, molecular sizes were calculated with respect to a reference DNA Ladder (MBI Fermentas, Germany) run together with the samples.

Determination of nucleic acid concentration

DNA and RNA quantification was performed using the spectrophotometric measurement (Ultraspec 2100 pro, Amersham Biosciences, Sweden) of UV absorption at wavelengths 260 and 280nm. Measures of DNA/RNA purity were determined by the A_{260}/A_{280} ratio. For DNA this ratio provides indications of protein contamination. A pure DNA solution has an A_{260}/A_{280} ratio around 1.8. For RNA the ratio A_{260}/A_{280} should be greater than 1.6.

Concentration calculations are based on the principle that A_{260} =1 is equivalent to 50μg/ml of double stranded DNA and 40μg/ml of RNA.

Reverse transcription PCR

First strand cDNA was generated from a RNA template using a High Capacity cDNA reverse Transcription Kit (AB Applied Biosystem). The cDNA reactions prepared using the High Capacity cDNA Reverse Transcription Kits can be used in a variety of applications, including: quantitatively converting up to 2 μg (for a 20-μl reaction) of total RNA to cDNA, generating single-stranded cDNA suitable for quantitative PCR

applications and generating single-stranded cDNA suitable for short- or long-term storage.

1 μg of total RNA per 50-μl reaction was used and eluted with sterile DEPC water to a final volume of 25 μl. 2X Reverse Transcription Master Mix for a final volume of 25 μl was prepared as followed: 5 μl of 10X RT Buffer + 5 μl of 10X RT Random Primers + 2 μl of 25X dNTP Mix (100 mM) + 2.5 μl MultiScribe™ Reverse Transcriptase + 10.5 μl Nuclease-free H2O. The reverse transcription reaction was carried out using the following conditions: 1st step at 25°C for 10min; 2nd step at 37°C for 3 hours; 3rd step at 85°C 5 min; 4th step 4°C.

The quality and yield of cDNA reverse transcription was checked by normal amplification of GAPDH (see next paragraph for details). Amplified GAPDH-PCR product were controlled by mixing 5 μl of the reaction mixture with 1 μl of 6x loading dye stock solution and subsequently run on an agarose gel.

Real time PCR

Quantitative real-time PCR was performed by an ABI Prism 7900 HT Sequence detection System (Applied Biosystem) using: TaqMan® Gold RT-PCR reagents, probes for *EGFR*, *OCT4*, *NANOG*, *NESTIN*, and *GAPDH* (endogenous control) (Applied Biosystem). The PCR reaction exploits the 5′ nuclease activity of AmpliTaq Gold® DNA polymerase to cleave a TaqMan® probe during PCR. The TaqMan probe contains a reporter dye at the 5′ end of the probe and a quencher dye at the 3′ end of the probe. During the reaction, cleavage of the probe separates the reporter dye and

the quencher dye, which results in increased fluorescence of the reporter. Accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence.

For each gene and cell line the analysis was performed in triplicate. The final volume for every single reaction was 20 μl. For each gene the mix for triplicate samples consist of: 3 μl of cDNA (25ng/μL) + 30 μl TaqMan Universal PCR Master Mix (2X) + 9 μl of sterile water. The samples were then loaded onto the MicroAmp® Optical 96-Well Reaction Plate and finally covered with Optical Caps. Analyses were performed using data analysis software (SDS software 2.2.2) with the ΔΔCT method for relative quantification (Applied Biosystems).

For gene expression analyses of ABC transporters we mixed 2 μl of single-stranded cDNA (equivalent to around 100 ng of total RNA) with 48 μl of nuclease-free water and 50 μl of TaqMan Universal PCR Master Mix. After loading 100 μl of the sample-specific PCR mixture into one sample port of the microfluidic cards (Human ABC Transporter Panel; Applied Biosystems), the cards were centrifuged twice for 1 min at 1500rpm and sealed to prevent well-to-well contamination.

The cards were placed in the microfluidic card Sample Block of an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems). The thermal cycling conditions were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 30 sec at 97°C and 1 min at 59.7°C. The assay for each

gene on the microfluidic card was carried out in triplicate, due to the design of this specific panel. The calculation of the threshold cycle (Ct) values was performed using the SDS 2.2 software (Applied Biosystems), after automatically setting the baseline and the threshold.

2.4 Biological and biochemical assays

In vitro proliferation rate

A431WT and A431SPH were seeded in standard medium in standard 6 well culture dishes and in sphere medium in 6 well low attachment culture dishes (Corning), respectively (10,000 cells/well in triplicate). Cell proliferation rate was monitored at day 4 and 7 after the seeding by cell counting. Proliferation of A431SPH was also performed in sphere medium in the absence of EGF (Sigma-Aldrich).

Cell cycle analysis

Cells were harvested and pelleted as described above. Collected cells were spun at 1200 or 1500 rpm for 5 minutes and the supernatant was removed. Ice-cold 70% ethanol was added dropwise to the pellet and the tube was flicked in order to resuspend and permeabilize the cell pellet. Generally 1ml for $1x10^6$ cells was sufficient. Permeabilized cells were stored at 20°C for at least 24 hours. Before sample analyses, cells were pelleted to remove ethanol and washed twice in ice cold PBS 1X. The pellets were then resuspended in PBS 1X with 0.5mg RNAse A (Sigma-Aldrich) and 50mg/ml Propidium Iodide (Sigma-Aldrich) and incubated at 37°C for 3 hours. Cells were spun and resuspended in cold PBS 1X. Flow cytometric analysis was performed using a FACS SCAN cytometer (Becton Dickinson, Franklin Lakes, NJ) and results were analysed with ModFit.

Western blot analysis

For preparation of total cell lysates of A431WT and A431SPH, cells were washed with ice-cold PBS and lysed in RIPA buffer (See Table 2.B)

containing 2% SDS and a protease inhibitor cocktail (Boehringer, Milan, IT) for 40 minutes in ice. After clearing by centrifugation (15 min at 13,000 rpm), lysates were quantified by the BCA Protein Assay Kit (Pierce, Rockford, IL). Aliquots containing 40 μg of protein were separated on precasted 4%–12% gels (Invitrogen) and transferred onto nitrocellulose membranes (Amersham Bioscience-GE Healthcare). After blocking with 5% non-fat dried milk (MERCK, New York, NY) in PBS plus 0.1% Tween 20, membranes were incubated with a rabbit monoclonal antibody anti-EGFR (Cell Signaling, Danvers, MA), a rabbit polyclonal antibody anti-Oct4 (Abcam, Cambridge, UK), a mouse monoclonal antibody anti-Nanog (Sigma-Aldrich) or a rabbit polyclonal antibody anti-Actin (Sigma-Aldrich). Binding of primary antibodies to membranes was detected by a peroxidase-conjugated secondary antibody and developed by ECL (Pierce, Rockford, IL). As a positive control for stem-protein expression we used MEL-1 (a human embryonic male stem cell line) whole cell lysates (ab27198, Abcam).

Flow cytometry

The expression of cell surface or intracellular molecules was evaluated by flow cytometry. For each sample, approximately $1x10^6$ tumour cells were harvested, washed twice in ice-cold PBS and resuspended in 200μl PBS 1X -0.03% BSA.

For nuclear or cytoplasmic staining, cells were permeabilized for 30 minutes on ice in 70% ethanol. Only live cells were used for the detection of molecules and 10 μg/ml of primary monoclonal antibody, or antibody concentration suggested by the manufacturer was added to each cell

suspension. As a negative control, 200 µl of PBS 1X with isotype matched control antibody was added in place of the primary antibody (See Table 2.C). Incubation was carried out for 30 minutes or 1 hour on ice depending on the characteristics of the antigen. Cells were washed twice with 300 µl PBS 1X by centrifuging at 1400 rpm for 5 minutes at 4°C. Anti-human primary antibody staining was detected with a fluorescein-labeled goat anti-mouse IgG + IgM (H + L) secondary antibody (KPL, Gaithersburg, MD). Samples were incubated in PBS 1X in the dark for 30 minutes on ice. For double staining with the ALDEFLUOR assay and CD44 APC (APC mouse anti human CD44, BD Bioscience) the CD44 antibody was added after 30 minutes of incubation with ALDH substrate +/- ALDH inhibitor. Stained cells were washed and resuspended in 0.3 ml PBS 1X + 1% formalin. Flow cytometry was performed using a FACS Calibur or a FACS ARIA or a FACS CANTO (Becton Dickinson) and the collected data were analyzed with Winmdi Software or FACS DIVA software (Becton Dickinson, CA).

Immunohistochemistry (on paraffin embedded and on frozen (cryostat-cut) tissues)

Immunohistochemistry on formalin-fixed paraffin-embedded sections was performed using the UltraVision LP large volume Detection System HRP Polymer kit (Thermo Scientific, Waltham MA). Formalin-fixed paraffin-embedded sections were de-paraffinized at 60°C (30-60min), treated twice with xylol for 1 min and hydrated by incubation with 99%, 95% and 75%

ethanol twice for 3 min each and then immersed in H_2O for 5 min. To block endogenous peroxidase, sections were incubated with methanol + 0.3% hydrogen peroxide solution for 30 min at RT. Antigen retrieval was carried out in 10mM citrate buffer pH=6 for 15 min at 95°C in a steam-cooker, then left at RT for 30 min.

Sections were washed 3 times with PBS (5 min each). Blocking solution BLOCK LP (UltraVision) was applied for 10 min at RT in a humidified chamber. Sections were then incubated with the primary rabbit polyclonal antibody anti-human CXCR4 (Biotrend) for 1h at RT in a humidified chamber and then washed 3 times with PBS (5 min each). The ENHANCER (UltraVision) was added and incubated for 30 min at RT in a humidified chamber. After 3 PBS washes, slides were incubated with HRP POLYMER (UltraVision) for 30 min at RT in a humidified chamber.

Slides were washed with PBS and the peroxidase activity was revealed by incubating sections in DAB (3-3'diaminobenzidine) (Sigma) for 5 min.

After washing with water, sections were counterstained with Gill's haematoxylin solution for 5 sec and washed again with water. Sections were passed through an ascending series of alcohols (75%, 80%, 95% and 100%), followed by xylol 5 min for 4 times, and then mounted with EUKIT resin.

Frozen sections were washed 3 times with PBS (5 min each). To block endogenous peroxidase, sections were incubated with PBS1X + 3% hydrogen peroxide solution for 30 min at RT. After PBS washing, sections

were incubated with PBS 1X + 2% normal blocking serum (depending on the animal species for secondary antibodies) or PBS 1X + 1% BSA for 30 min. After 3 washes with PBS 1X, sections were then incubated with the primary monoclonal antibody (overnight at 4°C) or with a primary polyclonal antibody (1h at RT) in PBS 1X + BSA 0.1% in a humidified chamber (EpCAM 1:35, Novocastra; FR 10µg/ml, homemade; CD44 10µg/ml, DAKO; CD133 1:10, Becton Dickinson; SSEA1 1:100, Chemicon). Sections were washed 3 times with PBS (5 min each) and incubated with either a polyclonal goat anti-mouse IgG/biotinylated secondary antibody 1:100 or with a polyclonal goat anti-rabbit IgG/biotinylated secondary antibody 1:200 (DAKO) in PBS 1X + BSA 0.1% 30 min at RT. After 3 washing with PBS 1X, sections were then incubated with Streptavidin/HRP 1:300 in PBS 1X for 30 min at RT. Sections were washed 3 times with PBS 1X (5 min each) and incubated with DAB for 5min. After washing with water, sections were counterstained with Gill's haematoxylin solution for 5 sec, washed again with water and then mounted with EUKIT resin.

Epifluorescence

A431SPH were collected after 3 or 4 days of culture, when the mean diameter was approximately 50µc, washed with PBS 1X to eliminate the entire culture medium and fixed with 4% formaldehyde in PBS 1X. Sphere cells were permeabilized with PBS 1X + BSA 1% and Triton-X 100 0.1% (Sigma-Aldrich) for 20min at RT. To mark the nuclei, spheres were firstly incubated with a rabbit polyclonal antibody anti-acetyl-Histone H3 or anti-acetyl-Histone H4 (Upstate) for 1 h at RT. After washing the cells 3 times

with PBS 1X, Alexa Fluor 546 goat anti- rabbit IgG was used as the secondary labelled antibody and incubated in PBS 1X and BSA 0.1% for 30min at RT in the dark. Spheres were then washed and incubated with one of the primary mouse monoclonal antibodies (see Table 2.C) in PBS 1X and BSA 0.1% for 1 hour at RT and then washed 3 times with PBS 1X. The Alexa Fluor 488 goat anti—mouse IgG secondary antibody was incubated in PBS 1X and BSA 0.1% for 30 min, at RT in the dark. Finally spheres were washed with PBS 1X and resuspended in 10µl of ProLong Antifade Reagent (Invitrogen), spread on glass slides and covered with a glass coverslip. After one night at RT, the mounting media was dry and confocal analyses were performed.

Animals and tumour models for *in vivo* assays

All protocols were approved by the Ethics Committee for Animal Experimentation of the National Cancer Institute of Milan and carried out according to institutional and international guidelines (347). Female SCID mice were obtained at 5-6 weeks of age from Charles River Laboratories (Calco, Italy) and left untreated for at least one week for acclimatization. After 1 week of stabilization, mice were xenografted subcutaneously in the flank region with 1×10^5 or 3×10^5 A431WT or A431SPH cells in a single cell suspension in 0.1 ml saline. Tumour growth was monitored on a regular basis and three dimension measurements were used to calculate tumour volume (V) using the formula $V = 1/6\ \pi\ D^3$, where D is the mean of the three diameters of the tumour. Animals were humanely sacrificed when the tumour volume reached 1 cm^3 or when the physical appearance of the

mouse was compromised. The explanted tumours were frozen at -80°C. From frozen tumours, 5 μm-thick sections were cut and stained with haematoxylin/eosin for histological analysis.

Tumours coming from patients either as solid masses or as ascitic fluids were disaggregated with the appropriate method (previously described). For the subcutaneous (s.c.) model, mice were xenografted in the flank region with either 0.5 x 10^6, 1x10^6, 2x10^6, 3x10^6 or 9x10^6 tumour cells in 0.1 ml of saline, and tumour growth was monitored on a regular basis and three dimension measurements were used to calculate tumour volume (V) using the formula $V = 1/6\ \pi\ D^3$, where D is the mean of the three diameters of the tumour. For the intraperitoneal (i.p.) models, mice were xenografted with different doses of tumour cells (see Result and Discussion, Chapter 3 and 4 for details) in 0.5 ml of saline. For each mouse, ascites growth was evaluated as percentage of body weight increment: the body weight measured at each time point after cell transplantation was related to the initial animal body weight at the time of cell injection [(Body Weight Measured/Body Weight at Day 0) x 100]. The humane end-points considered were: for s.c. models a tumour volume > 750 mm^3 and/or a body weight loss > 20%; for i.p. models a body weight increase > 20% or an i.p. tumour mass with an estimated volume > 500 mm^3. In these cases animals were humanely euthanized and tumours/ascites characterized.

Side population

A431WT and A431SPH cells were stained with 2.5 μg/ml of the DNA intercalating Hoechst 33342 dye (Invitrogen) in the absence or in the

presence of 50 μg/ml of verapamil, a BCRP inhibitor, for 90 min at 37°C, washed, and resuspended in cold PBS. Before flow cytometry analysis, 1 μg/ml propidium iodide (Sigma-Aldrich) was added to exclude non-viable cells. Side population (SP) cells were identified and electronically gated on a Digital Vantage or FACS ARIA cell sorter (Becton Dickinson) after excitation of the Hoechst dye with 150 mW of 350 nm UV light. SP fluorescence emissions were directed toward a 610-nm dichroic filter and captured simultaneously through both a blue (450-nm) band-pass and a red (675-nm) long-pass filter on a linearly amplified fluorescence scale.

Aldefluor assay

(ALDEFLUOR KIT, Stem Cells Technologies, Vancouver, BC).

Prior to the start, all necessary supplies were assembled and kit reagents were allowed to come to a room temperature (RT) of 18 to 22°C before use. Activation of the ALDEFLUOR reagent BAAA (BodipyTM-aminoacetaldehyde) was performed by adding 25 μl of DMSO (1.5 ml supplied by the kit) to the vial of dry ALDEFLUOR reagent (50 μg supplied by the kit), mixing well and incubating for 1 min at RT. Dry ALDEFLUOR reagent is an orange-red powder that changes to a bright yellow-green upon addition of DMSO. 25 μl of 2N Hydrochloric Acid (HCl) (1.5 ml supplied by the kit) was added and the new solution mixed well. This mixture was then incubated for 15 min at RT. The final step was the addition of 360 μl of ALDEFLUOR Assay Buffer (4 bottles of 25 ml each supplied by the kit) to the vial and mix. The activated reagent was kept at 2 to 8°C during use and at -20°C for its routine storage. For sample

preparation, cells were firstly detached, counted and then resuspended in ALDEFLUOR Assay Buffer to a concentration of $1x10^6$ cells/ml, and 5 μL of the activated ALDEFLUOR substrate was added to the cell suspension, mixed very well and immediately half of the mixture was transferred to the tube-control. The ALDH enzymatic reaction begins immediately upon addition of the activated substrate to the cell suspension. It is imperative that an aliquot of the ALDEFLUOR-reacted cells be added to the control-tube without delay. Five μl of the specific ALDH inhibitor diethylaminobenzaldehyde (DEAB) (1.5 mM in 95% ethanol stock solution supplied by the kit) was rapidly added to the tube-control, in order to inhibit the reaction. Both samples, the test-tube and the control-tube, were incubated for 60 min at 37°C in humidified incubators in the presence of 5% CO_2. Following incubation, all tubes were centrifuged for 5 min at 1200 rpm and supernatants were removed. Cell pellets were resuspended in 0.5ml of ALDEFLUOR assay buffer. Cells incubated with ALDEFLUOR substrate (BAAA) and the specific inhibitor of ALDH, DEAB, were used to establish the baseline fluorescence of these cells and to define the ALDEFLUOR positive region. After staining, cells were maintained on ice during all subsequent procedures. Flow cytometric analysis was performed using a FACS ARIA (Becton Dickinson, Franklin Lakes, NJ).

PKH assay

A431SPH cells were separately stained, using PKH26 Red Fluorescent or PKH67 Green Fluorescent Cell Linker Kit for General Cell Membrane Labeling (Sigma-Aldrich). Protocol was performed to obtain a final

concentration of 2 x 10^6 M PKH26 or PKH67 dye and 1 x 10^7 cells in Diluent C in 2 ml. All the steps ware performed at RT in polypropylene conical centrifuge tubes.

Prior to the start, sphere cells were disaggregated using TrypLE™ Express trypsin at 37°C for five minutes in humidified incubators in the presence of 5% CO_2. Approximately 20 x 10^7 cells obtained from a single cell suspension solution were washed once with complete medium and once using medium without serum and centrifuged five minutes at 1500 rpm. After centrifuging the cells, the supernatant was carefully aspirated, leaving no more than 25μl of supernatant on the pellet. Cells were resuspended, without vortexing in the residual medium and then diluted in 1ml of Diluent C (supplied by the kit). This will be a 2X cell solution. Prior to the staining, PKH26 RED or PKH67 GREEN 2X stock solutions were prepared: the mother solution dye 1x10^{-3} M (supplied by the kit in ethanol) was diluted in Diluent C 1:250. One ml of 2x cell solution was then rapidly added into 1 ml of 2X 4x10^{-6} M dye solution and mixed immediately by gentle pipetting. Cells were stained in the tube for 5 minutes at 25°C and periodically inverted. The reaction was stopped using 1% of BSA (Sigma-Aldrich). Cells were washed three times with complete medium and plated at 1,000 cells/ml in sphere medium (Lonza), in Ultra Low Attachment 6 well Plates (Corning) and monitored at the 4^{th} and 7^{th} days after seeding.

Morphological assay: analysis of cloning experiments in adherent conditions

For morphological analysis of adherent clones, single cells were collected, counted and seeded in standard medium in adherent conditions at 10 cells/ well in 24 well plates (Corning-Costar). After 7 days, the clones were counted and the percentage of each class of clone morphology (holo-, mero and para-clone) was determined, using the formula: (# clone type/ # total clones) x 100.

Sphere Forming efficiency (SFE)

Sphere forming efficiency of cancer cells (A431, SiHa and CaSki cell lines) was evaluated by low-density sub-cloning. Cells were detached and seeded in sphere medium at 1 cell/well in 96 well low attachment plates (Corning). Spheres with a mean diameter of 50-150 μm, were counted after 4/10 days, depending on the cancer cell line. Sphere Forming Efficiency (SFE) was determined using the formula: (# of spheres/# of cells plated) x 100.

CHAPTER 3

RESULTS and DISCUSSION

3.1 Tumour initiating cells: development and critical characterization of a model derived from the A431 carcinoma cell line forming spheres in suspension

Numerous tumours, including squamous cell carcinomas of the cervix, display characteristics of multilineage differentiation, an aspect of the normal stem cell compartments. This evidence supports the theory that these types of tumours are propagated and maintained by a population of Cancer Stem Cells/Tumour Initiating Cells (CSCs/TICs).

Very limited research is at present focused on cervical cancer CSC/TIC. The field is therefore open to new investigation.

To investigate the tumour fraction with CSC/TIC characteristics in this kind of tumour, we started with the A431 cancer cell line and we extended the study to two other cervical carcinoma cell lines, Caski and SiHa.

3.1.1 Sphere formation and ALDH activity of cell lines from squamous cell carcinomas of the cervix.

We compared three different cervical cancer cell lines, A431 and SiHa, both derived from primary SCC of the cervix, and CaSki, which is conversely established from a metastasis in the small bowel mesentery, for sphere forming efficiency and intrinsic ALDH enzymatic activity.

Cells from the three cell lines were grown in suspension at low density in serum-free sphere medium for 7-10 days. A431 and Caski but not SiHa cells were able to grow forming spheres in suspension (FIG. 3.1A). When tested in limiting dilution assay (1 cell/well), A431 cells showed a higher sphere forming efficiency (SFE; mean 65.5% ± SE 1.7 of four independent experiments) as compared to Caski (SFE ; mean 32.8 ± SE 6.8 of two independent experiments) (FIG. 3.1B).

To assess the presence and size of a stem-like population we tested the ALDH enzymatic activity of the same cell lines. The fluorescent ALDEFLUOR reagent freely diffuses into cells and is a non-toxic substrate for ALDH. The amount of fluorescent ALDH reaction product that accumulates in cells directly correlates to the ALDH activity in these cells. The cells incubated with ALDEFLUOR substrate together with the specific ALDH inhibitor were used to establish the baseline fluorescence and to define the ALDEFLUOR positive region. As shown in Figure 3.1C, A431 showed the highest fraction of ALDEFLUOR positive cells (mean % ± SE = 45.7 ± 6) as compared with Caski (mean % ± SE = 30.6 ± 10.7) and SiHa cells (mean % ± SE = 24.3 ± 6.7). On the basis of these results the A431 cell line was selected for further analyses (FIG. 3.1C and FIG. 3.1D).

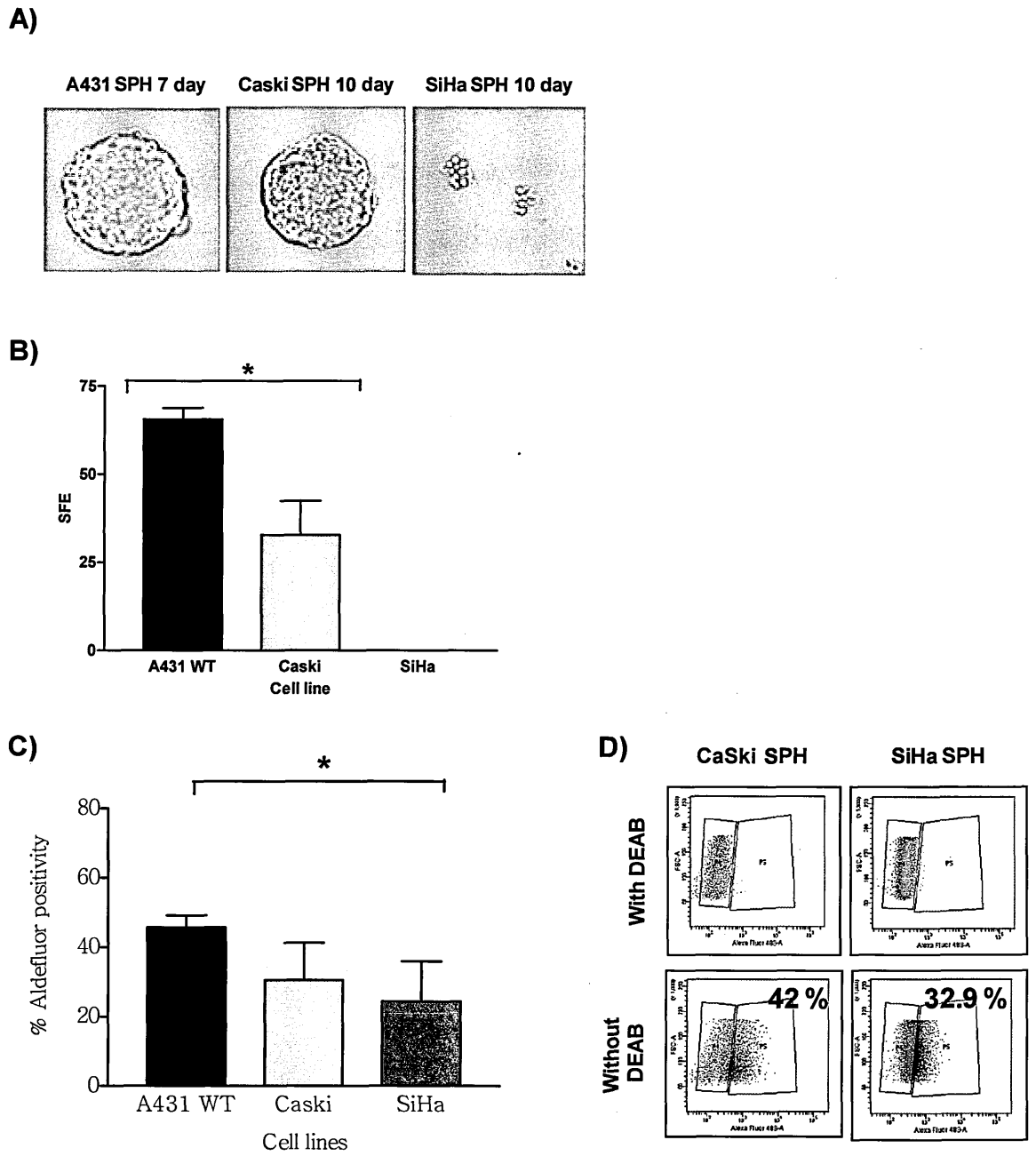


Figure 3.1: Sphere formation and ALDH activity of cell lines from squamous cell carcinomas of the cervix.

A) Example of spheres formed by A431, Caski and SiHa cells plated at limiting dilution in suspension and analyzed at day 7–10 after seeding. B) SFE of A431, Caski and SiHa cancer cell lines plated at 1 cell/well (A431: mean % ± SD of 4 independent experiments; Caski: mean % ± SD of two independent experiments). C) Percent of ALDEFLUOR positive cells evaluated in 3 independent experiments (mean % ± SD of 3 independent experiments). The difference in ALDH activity between the A431 and Caski were not statistically significant ($p = 0.2510$) but between A431 and SiHa they were statistically significant ($p = 0.0473$). D) Representative images of Aldefluor assay on Caski (10p vitro) and SiHa (10p vitro) cell lines. Aldefluor assay of A431 cell line was reported in FIG. 3.3A.

3.1.2 A431 sphere characterization: clonogenic and proliferative potential.

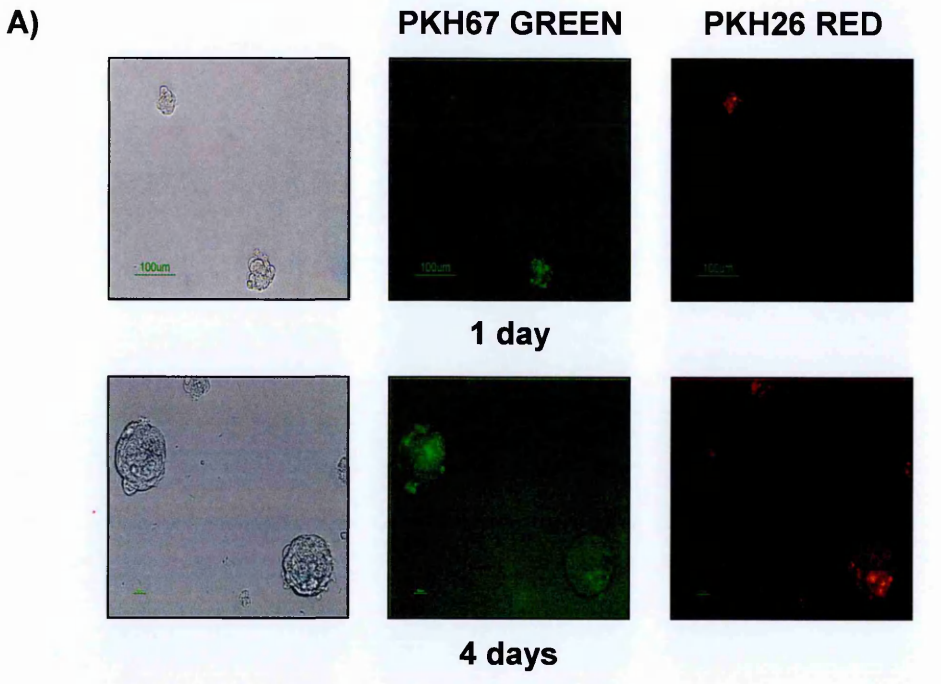
Single-cell suspensions of A431 sphere (A431SPH) cells were able to give rise to new spheres with a SFE similar to that of the parental A431 (A431WT) cells, but with a wider range of variability among the three different experiments performed (mean 55.5% ± SE 12.2). To demonstrate that spheres were derived from single cells and not from cell-aggregation, A431SPH cells were stained with PKH26RED or PKH67GREEN fluorescent dyes, mixed and plated at limiting dilution; this culture gives rise only to completely red or green spheres (FIG. 3.2A).

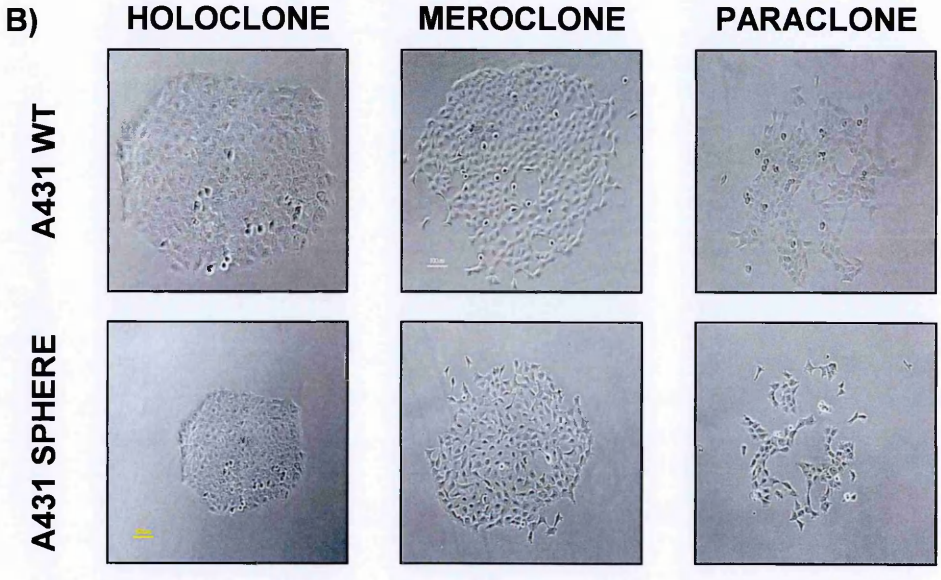
Cloning experiments, performed to evaluate the morphology of adherent clones derived from A431WT and A431SPH cells plated in FBS-containing medium, indicated that both parental and sphere cells were able to form holoclones. The number of holoclones, possibly derived from cells with stem-like characteristics, was higher in A431WT than A431SPH (mean % ± SE = 47.9 ± 7.1 and 36.4 ± 6.4, respectively). On the contrary, the number of more differentiated paraclones was significatively higher in A431SPH as compared to A431WT (mean % ± SE = 22.7 ± 4.3 and 4.85 ± 2.9, respectively). The number of clones with intermediate characteristics, called meroclones, was more or less similar in the two cell populations (mean % ± SE = 47.3 ± 4.3 and 40.95 ± 8.5, for A431WT and A431 SPH, respectively) (FIG. 3.2B and FIG. 3.2C).

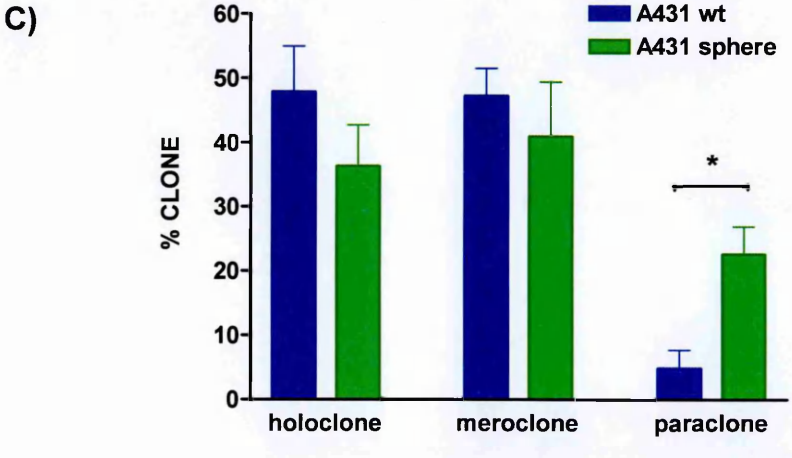
These results indicated that A431 cells have a high capability to grow in suspension as spheres and sphere-forming cells, and like TIC, displayed again consistent sphere-forming efficiency and therefore self-renewal capability in culture. However, the higher proportion of differentiated paraclones observed in A431SPH suggests that differentiation takes place in A431 cells in suspension much more than in adherent growth conditions.

Long-term cultures grown in suspension (more than 40 serial passages) were obtained from A431SPH cells, indicating that these cells maintain high proliferative potential. When compared both at early and late passages, the growth of A431SPH cells in suspension was exponential both at day 4 and 7, whereas A431WT cells proliferated more rapidly during the first 4 days and then more slowly, reaching a probable plateau phase of growth after 7 days of culture (FIG. 3.2D). Analysis of the cell cycle by staining the cells for DNA content revealed that the suspension cell culture seemed to select cells which had a better ability to proliferate. In contrast with the adherent parental cell, A431 spheres did not accumulate in the G1 phase of the cell cycle, with concomitant decrease in the S and G2/M phases (FIG. 3.2E).

The A431 cell line over-expresses EGFR due to amplification of the *EGFR* gene. Since sphere medium is supplemented by 20 nM EGF, this factor might be important to sustain A431 proliferation in non-adherent conditions. To verify the role of EGF, A431SPH were grown in sphere medium in the presence or absence of EGF. No difference in the proliferation rate was observed (FIG. 3.2F) suggesting that non-adherent growth of A431 cells is not dependent on EGFR stimulation.


A)
PKH67 GREEN
PKH26 RED
1 day
4 days


B)
HOLOCLONE
MEROCLONE
PARACLONE
A431 WT
A431 SPHERE


C)
A431 wt
A431 sphere
% CLONE
60
50
40
30
20
10
0
*
holoclone
meroclone
paraclone

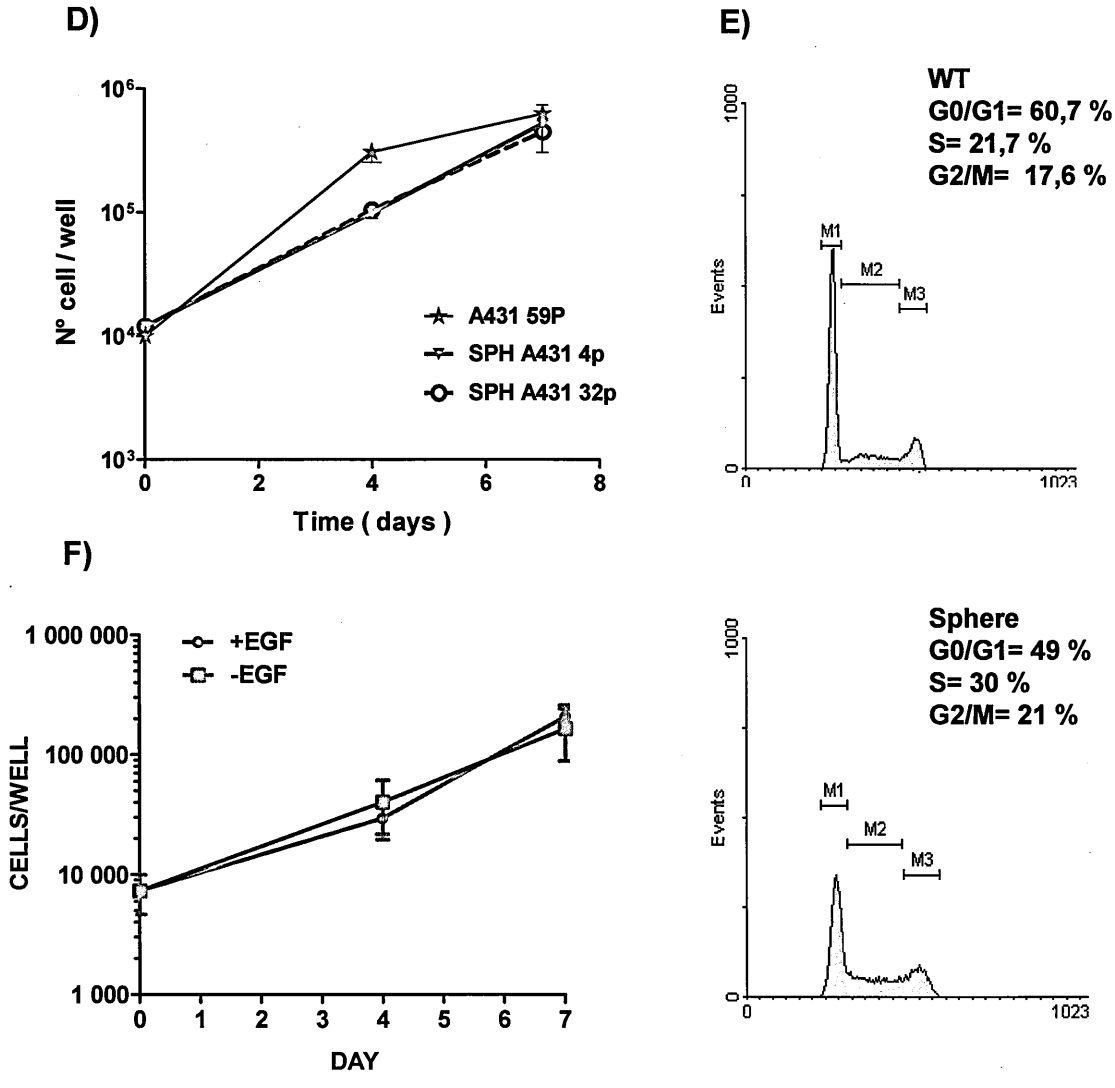


Figure 3.2: A431WT and A431SPH clonogenic and proliferative potentials.

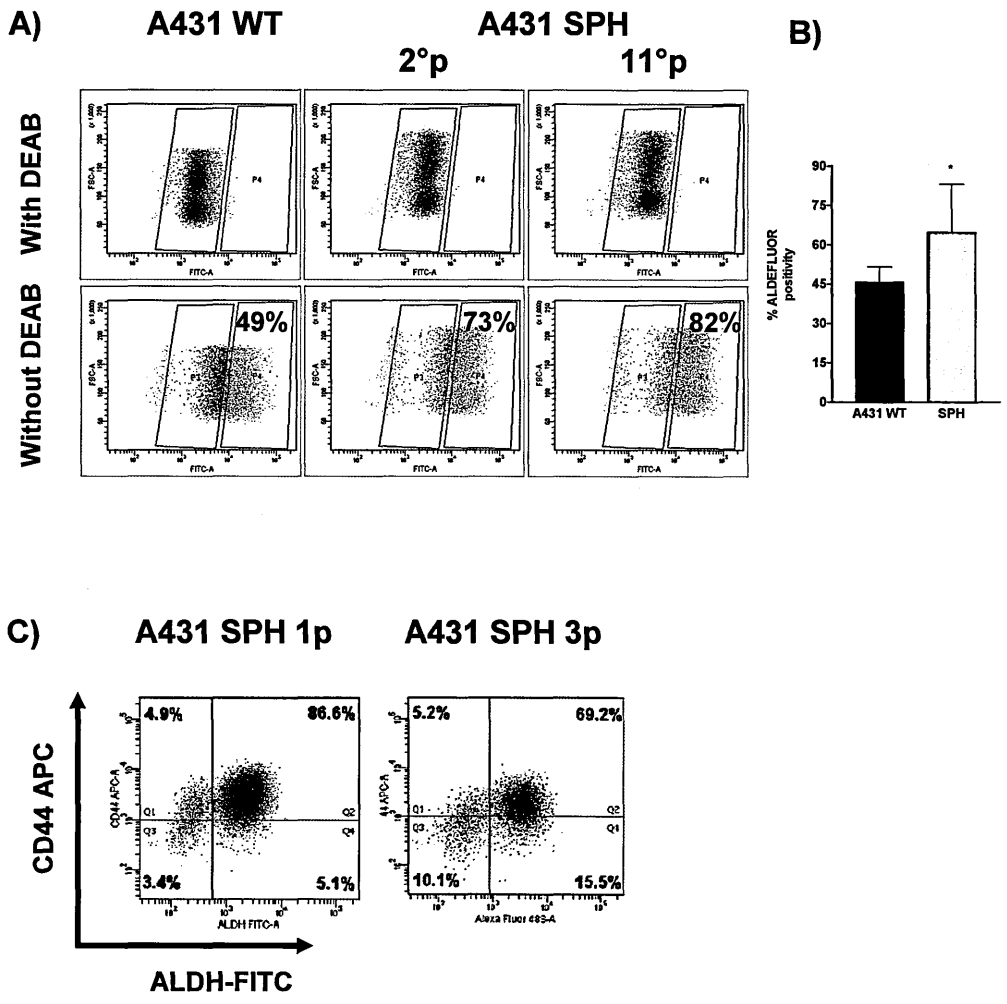
A) PKH67 green and PKH26 red staining of A431SPH, seeded in suspension at limiting dilution (1000 cell/mL) after the assay. B) Examples of clonal morphology from single-cell cloning in standard medium (Bar = 100 µm). C) Relative percentage of different clones from A431WT and A431SPH cells (mean ± SD). For A431WT cells, results are from 2 independent experiments, for A431SPH cells results are from 4 independent experiments. (*p = 0.0407 Paraclone). D) Proliferation rate of A431WT and A431SPH cells, at early and late passages. E) Cell cycle analysis of A431 WT and A431 SPH. (F) A431SPH were grown in sphere medium in the presence or absence of EGF. Mean ± SD of 3 different *in vitro* passages (1p, 5p, 13p).

3.1.3 Monitoring of ALDH expression and Side Population in A431SPH.

The ALDH enzymatic activity and Side Population (SP) phenotype of the A431WT and A431SPH cells were compared. Representative experiments for ALDH and SP are shown in Figure 3.3A and in Figure 3.3D, respectively. A431WT showed a sizeable fraction of ALDEFLUOR positive cells (mean % ± SE = 45.7 ± 6), but the proportion of ALDEFLUOR positive cells was significantly increased in A431SPH cells (mean % ± SE = 64.9 ± 7.5) (FIG. 3.3A and FIG. 3.3B). Since the CD44 marker was found to be associated to the Podoplanin positive stem cell-like subpopulation in the A431 cell line (290), we evaluated the possible correlation between $CD44^{high}$ and ALDH-positive cells. We found a high CD44 positivity both in A431WT and A431SPH cells, ranging from 88 to 95%. Moreover, in double staining experiments and FACS analysis, we observed a direct relationship and a clear overlapping between the two tested markers in both cell types (representative images are shown in FIG. 3.3C), suggesting that CD44 and ALDH do not identify in A431 cells a specific subpopulation with TIC properties.

SP is defined by Hoechst dye exclusion in flow cytometry. To determine the region containing $Hoechst^{low}$ cells (SP), corresponding to cells able to extrude Hoechst 33342 dye, A431WT and A431SPH cells were incubated with the dye alone or in combination with verapamil, a drug inhibiting the activity of ABC pumps. As consequence of verapamil treatment the SP positive fraction is expected to disappear.

Repeated FACS analyses demonstrated that the small fraction of Hoechst ^{low} SP cells present in A431WT (mean % ± SE = 0.13 ± 0.03) increased following sphere formation (mean % ± SE = 1.3 ± 0.32). Verapamil treatment reduced by 90% the A431SPH SP (0.08 ± 0.012) (FIG. 3.3D and FIG. 3.3E). These results indicate that the growth in suspension as spheres enriched the A431 cell lines in cells with detoxifying abilities that are hallmarks of the stemness phenotype.



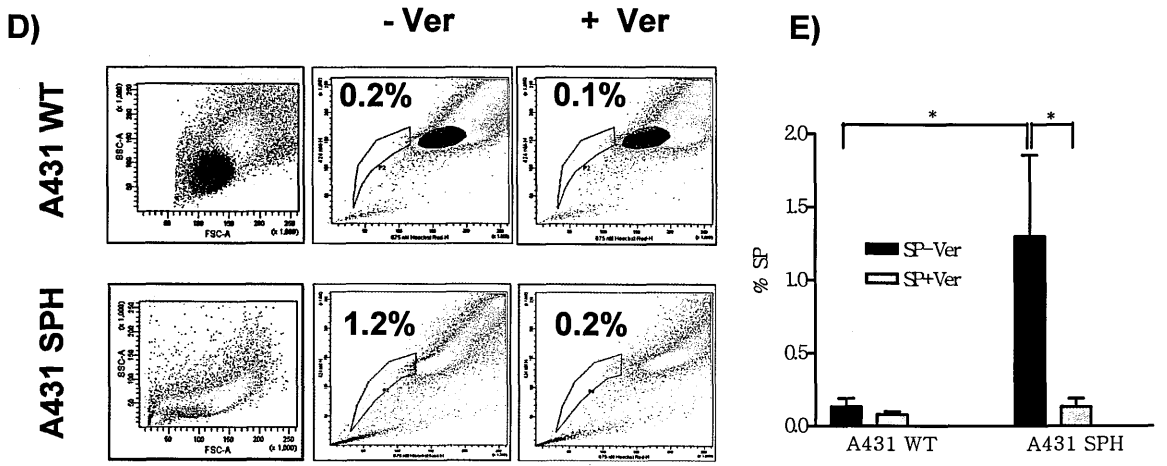


Figure 3.3: Monitoring of ALDH expression and Side Population.

A431WT and A431SPH cells were analyzed for the activity of ALDH1 (A and B) and for the side population (SP) phenotype (D and E). A) Plots from a representative experiment in presence (upper plots) and in absence (lower plots) of DEAB inhibitor. Baseline fluorescence is indicated as region P3 and the ALDEFLUOR positive region as region P4. B) Percent of ALDEFLUOR positive cells evaluated in 3 independent experiments (mean % ± SD of 3 independent experiments): the increase in ALDH1 positive cells was statistically significant ($p = 0.0002$). C) Representative images of A431SPH cells (at different *in vitro* passages) analyzed for the activity of ALDH1 and expression of CD44. D) SP plots from a representative experiment in absence (middle plots, -ver) or in presence (right plots, + ver) of verapamil. Morphological parameters of each population are described in left plots. E) Percentage of SP cells as evaluated in three independent experiments (mean % ± SD of 3 independent experiments). The differences between A431WT and A431SPH SP without verapamil and between A431SPH SP with and without verapamil were statistically significant ($p < 0.05$).

3.1.4 Modulation of stemness markers.

The expression of stem and differentiation markers in A431SPH cells was analyzed by Real Time PCR at the 4^{th}, 7^{th}, 10^{th} and 12^{th} *in vitro* passages using the A431WT as a calibrator (FIG. 3.4A). The growth in non-adherent conditions was found to up-regulate the expression of *NANOG* and *NESTIN*, with a less pronounced rise of *OCT4*. The *EGFR* levels were concurrently slightly up and down-regulated.

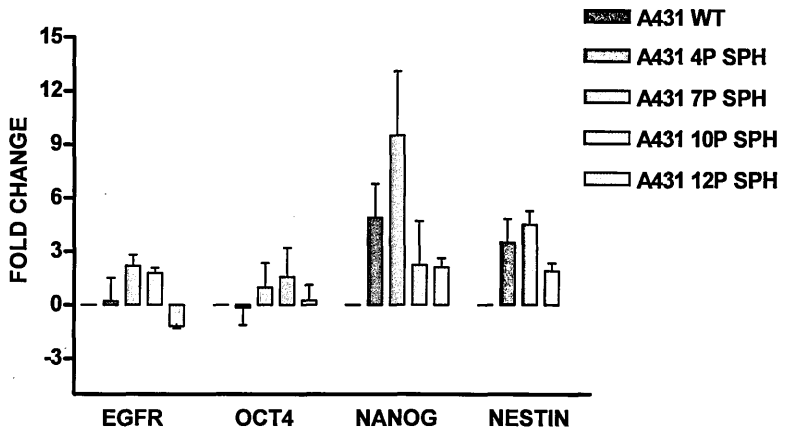
To verify if the RNA analyses correlated with the protein levels we analyzed whole protein cell extracts from A431WT and A431SPH cells, using as internal control a male embryonic cell extract. The results showed an increased Oct4 protein level in A431SPH as compared to the parental cells, whereas a similar Nanog protein level was detected in both cells (FIG. 3.4B, lanes 2 and 3). Overall, it seemed that the protein expression was not strictly related to mRNA level. Indeed the expression of genes linked to self-renewal was initially promoted at the RNA level by the cell grown as spheres, whereas their transcription became down-regulated in later passages.

We also evaluated expression levels and localization of epithelial, mesenchymal and stemness markers in A431SPH, using immunofluorescence analysis (FIG. 3.4C). Confocal analysis showed that CD44, a well known CSC marker in different solid tumours, was expressed homogeneously at a high level by all of the sphere-cells. The proliferation marker Ki67 seemed to mark few cells located at the external face of the

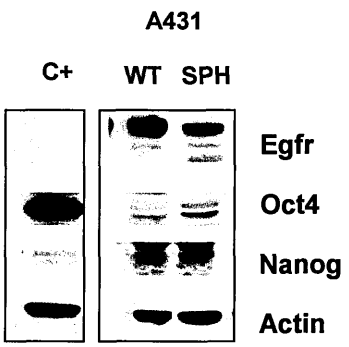
sphere, suggesting that the bulk of the spheroid could be composed of quiescent cells, which do not undergo asymmetric division, leading to the differentiation. We observed a weak or absent expression of N-Cadherin and vimentin, mesenchymal markers, but a strong expression of epithelial markers, such as E-Cadherin and Cytokeratin 8/18. We also tested the expression of the stem-germinal cell marker SSEA1, which was weakly expressed, and the expression of the POU-FAMILY factor OCT4 which was weakly expressed by a limited number of sphere-cells.

Expression of Podoplanin, a marker recently described to be associated with the stem-like population of A431 cells, was evaluated in A431SPH cells by FACS analysis. Based on the histograms shown in Figure 3.4D, Podoplanin is expressed in A431WT cells at very low levels. In contrast, sphere-derived cells displayed a distinct population of Podoplanin-positive cells, around 5.5%, as evaluated in two different sphere passages. This sub-population of Podoplanin positive cells increased during *in vitro* culture and they were distinctly smaller than the bulk sphere population. Moreover, a population of cells with this characteristic was not evident in A431WT (FIG. 3.4D, black arrow).

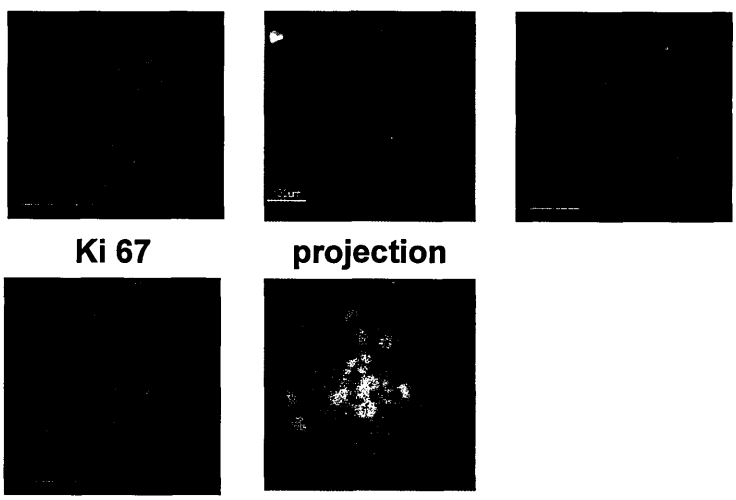
A)

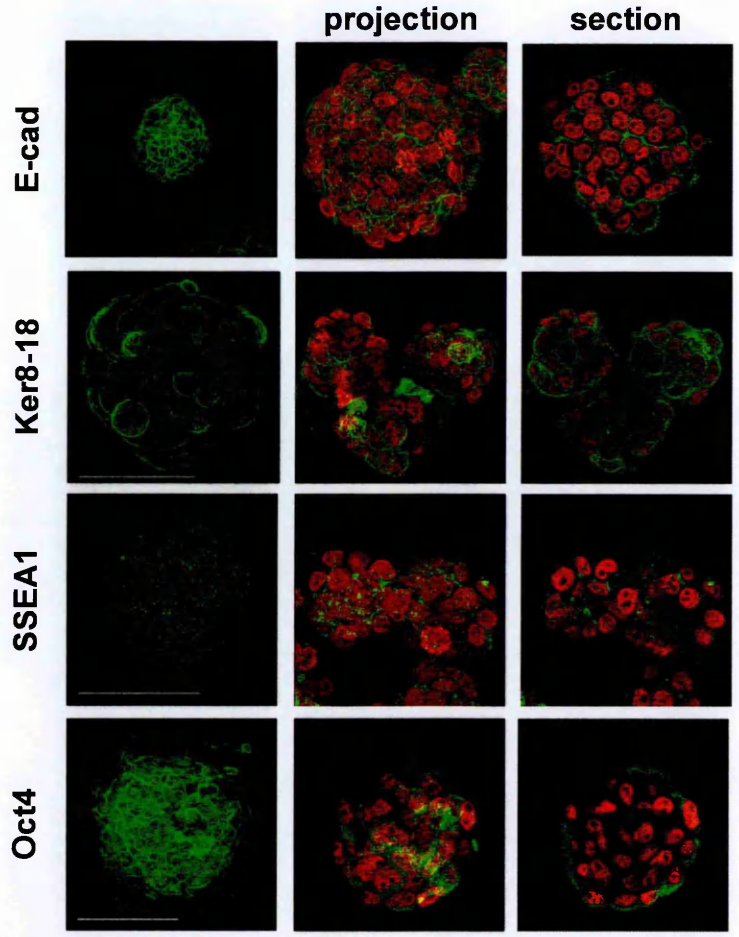

A431 WT
A431 4P SPH
A431 7P SPH
A431 10P SPH
A431 12P SPH
FOLD CHANGE
15
12
9
6
3
0
-3
EGFR
OCT4
NANOG
NESTIN

B)


A431
C+
WT
SPH
Egfr
Oct4
Nanog
Actin

C)


CD44
Vim
N-cad
Ki 67
projection


projection
section
E-cad
Ker8-18
SSEA1
Oct4

D)

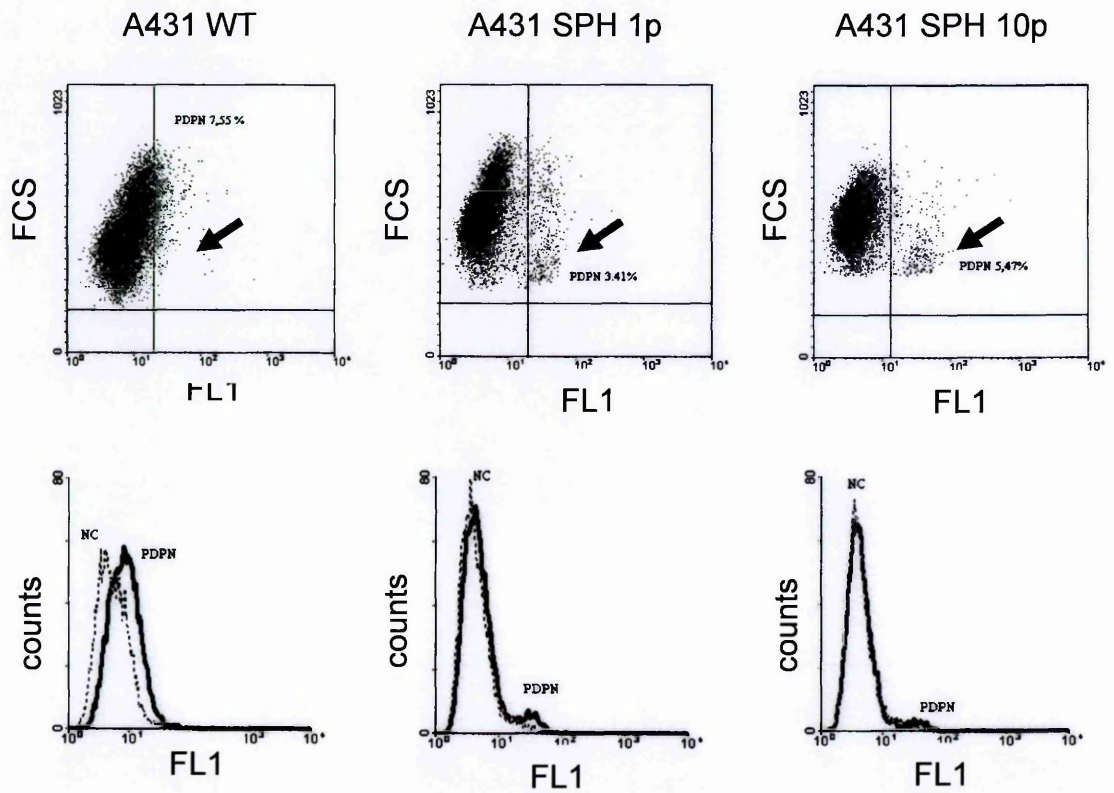

A431 WT
A431 SPH 1p
A431 SPH 10p
PDPN 7,55 %
PDPN 3.41%
PDPN 5,47%
FCS
FL1
counts
NC
PDPN

Figure 3.4: Modulation of "stemness" markers and evaluation of podoplanin expression in A431WT and A431SPH.

A) The expression levels of multiple "stem" genes in various (as indicated) *in vitro* passages of A431SPH cells were assessed by Real Time PCR using the A431WT cells as a reference. Results from 2 to 5 separate experiments, depending on the gene analysed, are shown. 4p, 7p, 10p, 12p indicates passage numbers. B) A representative Western Blot analysis of whole protein cell extracts of A431WT (WT) and A431SPH (SPH) using as positive control a male embryonic cell extract (C+). C) Representative pictures of confocal analysis on A431 sphere, after immunofluorescence for selected markers as indicated. Red fluorescence marked the nuclei whereas green fluorescence was specific for the annotated markers. D) Flow cytometry analysis of podoplanin expression in A431WT and A431SPH cells. Notice the presence of a subpopulation of podoplanin-positive cells composed of smaller cells, as demonstrated by their lower FCS parameter, in A431SPH cells (indicated by black arrow). Please note that black arrow also indicates the region where cells should appear on A431WT graph, but they are largely missing. (Abbreviations: NC = negative control; PDPN = podoplanin).

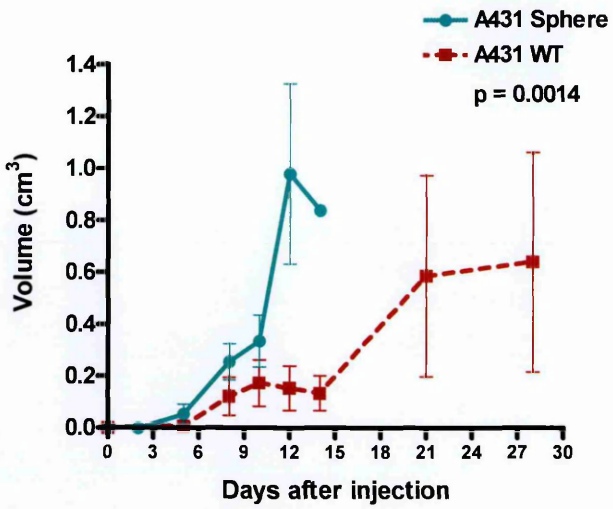
3.1.5 *In vivo* tumorigenicity.

The tumorigenic potential of A431SPH in comparison with A431WT cells was evaluated *in vivo* by s.c. injection of cells into athymic nude mice. At a dose of 3 x 10^5 cells, tumours developed in all A431SPH injected mice (100% take) (FIG. 3.5A). At this dose, the tumour onset was earlier and the growth faster than in tumours derived from A431WT cells, which also showed a lower tumour take (50%) (FIG. 3.5A and FIG. 3.5B). Indeed, the tumour volume doubling time was approximately 2 and 10 days for A431SPH and A431WT, respectively. At the lower dose of 1 x 10^5 cells, tumours from A431SPH cells grew in 2 out of 6 mice (33% take). In contrast, no tumours were observed in six mice injected with the same dose of A431WT cells (FIG. 3.5A). A431SPH tumours could be serially transplanted for at least four *in vivo* passages at the dose of 3 x 10^5, propagating their original tumour phenotype and *in vitro* they gave rise to new sphere formation. Histologically, the parental A431WT-derived tumours were poorly differentiated squamous epidermoid carcinomas (FIG. 3.5C). A431SPH-derived tumours initially maintained the same histological characteristics but became much more undifferentiated after a few *in vivo* passages. These results indicate that, despite the modest increase in tumorigenic potential of A431SPH as compared to A431WT, A431SPH cells demonstrated a higher proliferative potential that strongly contributed to the rapid development of the tumour mass.

A)

Subcutaneous tumor take in Nude mice		
Dose (cells)	A431 WT	A431 sphere
$1x10^5$	0/6	2/6
$3x10^5$	4/8	6/6

B)



C)

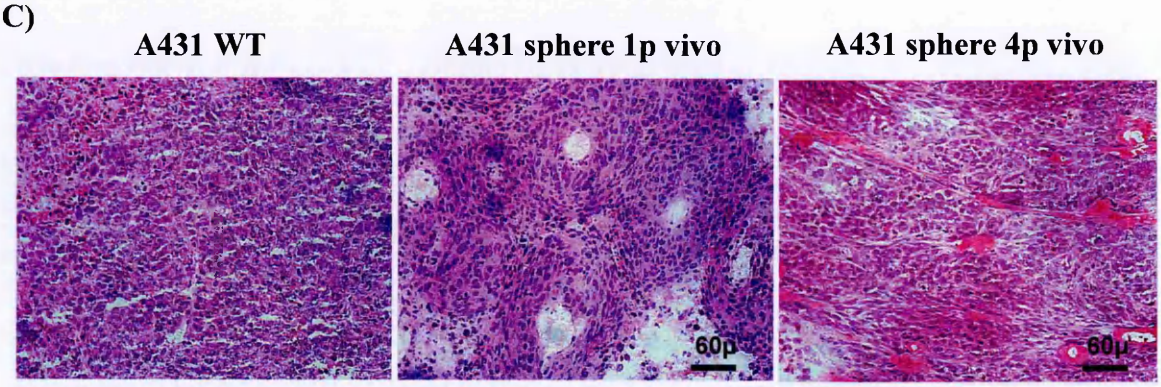


Figure 3.5: *In vivo* tumorigenicity.

Two doses (1 x 10^5 and 3 x 10^5) of A431WT and A431SPH cells were s.c. injected in nude mice and tumour development evaluated as A) take rate at the two doses of cells; B) tumour volume at different times after injection of 3 x 10^5 cells; C) H&E staining of representative frozen sections from A431WT and A431SPH tumours.

3.2 Discussion

Established tumour cell lines, even after long-term propagation *in vitro*, can maintain the heterogeneity of the original tumour, including cells with different morphological phenotypes, variable proliferative and clonogenic potential together with the presence of cells with stem-like characteristics. For these reasons, tumour cell lines may also represent useful models to study the CSC/TIC component *in vitro*. In this study, among three cell lines derived from squamous cervical carcinoma, A431 cells exhibited the highest fraction of ALDH-positive cells and a good correlation between ALDH positivity and sphere forming efficiency (FIG. 3.1). Therefore, we selected the A431 cell line as a model suitable for a critical evaluation of the experimental procedures adopted for the characterization of CSCs in tumour cell lines.

Forced growth in suspension in the presence of specific growth factors was found to be a useful tool for the isolation of undifferentiated multipotent neural and breast stem cells (348) (327). Under these conditions, most of the cells die and surviving cells give rise to floating spherical colonies (spheres). One of the most important characteristics of stem/progenitor cells is the ability to self-renew. Only cells with a self-renewal capability are able to sustain the growth in suspension giving rise to non-adherent colonies. The growth in suspension has also been applied to the isolation and propagation of putative tumour stem cells from tumour cell lines and from patient tumour specimens (349) (308) (350) (351). In suspension, the A431 parental cells (A431WT) showed a very high SFE, which was

essentially maintained by the cells derived from single sphere cloning, indicating that both the A431WT and sphere-derived cells have self-renewal properties (FIG 3.1B).

Increased ALDH activity has been found in stem cell populations in multiple myeloma and acute myeloid leukaemia (351), and also in normal and cancer stem cells of the breast (137;352). Our analysis on cervical cancer cell lines showed the presence of ALDH positive cells in all tested cell lines in a range between 30 to 49%, and a good correlation between ALDH activity and sphere forming efficiency was also observed, especially in the A431 cell line (FIG. 3.1B and 3.1C). Moreover, in the A431 model, cells exhibited a high fraction of ALDH-positive cells (49%) which increased up to 80% in sphere cells (FIG. 3.3A). Overall, our data are in agreement with the wide range of ALDH enzymatic activity recently reported in breast cancer cell lines, where ALDEFLUOR positive cells showed higher sphere-forming capacity compared to ALDEFLUOR-negative cells (353).

The high ALDH activity and SFE of the selected cell line correlated with the formation, upon cell adhesion, of a high number of clones with compact round morphology (holoclones) both by A431WT and A431SPH. Clones with these characteristics are considered enriched in stem cells not only in keratinocytes, where they were originally described (343), but also in several carcinoma cell lines (285) (354) where a holoclone fraction, ranging from less than 5% to about 30%, has been described. Interestingly, the percent of holoclones formed by A431WT approached 50%, representing the highest among those reported (285). Holoclones are characterized by a

high proliferative potential (355) and this property can explain why A431WT and spheres showed quite similar proliferation rates when evaluated in the exponential growth phase (seven days growth) and also the maintenance of the proliferative potential for more than 40 passages (FIG. 3.2D). However, as evaluated after a few passages in suspension, A431 sphere-derived cells formed slightly lower numbers of undifferentiated holoclones as compared to parental A431 cells, whereas the number of differentiated irregular colonies (paraclones) appeared significantly increased (FIG. 3.2B and 3.2C). We conclude that, in agreement with that described in other tumour models (308), in our model system the growth in suspension sustained the cell fraction with stem-like characteristics but at the same time also induced differentiation inside the spheres.

We found that the SP, originally representing a very small fraction of A431 parental cells (0.2%), increased to about 1.2% in sphere-derived cells but remained far smaller than the percentage of ALDH-positive cells (FIG. 3.3 A, B, D and E). Similarly, in human haematopoietic cells, ALDH-positive cells only partially overlapped with the SP (356). The SP fraction, identified by Hoechst dye exclusion, represents only 0.16% of the main population on short-term culture of human keratinocytes (31), whereas in oral squamous cell carcinoma cell lines and primary tumours, the SP was highly variable (from 0.1 to about 10%) (357).

Podoplanin, in association with $CD44^{high}$ has been recently reported as a new marker of epidermal carcinoma cancer stem cells in the A431 cell line (290). Our FACS analysis revealed that a population of small sized cells,

representing about 5% of the total count, emerged in sphere cells with a higher Podoplanin mean fluorescence (FIG. 3.4D). Interestingly, by flow cytometric analysis we also observed that small-sized cells constitute the SP fraction of A431SPH cells (FIG. 3.3D). Similar to murine keratinocytes, where SP is suggested to constitute the most primitive blast-like subset of any epidermal population (358), small sized Podoplanin-positive and SP cells might represent the real stem-like cell component of A431 cells, which was highlighted by the growth in suspension. CD44 was highly expressed in both A431WT and A431SPH cells, suggesting that this molecule does not identify in A431 cells a specific subpopulation with TIC properties.

Since the CD44 marker was found to be associated with the Podoplanin positive stem cell-like subpopulation in the A431 cell line (290), we evaluated the possible correlation between $CD44^{high}$ and ALDH-positive cells. We found a high CD44 positivity both in A431WT and A431SPH cells, ranging from 88 to 95% (FIG. 3.3C). Moreover, in double staining experiments and FACS analysis, we observed a direct relationship and a clear overlap between the two tested markers in both cell types, suggesting that both CD44 and ALDH do not identify in A431 cells a specific subpopulation with TIC properties.

By epifluorescence analyses, the expression pattern of the proliferation marker Ki67 indicates that the more quiescent cells, which do not undergo asymmetric division, leading to their differentiation, are located in the bulk of the spheroid, whereas proliferating TA-cells are placed at the external

face of the sphere. The absence of vimentin expression, the low levels of N-Cad and the homogeneous expression of epithelial markers, such as E-Cad and cytokeratins, indicated that spheres may be more prone to acquire an epithelial phenotype than a mesenchymal one (FIG. 3.4C). We also tested the expression of the stem-germ cell marker SSEA1, which is weakly expressed in the spheres, and the expression of the POU-FAMILY factor OCT4 which is only weakly expressed by sphere-cells located at the external face of the spheroids (FIG. 3.4C). These data confirm our previous Western Blot and Real time analyses and highlight the potential stemness phenotype of A431 spheres.

The presence of a subpopulation with stem-like properties was confirmed by Real Time PCR analysis. mRNA levels of 'stemness' genes, in particular *NANOG* and *NESTIN*, were initially up-regulated during growth of spheres in suspension, supporting the notion that this kind of growth allowed the initial expansion of a pool of cells with stem-like properties (FIG. 3.4A). These data have been partially confirmed by protein analysis of selected markers. In particular, since no change in Nanog protein expression had been detected by Western blot analysis, we suppose that other mechanisms of post-transcriptional regulation could affect the protein production in A431SPH cells and in the A431WT cells (FIG. 3.4B). The observed transcriptional activation of stem cell markers tended to be down-regulated in higher passages, in agreement with the observed increase in more differentiated paraclone formation in adherent conditions.

The tumorigenic potential *in vivo* defines the intrinsic 'stemness' of cancer cells, and we found that A431WT cells, which were 100% tumorigenic at 1 x 10^6 cells dose (data not shown), were unable to establish tumours after injection of 0.1 x 10^6 cells. A431SPH, fully tumorigenic at 0.3 x 10^6 cells, developed rapidly growing tumour masses in a shorter period of time than parental cells, but were only partially able to form tumours (2/6) after injection of 0.1 x 10^6 cells (FIG. 3.5). The number of A431SPH cells required for tumour formation was quite high when compared to that sufficient to induce tumours in other tumour models (263) (349). However, the assays were carried out in not completely immunocompromised animals and without Matrigel support. Nevertheless, we suggest that the increased tumorigenic potential, transplantability and aggressiveness of the sphere-growing population support our contention that we are enriching for cells with stem/progenitor-like properties.

In conclusion, this study has illustrated that A431 cells represent a useful tool for studying many aspects of cancer stem cell biology that are probably applicable to most, if not all, human cancers. Together our results support the hypothesis that the growth in suspension is essential for the emergence of a small population of tumour cells with CSC properties (SP, presence of Podoplanin positive cell, small cellular dimension) inside a wider "sphere" cell population characterized by "less stringent" stem cell features, such as high ALDH activity and high levels of expression of putative stemness markers, such as CD44, that, as in the case of A431, were frequently already present in adherent parental cells.

CHAPTER 4:

RESULTS and DISCUSSION

4.1 Isolation of cells with characteristics of stem/progenitor cells from epithelial ovarian cancer.

Recent studies have shown that epithelial ovarian CSCs could be isolated on the basis of their ability to grow in suspension as spheres, or by their capacity to exclude the Hoechst dye (Side Population phenotype), or by the expression of CD44/CD117 or CD133 markers (See Chapter 1.10.3).

To identify and isolate ovarian CSCs, we applied different experimental procedures 1) establishment of *in vitro* cultures from solid tumour specimens or ascitic cells in non-adherent condition using a specific culture medium (See Chapter 'Material and Methods'), 2) establishment of transplantable *in vivo* tumour cell lines, starting from solid tumour specimens or ascitic cells and their characterization by identification of specific marker expression and their possible correlation with a more aggressive phenotype; 3) Sorting of subpopulations expressing the selected markers and phenotypic and functional characterization of the sorted subpopulation for stemness properties (See Chapter Material and Methods).

4.1.1 Setting up the culture conditions for in vitro expansion of ovarian cancer cells

In vitro culture of ovarian cancer cells isolated from solid tumour specimens or ascites was adopted for two main reasons: the expansion of tumour cells in adherent conditions in order to obtain sufficient material for further characterization and the selection of tumour cells able to grow in suspension as spheres.

Following solid tumour or ascitic cell aggregate digestion, the isolated cells were seeded in adherent conditions for two hours to allow inflammatory and/or stromal cell adhesion to the plastic. After this initial adhesion-purification step, the supernatant was collected and cells were seeded in different culture conditions.

Aliquots were plated in the presence of different concentrations of serum in standard conditions. The use of different media (RMPI 1640 or MCDB 105/MEDIUM 199 1:2) and different concentrations of serum (10%, 1% and 0.1%) did not improve the growth of ovarian cancer cells, recovered from both solid tumours and ascites. Often a strong contamination of normal-stromal infiltrating cells was present in tumour cultures and this became predominant during the passages (representative examples are shown in FIG. 4.1A). When tumour cells survive, after several weeks of culture they form small colonies, composed of slow cycling tumour cells, which do not proliferate even after the addition of different growth factors.

We have also tried to plate the purified tumour cells in Sphere medium, using appropriate low attachment culture flasks, which permit the selection of those cells able to grow in suspension as spheres (See Chapter 2 'Material and Methods'). From both solid tumours and ascites, some spheroids or cell aggregates appeared after a few days of culture. After the mechanical or enzymatic disaggregation of spheroids, growth of new spheroids was observed only for one or two *in vitro* passages. The poor sphere formation and the lack of proliferation or self-renewal were not improved by the addition to the culture media of different growth factors, such as Lysophosphatidic acid (LPA) (359) and bFGF (representative examples are shown in FIG. 4.1B). On the contrary, LPA did not stimulate cell proliferation and seemed to induce cell death. Similarly tumour cells seeded in the sphere medium plus bFGF were not able to form spheroids and showed a highly vacuolated cytoplasm.

The establishment of *in vitro* culture of cells derived from thirty-eight tumours and thirty ascitic samples was not achieved either in adherent conditions or in specific stem cell medium.

Therefore our efforts were directed towards stabilization of *in vivo* transplantable cell lines from patient-derived tumour cells. This alternative approach led us to obtain three transplantable ovarian cancer cell lines, which were further characterized for potential stemness properties.

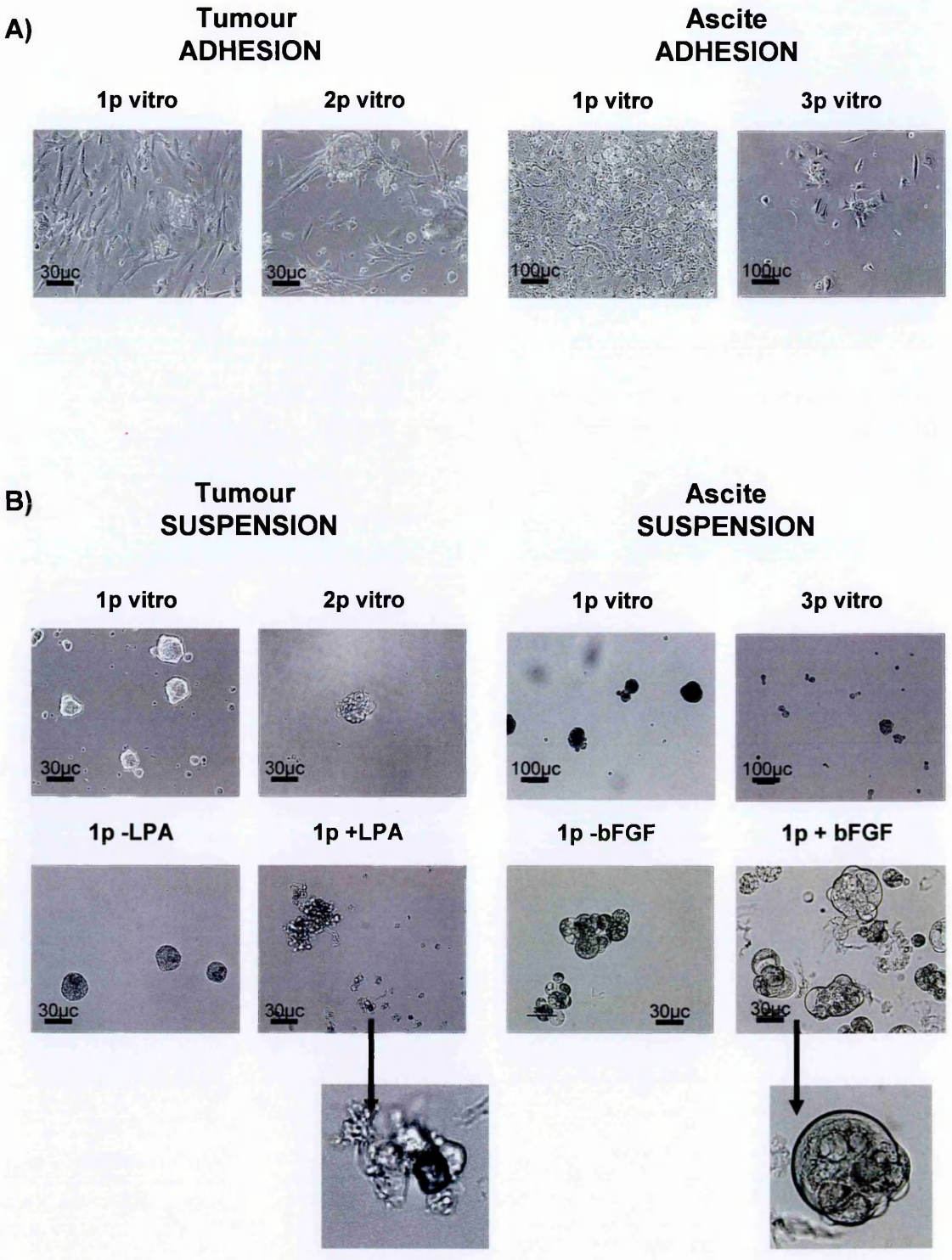


Figure 4.1: Setting up the culture conditions for expanding cancer cells:

A) Representative images of tumour cells, obtained after digestion of solid tumours or ascites clumps and *in vitro* maintenance in adherent conditions. After a few *in vitro* passages tumour cells did not proliferate and stromal cells became predominant. B) Representative images of tumour cells obtained after digestion of solid tumours or ascitic clumps and *in vitro* maintenance in suspension in Sphere medium. Growth of new spheroids was observed only for a few *in vitro* passages and was not improved by the presence of LPA (10 µM) and bFGF (10nM).

4.1.2 Ovarian cancer sample characterization: evaluation of specific marker expression

The expression of specific antigens, correlated with a stemness phenotype, is the principle approach adopted to isolate and characterize the CSCs/TICs from a wide range of haematopoietic and solid tumour types.

The chemokine receptor CXCR4, expressed by CSCs of different solid tumours, has been reported on a subset of ovarian tumour cells, but not yet described as a CSC marker in EOC. We aimed to investigate whether, like CD133, CXCR4 expressing cells would also identify a subpopulation with characteristics of cancer stem/progenitor cells in human primary EOC and ascites.

In the period from January 2007 to June 2010, we obtained at the time of surgery 69 ovarian solid tumour specimens and 106 ovarian carcinoma ascites.

Fifty-five cases of solid tumours were processed as described in the Chapter 'Material and Methods'. After processing, depending on FACS limiting factors, such as cell viability, the number of available cells and CD45 positivity, the cells obtained from 39 tumour samples were phenotypically characterized by FACS analysis. The expression level of CD45 was evaluated for monitoring the percentage of inflammatory cells present in the samples. Nine cases with a low percentage of CD45 positive cells (median 4.2%) were selected and used for further analysis (FIG. 4.2A).

In these cases, EpCAM expression was high (median 74.6%) while CD44 and CXCR4 showed a variable degree of expression (median 12.9% and 26.6% respectively) with a low mean fluorescence. CD133 expression was found on a low number of the cells in the tumour samples with a range of expression between 0-16.4% (median 1.4%) (FIG. 4.2A).

To confirm these results and to verify expression and localization of stemness markers; we analyzed, by immunocytochemistry on cryostat samples collected at the time of surgery, 18 ovarian carcinomas that were poorly differentiated. All the samples were positive for the epithelial marker EpCAM and for the ovarian carcinoma marker Folate Receptor (FR). All cases showed a focal reactivity for CD44, indicating that this antigen is expressed by a subpopulation of tumours cells. Among 17 cases analysed for CD133 expression, 10 showed focal tumour positivity, this being small groups of cancer cells positive for this marker. Interestingly, in one case we noted that CD133 positive cells were located near endothelial cells, surrounding blood vessels. For two cases we were able to monitor CXCR4 expression on paraffin embedded tumour samples (FFPE), depending on the specificity and immuno-reactivity of the CXCR4 monoclonal antibody used for this approach. As with CD44 and CD133 expression, CXCR4 positive cells were organized in small groups of tumour cells, close to the tumour invasive front (representative images are shown in FIG.4.2B).

Among 106 cases of ovarian carcinoma ascites, most of which were derived from ovarian serous adenocarcinoma, almost half (51) of the samples were characterized by the absence of tumour cells. The other 50% were

classified either as samples with a high or low number of cancer cells. These cases were phenotypically characterized by FACS analyses. FIG 4.2C illustrates some of these cases, depending on FACS limiting factors, such as cell viability, the number of available cells and CD45 positivity. The expression level of CD45 was assessed to check the range of inflammatory cells present in the samples. Only cases with a low percentage of CD45 positive cells were chosen and considered for further analysis (median 8.5%).

In these cases EpCAM positivity ranged from 15% to 82% (median 35.2%). CD44 and CXCR4 expression levels were similar to those observed in solid tumour samples. Both markers had a variable degree of expression with a low mean fluorescence, suggesting that most of the ascitic cells expressed few levels of these two molecules (median 7.3% and 24.2% respectively). Also the expression level of CD133 was similar to that observed in solid tumour samples with a range of positivity from 1% to 20% (median 2.7%) (FIG. 4.2C).

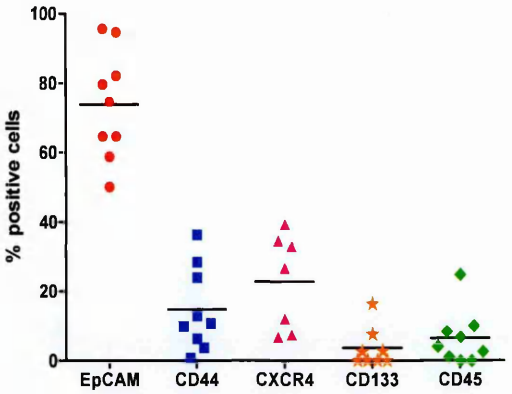
Cells obtained from four solid tumour cases were intraperitoneally injected in SCID mice and from none of them did we obtain a transplantable tumour cell line.

On the other hand, 18 cases of cells obtained from patient ascitic tumours were injected intraperitoneally in SCID mice and 33% (6) were able to grow *in vivo* for at least one passage. From three of them we obtained a transplantable primary ascites tumour cell line which could be phenotypically and functionally characterized for stemness properties.

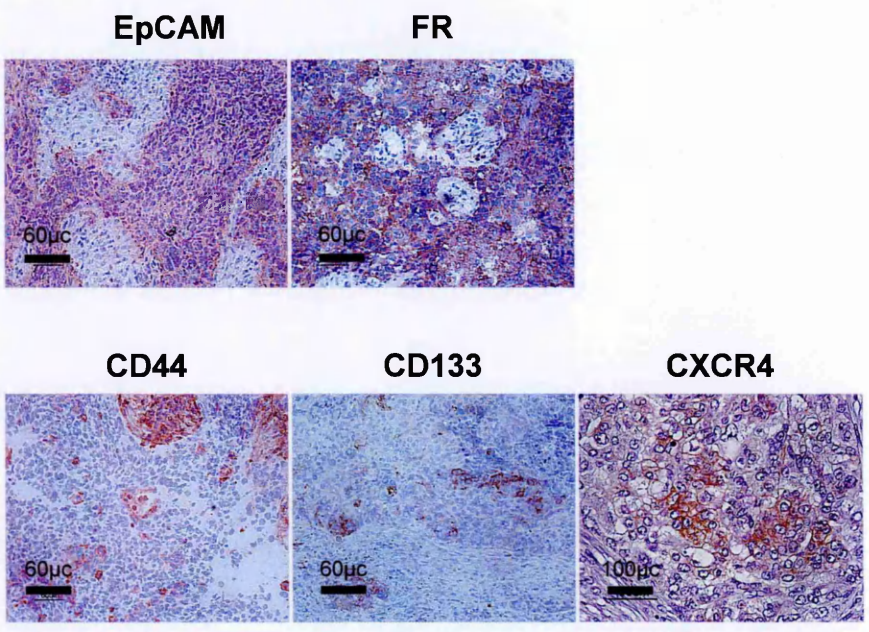
These results indicated that in a wide range of EOC primary tumours and ascitic samples the expression of CD133, CD44 and CXCR4 was restricted to a subpopulation of ovarian cancer cells. We thus focused on CD133 and CXCR4, both found expressed in other solid tumours, as potential ovarian CSC/TIC markers.

A)

MARKER	N° CASES	% POSITIVE CELLS (MEDIAN)
EpCAM	9	74.6
CD44	9	12.9
CXCR4	7	26.6
CD133	6	1.4
CD45	9	4.2



B)



C)

MARKER	N° CASES	% POSITIVE CELLS (MEDIAN)
EpCAM	7	35.2
CD44	7	7.3
CXCR4	7	24.2
CD133	5	2.7
CD45	7	8.5

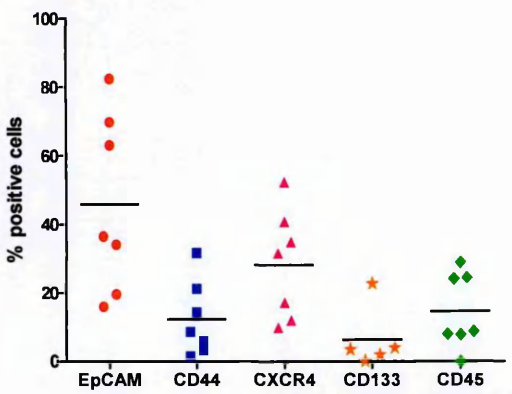


Figure 4.2: Ovarian cancer sample characterization: identification of specific marker expression.

A) and C) The table and graph summarize the % of positive cells (mean) for the indicated markers among ovarian tumours A) and ascites C) analyzed by FACS analysis. B) IHC representative images of tumours samples for specified markers

4.2 Isolation of cells with characteristics of stem/progenitor cells from epithelial ovarian cancer: selected case characterization

4.2.1 Clinical features and *in vivo* transplantability of selected cases

From three cases of ovarian carcinoma ascites (named ascites #1, ascites #2 and ascites #3) we were able to establish by intraperitoneal injection of ascitic cells, transplantable primary ascites tumour cell lines that were phenotypically and functionally characterized for CSC properties.

Ascites #1 was derived from a 47 year-old woman, with a poorly differentiated serous adenocarcinoma. The cells were transplanted in Nu/Nu mice for two *in vivo* passages but then its tumorigenic potential decreased (FIG. 4.3A). We received the sample at the time of primary surgery. The patient underwent a front-line treatment of standard chemotherapy and was lost at the follow-up.

Ascites #2 gave rise to a stable transplantable cell line for up to 9 *in vivo* passages in SCID mice. It originated from a 61 year-old patient with a serous adenocarcinoma stage IIIC, who had primary surgery followed by a debulking surgery within two months. We received the sample at the debulking surgery. At this time point the tumour had spread to the peritoneal cavity, most of the lymph nodes were positive and the ascites was characterized by a large number of tumour cell nests. After surgery the patient underwent a front-line treatment of six cycles standard

platinum-taxane based chemotherapy and was lost at the follow-up (FIG 4.3B).

The third case, ascites #3, was successfully transplanted for five *in vivo* passages in SCID mice. The patient had a previous clinical history of breast cancer. At 34 years old she was diagnosed with a triple negative breast cancer, which was chemotherapeutically treated. After three years she developed an endometrioid ovarian cancer stage III and received a standard neo-adjuvant treatment for three months and primary debulking surgery. After surgery the patient underwent a front-line treatment (six cycles of platinum-taxane based chemotherapy). After a disease-free period of about two years (platinum-sensitive) the tumour relapsed and we received the sample at the time of the second surgery. At this time point the tumour had spread into the peritoneal cavity, and the ascites was characterized by a large number of cancer cells and most of the lymph nodes were infiltrated by tumour cells (FIG 4.3C).

Among these three cases we could observe diversity in terms of histopathology, response to treatment and clinical features. Nevertheless all of them shared some common properties related to a stemness profile, as reported in the following chapters.

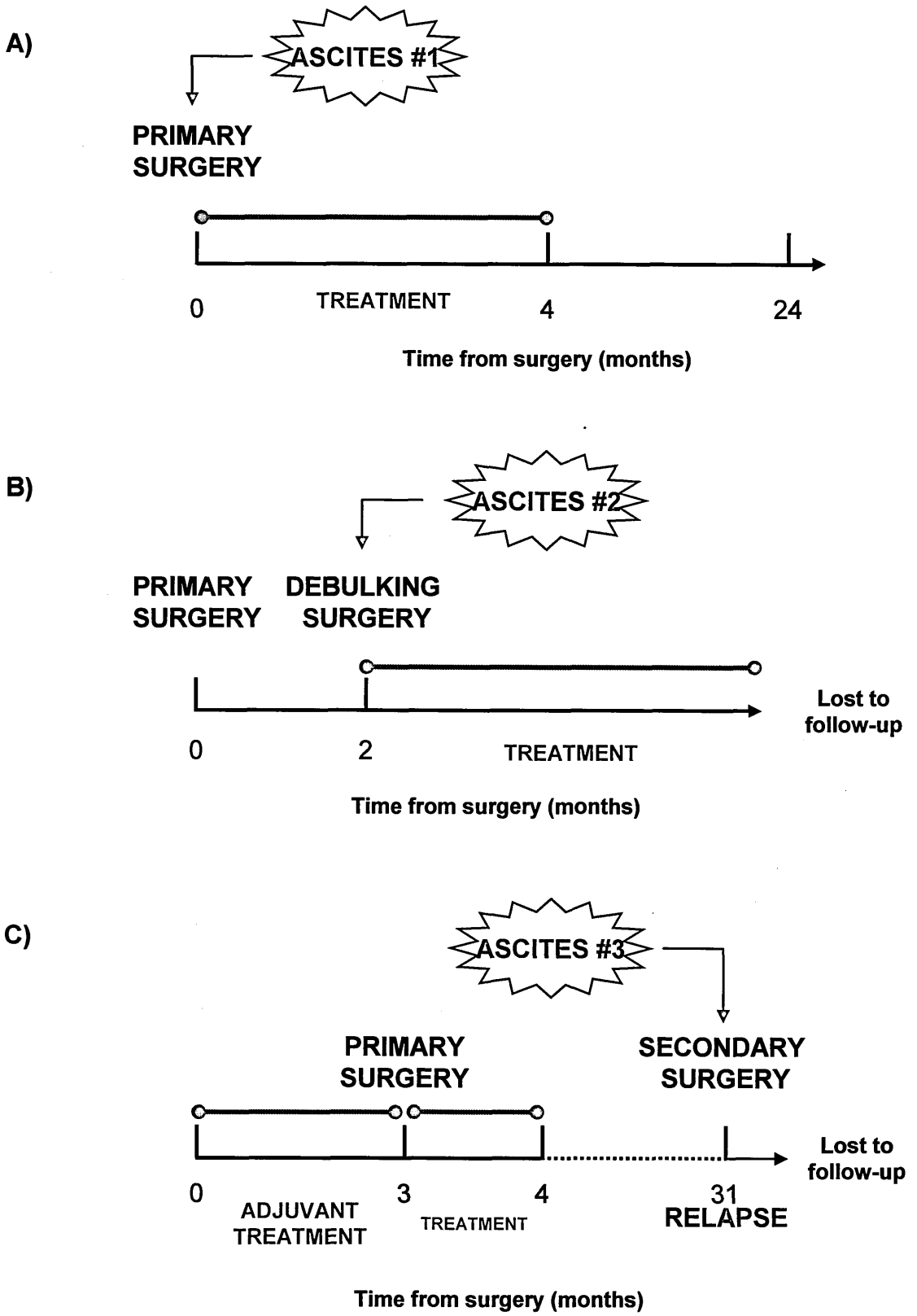


Figure 4.3: Establishment of transplantable primary ascites tumour cell lines: Clinical features of selected cases. Clinical history of patients from whom ascites #1 A), #2 B) and #3 C) were collected.

4.2.2 Ascites #1: stem cell-like phenotype of xenograft-derived ascites.

The first attempt to isolate a subpopulation of CSCs/TICs with stem-like properties was based on culture selection of cancer cells able to grow *in vitro* as spheres. Tumour cells derived from Ascites #1 and from xenograft-derived tumours/ascites were cultured *in vitro* in specific media, as described previously. We did not observe sphere formation in suspension culture or cancer cell growth in adherent conditions.

FACS analyses of ascites #1 revealed a strong positivity for the epithelial markers EpCAM and for CD44 (% positive cells 90.04% and 30.55% respectively). Positivity was also observed for the stem cell antigen CD133 (15.85%) and the embryonal stem cell antigen SSEA1 (15.27%) (FIG. 4.4A). These results were then confirmed by immunocytochemistry analyses on ascites #1 cryostat-cut solid tumour collected at the time of surgery. The patient tumour expressed high levels of EpCAM and CD44, showed a focal positivity for both CD133 and SSEA1 while CXCR4 expression was absent (FIG.4.4B).

Tumour cells from ascites #1 were injected intraperitoneally (i.p) in Nu/Nu mice and were successfully transplanted for 2 *in vivo* passages. Xenograft -derived tumours and ascites from the first and second transplantations were phenotypically characterized. We analyzed the xenograft-derived ascitic cells by FACS analysis and the xenograft-derived solid masses by immunocytochemistry on cryostat-cut sections (FIG. 4.4C and FIG. 4.4D).

The epithelial origin was confirmed by high expression of the EpCAM marker both in the xenograft ascitic cells (% positive cells: 95.9%) and the xenograft tumour. CD44 expression decreased in xenografted ascitic cells (% positive cells: 12.1%), whereas it seemed to be maintained at the same levels in xenograft-derived tumours.

On the contrary, the expression levels of CD133 (% positive cells: 96.7%) and SSEA1 (% positive cells: 96.6%) strongly increased in the xenograft-derived tumour and ascites. CXCR4 expression was found in a small subpopulation of ascitic cells (% positive cells: 4.1%). Its expression was not analyzed on cryostat sections from xenograft-derived solid masses (FIG. 4.4C and FIG. 4.4D).

The high expression level of stem cell markers observed in ascites #1 xenograft-derived sample was not sufficient to guarantee the *in vivo* transplantability of this case. Nevertheless, these results suggested that ovarian tumour samples could be characterized by the presence of a subpopulation with a stem cell-like phenotype.

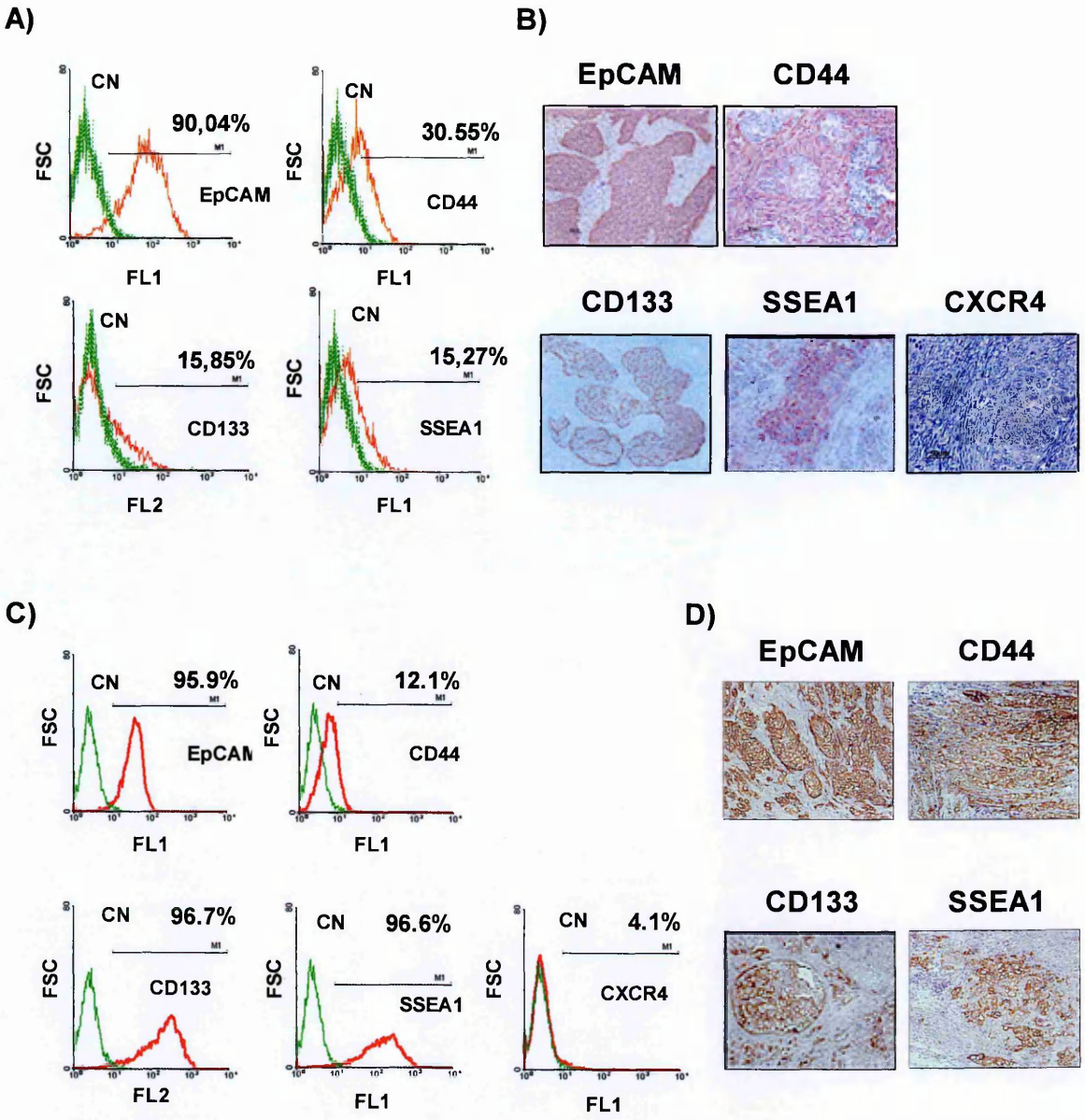


Figure 4.4: ascites #1: phenotypic characterization and stem cell-like phenotype of xenograft-derived solid tumours and ascites.

A) and C) FACS and B) and D) immunocytochemistry analyses of patient's surgical sample (A and B) and xenograft derived samples (C and D). The percentage of positive tumour cells for the indicated markers are shown; CN= negative control.

4.2.3 Ascites #2: stem cell-like phenotype of xenograft-derived ascites and *in vivo* tumorigenicity

As reported in Figure 4.5A and 4.5B, the patient's solid tumour showed a strong positivity for the epithelial marker EpCAM, while the ascitic cells were only partially positive for this antigen (% positive cells: 34%). CXCR4 expression was localized in a few tumour cell foci (FIG. 4.5A) whereas it was highly expressed in ascitic tumour cells (% of positive cells: 40.6%) (FIG. 4.5B). In both tumour and ascites, the CD44 antigen was expressed at a very low level. In the tumour sample, focal expression of CD44 was observed and only 1.4% of ascitic cells were positive for this marker.

CD133 showed a peculiar expression range. In the solid tumour we did not detect any CD133-positive cells while in ascitic cells its expression was present on 22.7% of tumour cells. Finally SSEA1 expression was detected only in ascitic cells at a very low level (% positive cells: 1%).

These results revealed that, in the same patient, the solid tumour and the ascitic cells may have a different CSC marker profile, suggesting further heterogeneity within the same population of cancer cells. Alternatively, some protein expression could be acquired late during tumour development, possibly as an effect of growth in non-adherent conditions in the ascitic fluid, such as CD133, or could indicate specific features of the subpopulation of positive cancer cells, such as CD44 and CXCR4.

To identify another possible marker for ovarian CSC characterization and isolation, we performed the Aldefluor assay, which is based on the

expression of the ALDH enzymes. This analysis revealed that only 1.9% of the ascitic cancer cells possessed the ability to metabolize the Aldefluor reagent. This subpopulation of ALDH+ cells was only partially inhibited by the presence of the DEAB inhibitor (0.6%) (FIG. 4.5C). Notably we detected a high mortality of tumour cells after the Aldefluor staining procedure, suggesting that the low ALDH positivity observed may have been due to the high percentage of dead cancer cells.

Ascites #2 was successfully transplanted in SCID mice for more than eight *in vivo* passages, developing mainly xenograft-derived ascites and showing a high tumorigenic potential. The tumorigenic potential of ascites #2 was evaluated *in vivo* by both intraperitoneal and subcutaneous injection in SCID mice.

After i.p injection, the xenograft-derived ascitic cell growth was assessed as a body weight increment (g) and compared to same age non-xenografted mice, used as control. Initially high tumour cell doses were injected to ensure tumour engraftment. At a high dose of $60x10^6$ cells, ascites developed in all mice treated (100% tumour take) and in all *in vivo* passages analyzed (FIG. 4.6A). Moreover the body weight increment was exponential, suggesting that the *in vivo* growth may have selected for a subpopulation of tumour cells with a higher proliferative potential. These data were also confirmed by the decrement observed in the tumour onset. At the 5^{th} *in vivo* passage ascitic cells grew more rapidly than at the 1^{st} *in vivo* passage, as the time necessary for ascites growth decreased from 75 days (1^{st} passage) to 40 days (5^{th} passage) (FIG 4.6A and FIG. 4.6B). These

results indicated that cancer ascitic cells with a higher tumorigenic and proliferative potential were selected during *in vivo* transplantation.

To better evaluate cancer cell growth and confirm the tumorigenic potential of ascites #2 s.c injections in SCID mice were performed.

As shown in Figure 4.6C, injection of 9 and 3 x 10^6 cell doses induced weight loss and cachexia in treated mice, causing animal death within 20 days after injection. The injection of lower cell doses of $2x10^6$, $1x10^6$ and 0, 5 x 10^6 cells generated xenograft-derived solid tumours in almost all mice treated (tumour take: 100% at $2x10^6$, 66% at $1x10^6$ and 66% at 0, 5 x 10^6). Tumour take increased in proportion to cell dose but tumour masses were not easily measurable, these being characterized by small volumes even after three months from injection. Xenografted solid tumours showed a cystic morphology, with the presence of necrotic or inflammatory infiltrate (FIG. 4.6C).

These data indicate that, injection of ovarian cancer cells isolated from ascites #2 was able to propagate cancer cells much more efficiently if injected intraperitoneally instead of subcutaneously. After i.p. injection of patient's ascites we did not detect the formation of solid tumour masses, since it only developed xenograft-derived ascites in all *in vivo* passages. Moreover these results suggest that within the heterogeneous cancer cell population, there may exist a pool of CSCs that strongly contributed to the rapid development of the tumour mass.

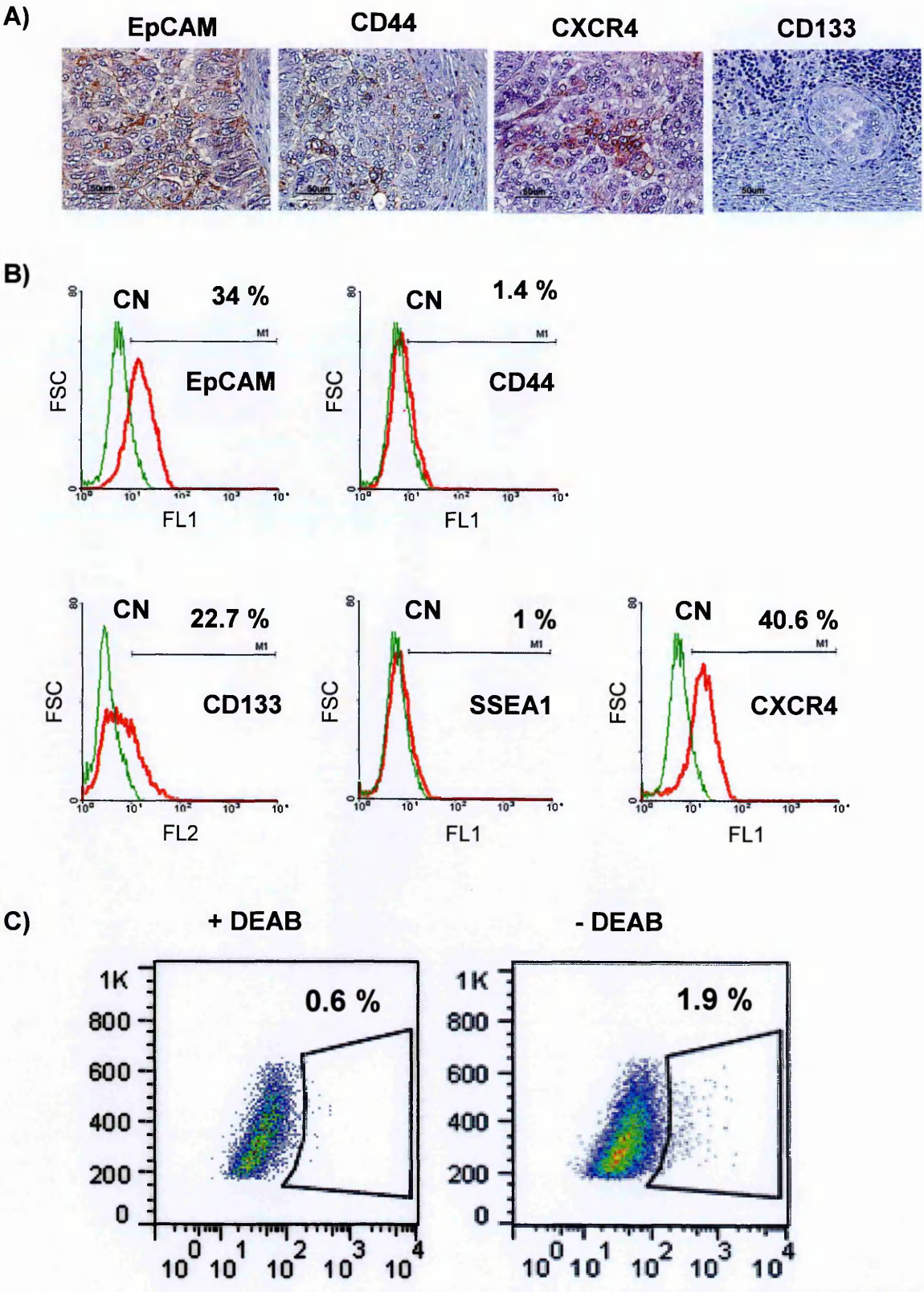


Figure 4.5: ascites #2 phenotypic characterization.

A) Immunocytochemistry analyses of the patient's solid tumour (Bar: 50μc, 20X Epcam, 40X CD44, CXCR4, CD133). B) FACS analysis of ascites #2. The percentage of positive tumour cells for indicated markers are shown. C) Aldefluor analysis of ascites #2 treated or not with ALDH inhibitor DEAB. The percentage of ALDH positive tumour cells is indicated.

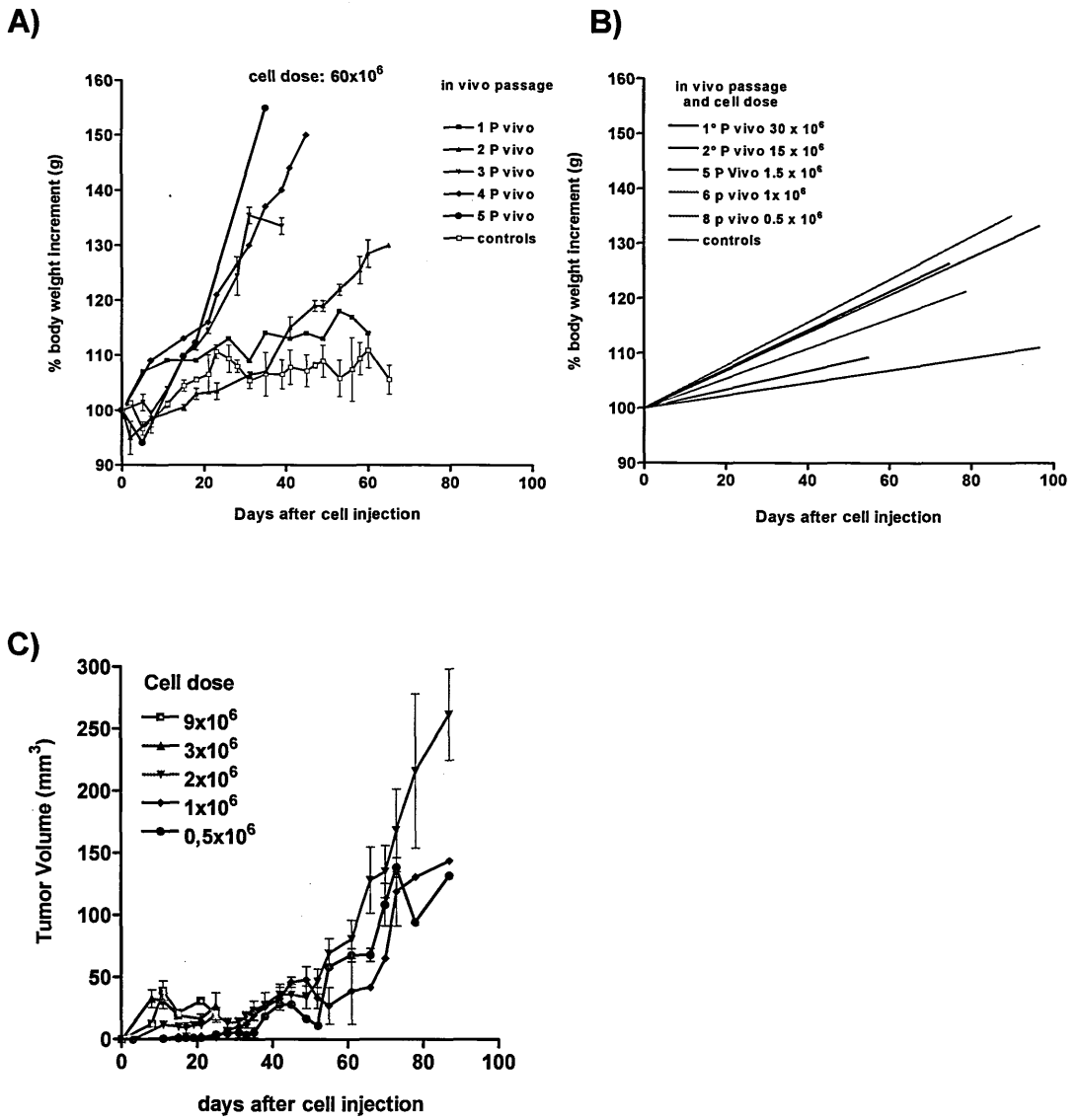


Figure 4.6: ascites #2 *in vivo* tumorigenicity

A) One cell dose ($60x10^6$) of ascites #2 was intraperitoneally (i.p) injected in SCID mice and xenograft-derived ascites growth was evaluated as % of body weight increment. Non-treated mice were reported as control for normal body weight increment. Ascites #2 tumour up-take and tumorigenic potential increased during *in vivo* transplantation. B) Statistical analysis (linear regression) at different *in vivo* passages of ascites #2 at decreasing cell doses. C) Ascites #2 subcutaneously (s.c) injected at decreasing cell doses in SCID mice. Tumour growth was evaluated as tumour volume (mm^3).

4.2.4 Ascites #3: Stem cell-like phenotype of xenograft-derived ascites and *in vivo* tumorigenicity

Ascites #3 was phenotypically characterized for stemness properties and successfully transplanted in SCID mice for five *in vivo* passages.

FACS analysis of the patient's sample revealed that only 16% of ascitic cells expressed the epithelial marker EpCAM and the 8.6 % of tumour cells were positive for CD44. Ascites #3 CXCR4 positivity was lower than that observed for the previous case (ascites #2), but more or less similar to those of ascites #1. We did not detect any significant positivity for SSEA1 and CD133, with expression levels very low (2.6% and 3% respectively) (FIG. 4.7A).

The tumorigenic potential of ascites #3 was assessed by intraperitoneal injection in SCID mice at different cell doses ($100x10^6$, $50x10^6$ and $30x10^6$). In the first and second transplantations, mice developed both ascites and solid tumours, while in the subsequent *in vivo* passages we detected only ascites formation. Xenografted solid tumours recapitulated the original tumour heterogeneity, with wide areas of necrosis and endometrioid features. At the dose of $30x10^6$ cells, ascites developed in all mice treated (100% tumour take) and in all *in vivo* passages analyzed (FIG. 4.7A). As for the previous case, the percentage of body weight increment was exponential whereas the tumour onset decreased during *in vivo* transplantation. At the 5^{th} *in vivo* passage, ascitic cells grew more rapidly

than at the 1st *in vivo* passage, as the time necessary for ascites growth decreased from 200 days (1st passage) to 50 days (5th passage) (FIG. 4.7B). These results indicated that *in vivo* transplantation has again selected for those cancer cells with the highest tumorigenic and proliferative potential.

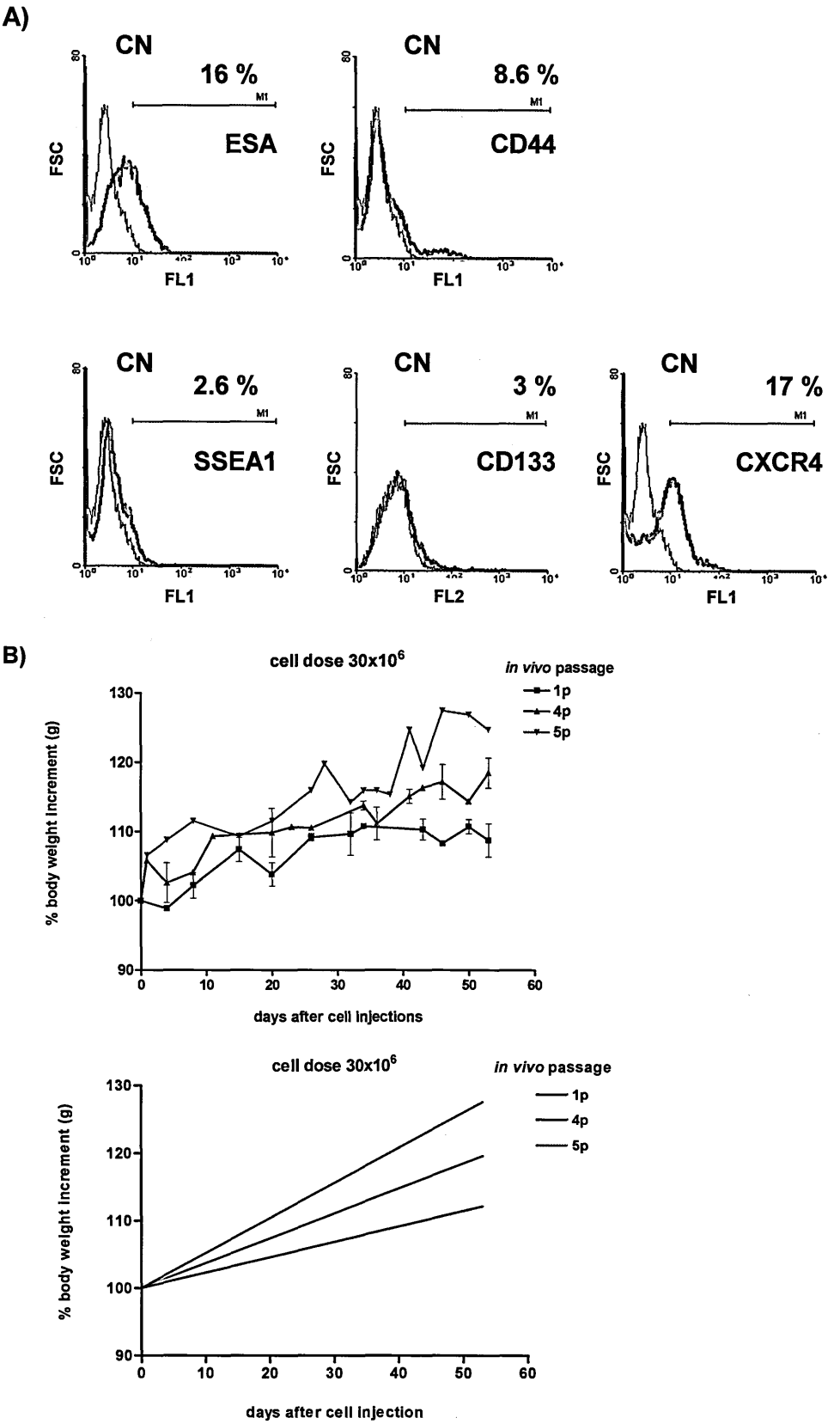


Figure 4.7: phenotypic characterization and i*n vivo* tumorigenicity of ascites #3.

A) FACS analysis of ascites #3. The percentage of positive tumour cells for indicated markers are shown. B) The cell dose $30x10^6$ of ascites #3 was intraperitoneally (i.p) injected in SCID mice and ascites growth was evaluated as % of body weight increment. Statistical analysis (linear regression) at different *in vivo* passages of ascites #2 after injection of $30x10^6$ tumour cells.

4.3 Characterization of *in vivo* stabilized cell lines.

4.3.1 Ascites #2: phenotypic characterization of xenograft-derived ascites

Ascites #2 was successfully transplanted in SCID mice for more than eight passages, as described in 4.2.3 paragraph. After xenograft-derived ascitic fluid withdrawal, ascitic cells were phenotypically characterized by FACS analyses in order to check if the expression of selected markers could be modulated after *in vivo* transplantation, furthermore to verify if *in vivo* growth could have selected for a subpopulation of cancer cells with a specific antigenic profile.

Epithelial phenotype, assessed by the expression of the EpCAM marker ranged from 46.9 % to 96.1% (FIG. 4.8A and FIG. 4.8B). The high percentage of EpCAM positivity confirms that the majority of the cancer cells analyzed were both epithelial and human in origin, excluding possible contamination of mouse cells.

CD44 expression levels increased rapidly after the 1st *in vivo* passage, suggesting that *in vivo* growth selectively stimulated the proliferation of CD44 positive cells. Indeed, starting from a positivity of 8.3% (1st passage), we observed that almost 50% of ascitic cells expressed this antigen in the subsequent *in vivo* passages (FIG. 4.8A and FIG. 4.8B).

CD133 and CXCR4 positivity were also evaluated, considering only the brightest cells to exclude possible false-positives due to fluorochrome internalization. As reported in Figures 4.8A and 4.8B, CD133 was

expressed by a small percentage of ascitic cells with a range of positivity from 0.2% to 10.8%. Compared to the patient's ascitic phenotype (FIG. 4.5B), CD133 expression decreased *in vivo*, achieving a stable expression level of about 1% (FIG. 4.8B). Considering the very low levels of CD133 positivity and to exclude probable CD133 false-positives, epifluorescence analyses on FACS stained samples were performed and we demonstrated that only a small number of xenografted ascitic cells specifically expressed this molecule on the cell surface (representative image is shown in FIG. 4.8C).

CXCR4 expression did not change significantly between the patient sample and the xenografted ascitic cells. This marker was expressed in all *in vivo* passages examined and almost 30% of ascitic cells expressed this receptor (FIG. 4.8A). Indeed, the CXCR4 expression level was very heterogeneous in xenograft derived ascites and we noted the co-existence of CXCR4 low and bright cells in the same cancer cell population. For this reason, we considered only the subpopulation of CXCR4 bright cells as being the tumour cells positive for this antigen. Except for a few cases, CXCR4 positivity ranged from 4% to 20% (FIG. 4.8B). By epifluorescence analyses on FACS stained samples, we confirmed that only a minority of xenograft-derived ascitic cells specifically expressed this marker (representative image is shown in FIG. 4.8C).

Since the CD133 marker was reported to be associated with the ovarian CSC phenotype, we evaluated the possible correlation between CD133 and CXCR4. In double staining experiments and FACS analyses, we did not

observe a clear overlap between the two tested markers in all the xenograft-derived ascites examined (FIG. 4.8A). The existence of a double $CXCR4^{bright}/CD133^{bright}$ positive subpopulation was not easily detectable due to the variable degree of expression of both antigens. FACS analyses showed that double positive cells ranged from 0.1% to 4%, but epifluorescence analyses did not confirm these data (data not shown).

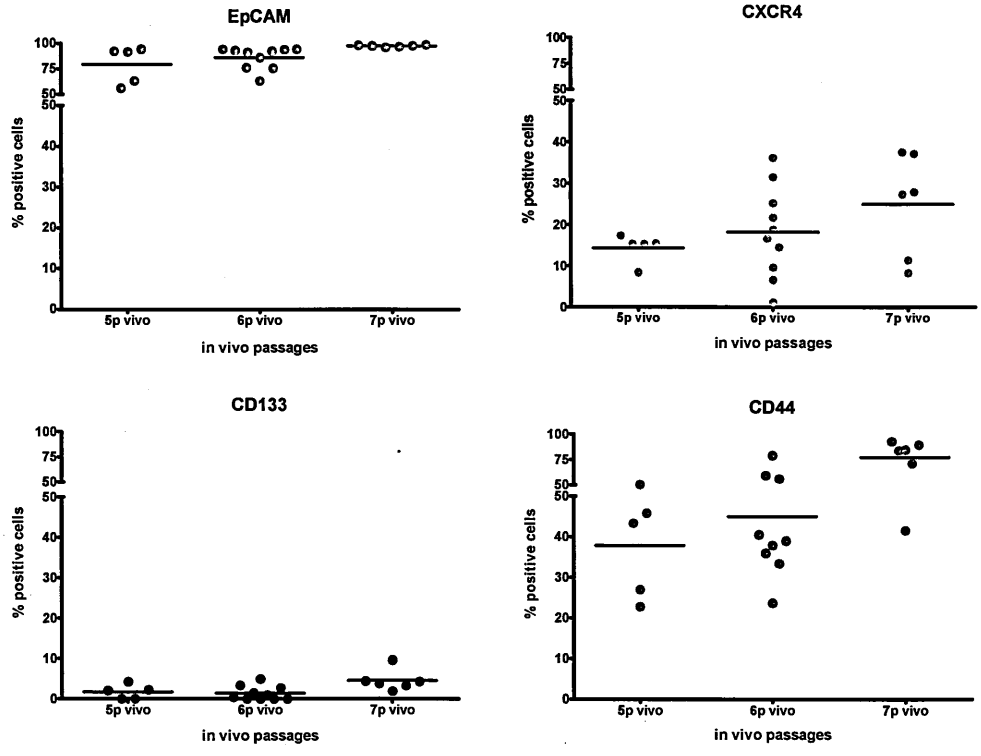
As shown in Figure 4.8D, we also tried to evaluate the differences in term of morphological parameters between CXCR4+, CD133+ and CXCR4+/CD133+ tumour cells. Whereas CD133 expression was correlated with cancer cells with the highest SSC and FSC parameters (FIG. 4.8D red arrow), the CXCR4 positive subpopulation was composed of the smallest cancer cells (FIG. 4.8D yellow arrow). As expected, the double positive population was formed by those cells with intermediate FCS and SCS values (FIG. 4.8D orange arrow). Nevertheless the existence of a double positive ovarian cancer cell population may correspond to the most metastatic and chemoresistant subpopulation.

The Aldefluor assay was performed on the xenograft-derived ascites from patient #2 to evaluate the possible *in vivo* selection and amplification of ALDH-positive cells. A notable increase in ALDH positivity was observed after *in vivo* transplantation as compared to the patient sample (FIG. 4.5C). In fact almost 60% of xenograft-derived ascitic cells at the 3^{rd} *in vivo* passage were found positive for this marker (FIG. 4.8D).

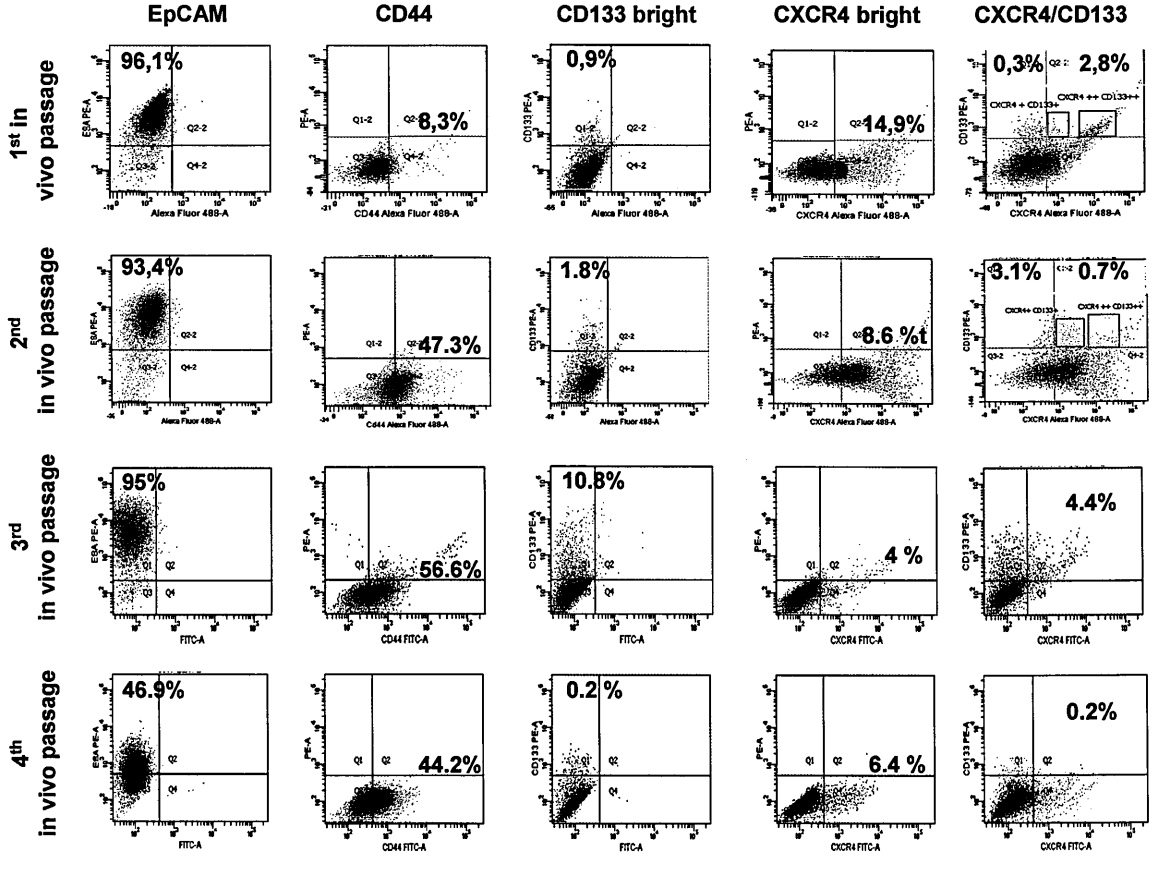
These results may indicate a possible role for CD133 and CXCR4 in the ovarian CSC phenotype. These markers could be co-expressed together

with ALDH by a subpopulation of ovarian cancer cells and these cells may be responsible for the tumour relapse, chemoresistance and metastasis.

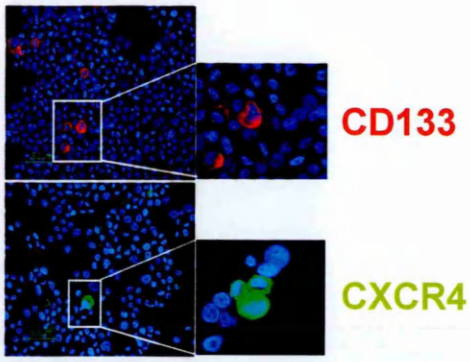
A)


EpCAM
CXCR4
CD133
CD44
% positive cells
5p vivo
6p vivo
7p vivo
in vivo passages

B)

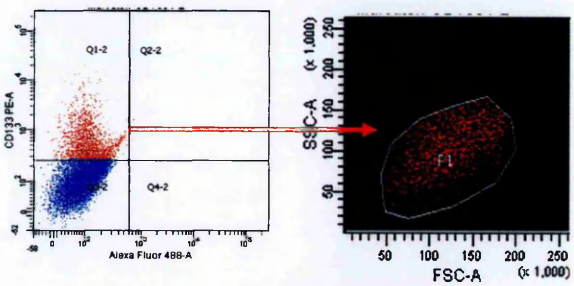

EpCAM
CD44
CD133 bright
CXCR4 bright
CXCR4/CD133
1st in vivo passage
2nd in vivo passage
3rd in vivo passage
4th in vivo passage
96,1%
8,3%
0,9%
14,9%
0,3%
2,8%
93,4%
47.3%
1.8%
8.6 %t
3.1%
0.7%
95%
56.6%
10.8%
4 %
4.4%
46.9%
44.2%
0.2 %
6.4 %
0.2%

C)

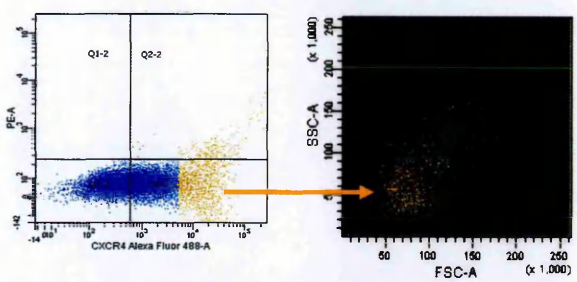


D)

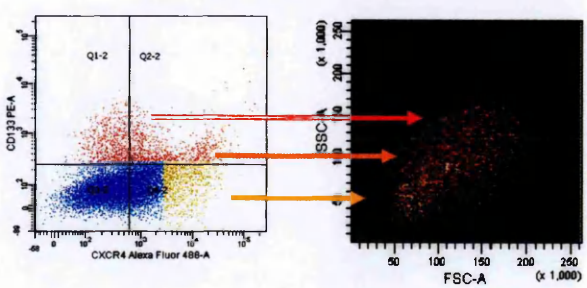
CD133 11,2%



CXCR4 8.8 %



CXCR4+/CD133+ 6.8 %



E)

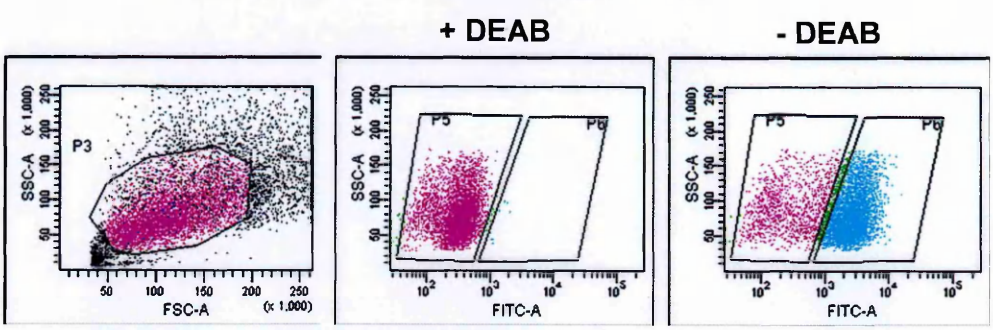


Figure 4.8: Ascites #2: characterization of xenograft-derived ascites

A) Expression levels of indicated markers were monitored at the 4^{th}, 5^{th}, 6^{th} and 7^{th} *in vivo* passages of ascites #2. B) FACS analysis of xenograft-derived ascitic cells. The percentage of positive tumour cells for indicated markers is shown. C) Representative epifluorescence images for CD133+ and CXCR4+ cells were reported. D) Example of FACS analysis of xenograft-derived ascitic cells for CD133+, CXCR4+ and CD133+/CXCR4+ cells. For each of these subpopulations are reported the morphological parameters; the analysis underlined the differences in terms of cell granularity and size among the three subpopulations. E) Aldefluor analysis of xenograft derived ascitic cells at the 3^{rd} *in vivo* passage.

4.3.2 Ascites #2: correlation between selected markers and tumorigenic potential

Xenograft-derived ascitic cells were sorted for CD133 or CXCR4 and positive and negative fractions were again i.p injected in SCID mice.

Three sorting experiments for CXCR4 at 5^{th}, 7^{th} and 8^{th} *in vivo* passages were performed and sorted cells were i.p injected in SCID mice at a dose of 0.5×10^6 for each subpopulation. Two sortings for CD133 were carried out at 5^{th} and 7^{th} *in vivo* passages and sorted cells were i.p injected in SCID mice at a dose of 0.5×10^6. The obtained results are summarized in Figure 4.9. CXCR4 positive and negative cells demonstrated a similar *in vivo* take (100% of injected mice) and similar time of ascites onset and ascites growth, expressed as the percentage of body weight increment (FIG. 4.9A). On the contrary, the unsorted population grew more rapidly than either one of sorted subpopulations and gave rise to a more abundant ascites. In addition, the CD133 positive and negative cells had a similar growth rate and did not differ significantly in terms of tumour take (100%) and time of tumour onset (FIG. 4.9B). Unsorted cancer cells had a similar growth potential to sorted CD133 subpopulations.

Taken together these results suggest that both CD133 and CXCR4 do not identify ovarian cancer cells with a higher tumorigenic potential. It could be possible that, within the heterogeneous cancer cell population, these two markers are linked to other CSC properties but not to tumour initiating capacity.

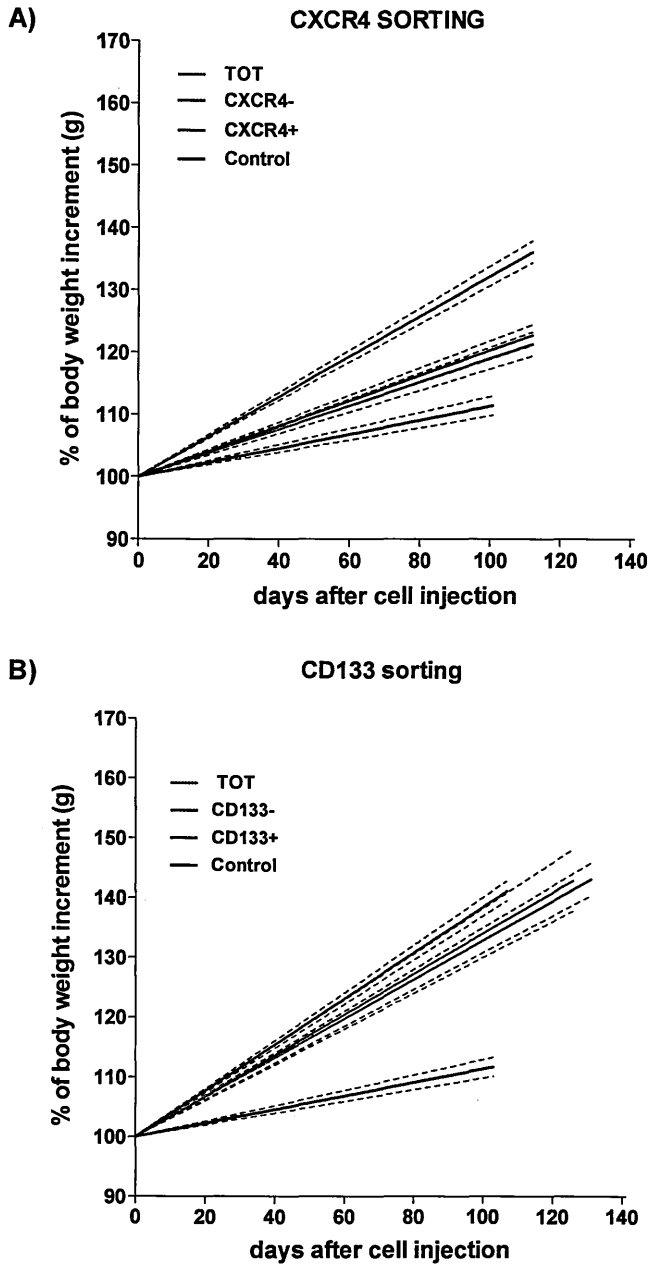


Figure 4.9: Ascites #2: correlation between selected marker expression and tumorigenic potential.

After sorting ascites #2 xenograft-derived ascitic cells for CXCR4 (mean of three experiments, four mice each group) A) and CD133 (mean of two experiments, three mice each group) B), positive, negative and unsorted populations were evaluated for their tumorigenic potential. $0.5x10^6$ cells were i.p injected. Tumour growth was evaluated as % of increased body weight and non-treated mice were reported as control for normal body weight increment. The data are reported as linear regression analysis.

4.3.3 Ascites #2 and #3: correlation between selected markers and expression of stemness markers

Cancer stem cells share some characteristics with normal stem cells (see Chapter 'Introduction'). One of them is the expression of similar surface receptors (e.g. CXCR4, CD133, CD117 and CD44) that are either identified as stem cell markers or associated with homing and metastasis. Moreover, CSCs have in common with their normal counterparts the expression of transcription factors such as Oct4 and Nanog, which maintain a functional plasticity by promoting pluripotency and immortality.

Based on this assumption, we evaluated the possible correlation between CXCR4 or CD133 expression and those of different genes linked to a stemness profile.

Xenograft-derived ascites from case #2 were sorted for CXCR4 at the 4^{th}, 5^{th} and 6^{th} passages and for CD133 at the 3^{rd}, 5^{th} and 7^{th} *in vivo* passages. Sorted CXCR4 or CD133 positive tumour cells were evaluated by Real Time PCR for stem cell-associated genes (*OCT4*, *NANOG*, *CD133* and *NESTIN*). For both ascites #2 and #3 the analyses were performed using the negative fraction as an internal calibrator and considering significant only those expression differences whose fold change was ≥ 2 (FIG. 4.10A). In two out of three sorting experiments, ascitic cells, that were CXCR4 bright at the cell surface, exhibited significantly higher *CXCR4* and *CD133* mRNA levels than the CXCR4 negative cells. In all three sorting experiments, the expression level of *OCT4* was up-modulated in CXCR4 positive cells and in

two out of three sorting experiments *NANOG* and *NESTIN* expression levels were higher in the CXCR4 bright cells than in the negative fraction. These results were confirmed by real time analysis of ascites #3. Also in this case, CXCR4 positive cells (sorted at the 3rd *in vivo* passage) expressed higher mRNA levels of stem cell-associated genes than the negative fraction (FIG. 4.10B). Association of increased expression of mRNA levels of *OCT4*, *NANOG* and *NESTIN* to CXCR4 seemed to be much more evident in ascites #2 than that in ascites #3.

CD133 positive cells showed in all three sorting experiments significantly higher levels of *CD133* mRNA, while the *CXCR4* mRNA level was increased in two out of three experiments. mRNA levels of *OCT4* and *NANOG* were up-modulated in one out of three sorting experiments and the mRNA level of *NESTIN* did not significantly change between CD133 positive and negative cells (FIG. 4.10C).

In conclusion, we found an enrichment in mRNA levels of stem cell-associated genes, with a good reproducibility, only in the sorted CXCR4 positive cells, whereas in the CD133 positive cells these genes seemed to be enriched to a very variable degree.

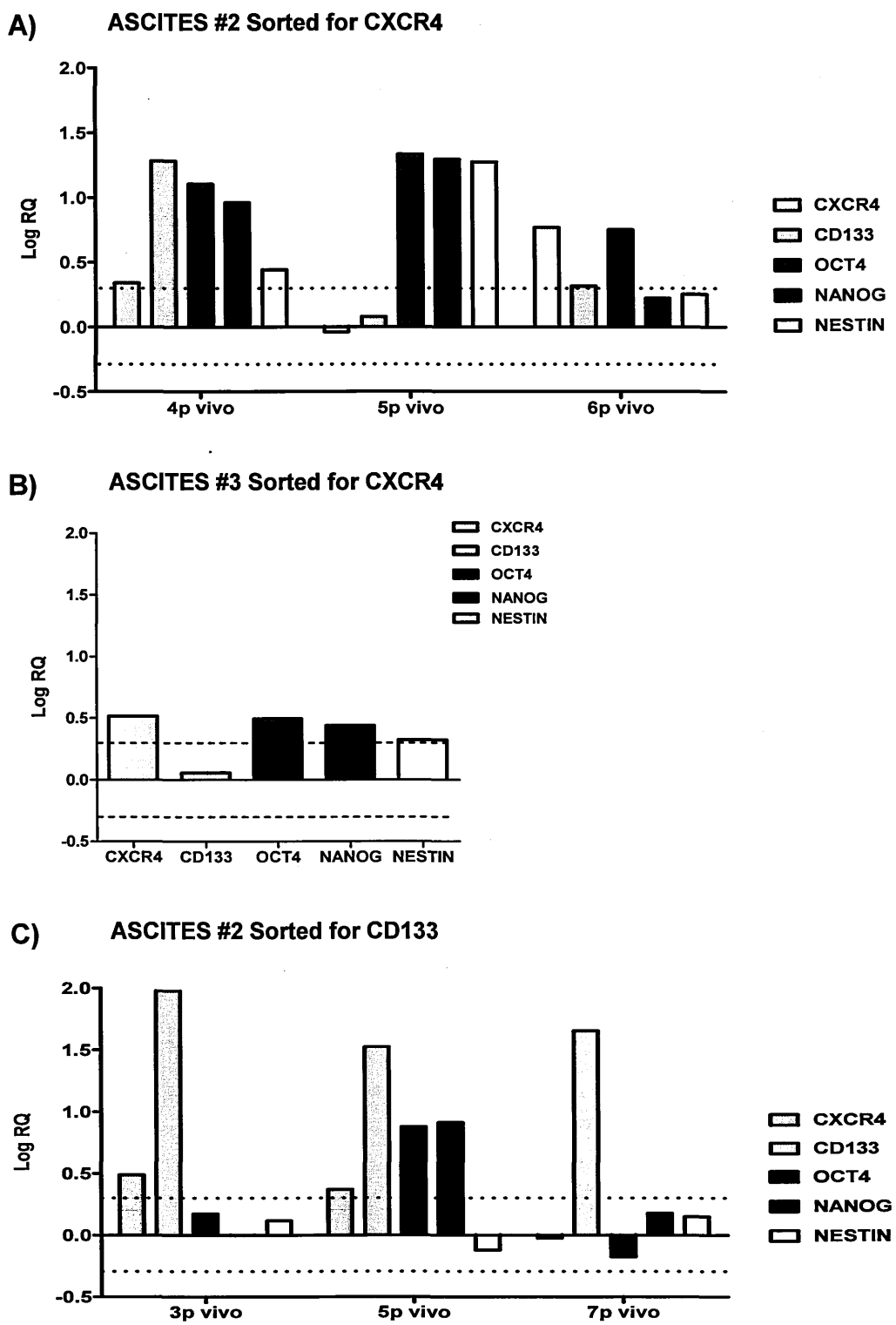


Figure 4.10: Ascites #2 and #3: correlation between selected subpopulations and expression of stemness markers.

Real time analysis of sorted CXCR4 A) and CD133 C) positive and negative cells of ascites #2 at different *in vivo* passages. B) Real Time analysis of sorted CXCR4 positive and negative cells of ascites #3. Fold change for each sample and gene were relative to the internal calibrator (negative cells) and were indicated as Log RQ (relative quantification). Fold change of two, corresponding to Log RQ= ± 0.3, was considered significant in terms of differential gene expression profile.

4.3.4 Ascites #2 and #3: correlation between selected markers and expression of ABC transporters

Multi-drug resistance of tumour cells is an important element which leads to the ineffectiveness of chemotherapeutics. The identification of subpopulations of cancerous ovarian cells expressing multi-drug resistance factors and exhibiting cancer stem cell properties has recently become a major research interest (See Chapter 'Introduction'). Drugs are effluxed from the cell by numerous cell surface transporters, the majority of which belong to the ABC (ATP binding cassette) transporter super-family. ABC transporters comprise seven families (ABCA-B-C-D-E-F and G) which include members involved in the efflux of structurally-unrelated drugs. ABCG1, ABCG2 and MDR1 (ABCB1) have been reported to be up-regulated in CSC/TIC of numerous tumour types (127) (360) (128).

To verify if CXCR4 and CD133 expression could identify tumour subpopulations, expressing markers linked to chemoresistance, CXCR4 and CD133 sorted fractions were analyzed by real time PCR for the expression of all known drug transporters of the ABC superfamily. For both ascites #2 and #3 the analyses were performed using the negative fraction as an internal calibrator and considering significant only those genes with a fold-change ≥ 2.

CXCR4 positive cells of ascites #2 xenograft-derived ascitic cells showed a variable pattern of modulation of selected ABC transporter mRNA levels, depending on the *in vivo* passage considered. In two out of three or in all *in*

vivo tested passages, and for only a small number of ABC transporters, was there a concordant increase (*ABCA10*, *ABCA12*, *ABCB10* and *ABCF2*) or decrease (*ABCC2* and *ABCA1*) in the positive fraction as compared to the negative one (FIG. 4.11A).

Ascites #3 was sorted for CXCR4 at the 3rd *in vivo* passage and sorted subpopulations were analysed. In this case, mRNA expression levels of all ABC transporters were lower in the CXCR4 positive cells as compared to the negative subpopulation (FIG. 4.11B).

Ascites #2 sorted for CD133 at two *in vivo* passages (3rd and 5th) was also analysed by Real time PCR for ABC transporter mRNA levels. The mRNA levels of the majority of the examined genes were more strongly expressed in the positive cells, especially those of the ABCA and ABCB sub-families. A moderate increase of transcripts of the ABCC and ABCG sub-families members was also observed (FIG. 4.11C).

Overall, in sorted subpopulations from both ascites #2 and #3: *ABCG1* appeared to be expressed at lower levels in CXCR4 positive cells and at higher levels in CD133 positive cells; expression of *ABCG2* mRNA was at a lower level in CXCR4 positive cells from ascites #3, while the gene expression level was similar between positive and negative fractions from ascites #2. Similarly, the fold-change of *ABCG2* between CD133 positive and negative cells was not significantly different (FIG. 4.11A-C).

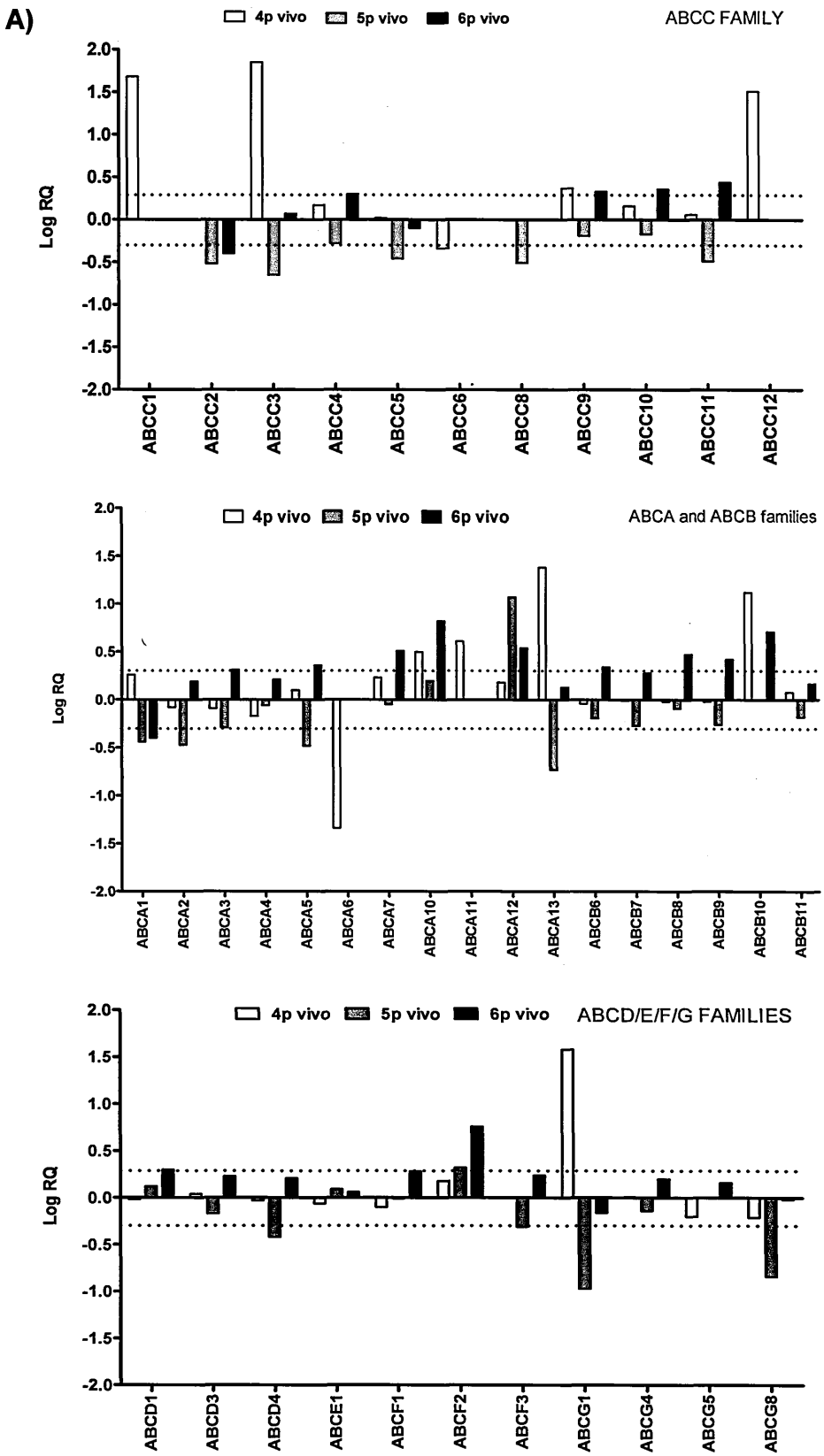
Modulation of the expression profile of *ABCB1*, also known as *MDR1*, was not observed in ascitic cells expressing CXCR4, because no significant difference was found in CXCR4 positive cells as compared to the negative

fraction (FIG. 4.11A and FIG. 4.11B). Conversely, in one out of two *in vivo* passages, sorted for CD133, a strong up-modulation of *MDR1* was observed in CD133 positive cells (FIG. 4.11C).

These results suggest that CXCR4 expression in ovarian carcinoma or ascites may not be correlated to the chemoresistant phenotype of ovarian TICs. On the contrary, the strong up-modulation of ABC transporter observed in CD133 positive cells suggests that this marker is probably expressed by those cells with a major ability to exclude chemotherapeutic agents.

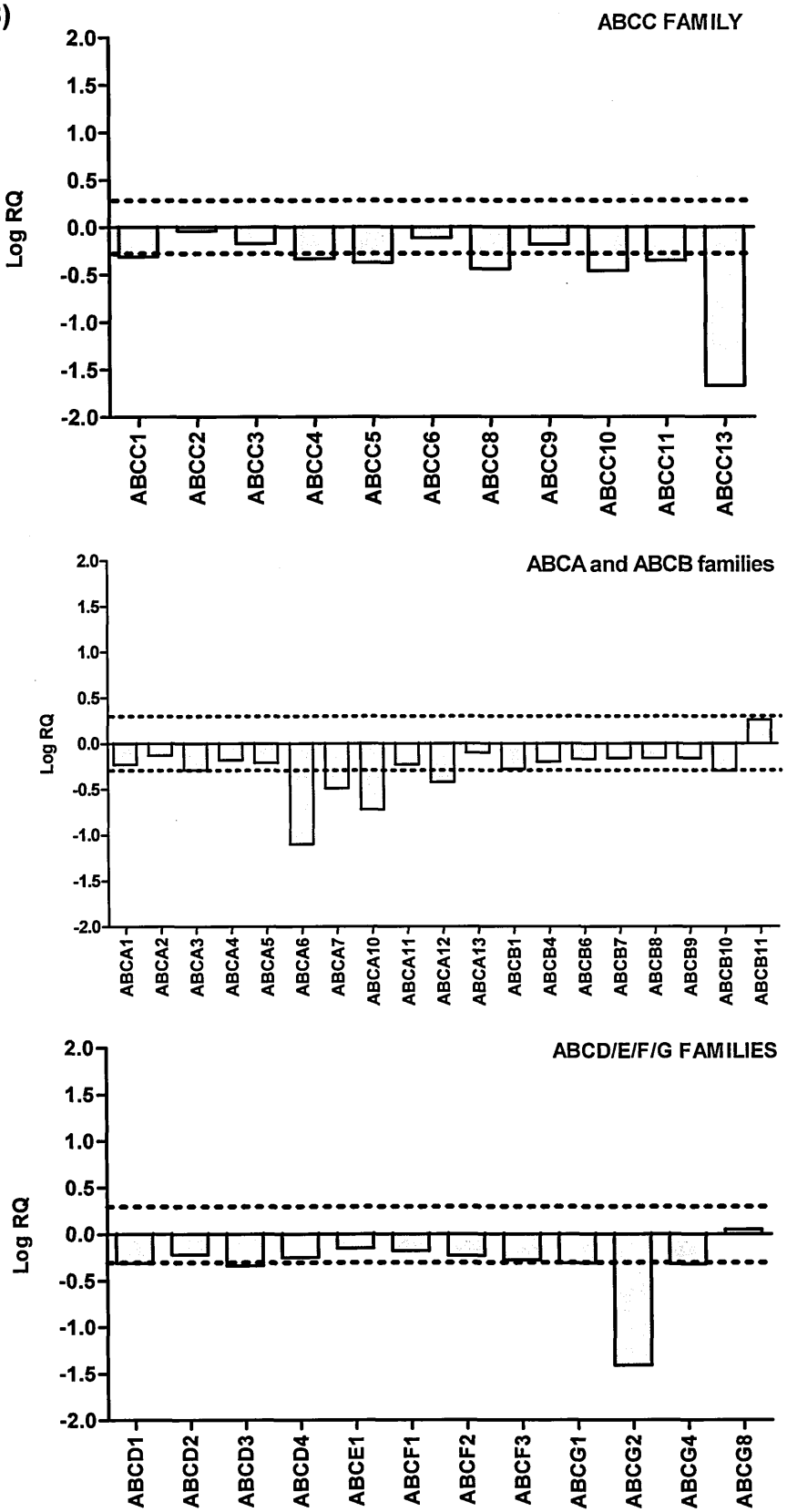
ASCITES #2 sorted for CXCR4

A)



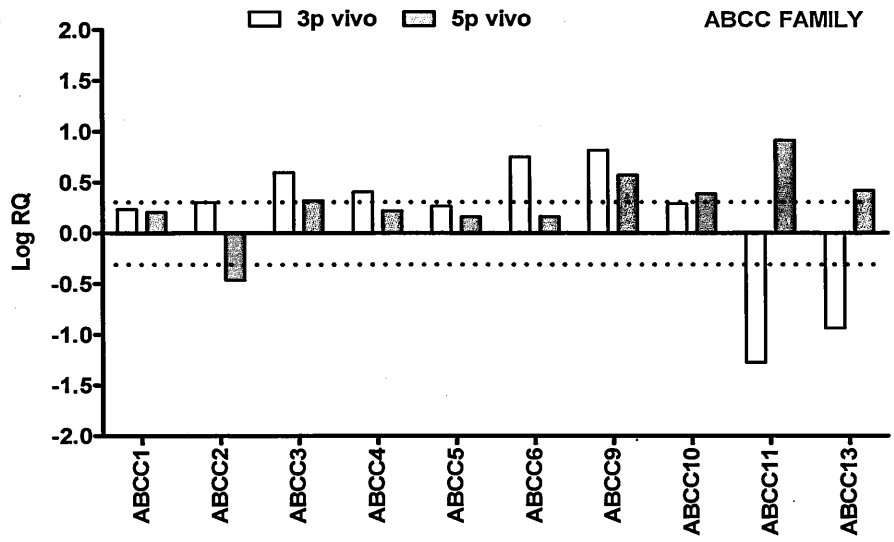
ASCITES #3 sorted for CXCR4

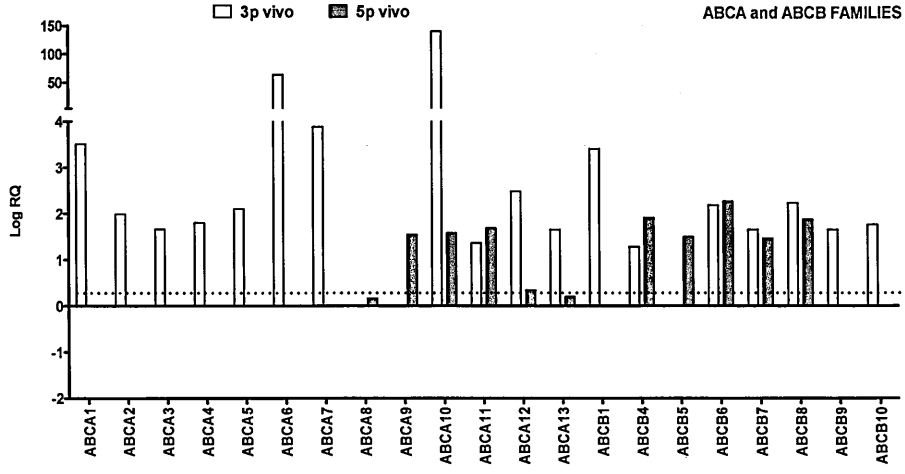
B)



ASCITES #2 sorted for CD133

C)





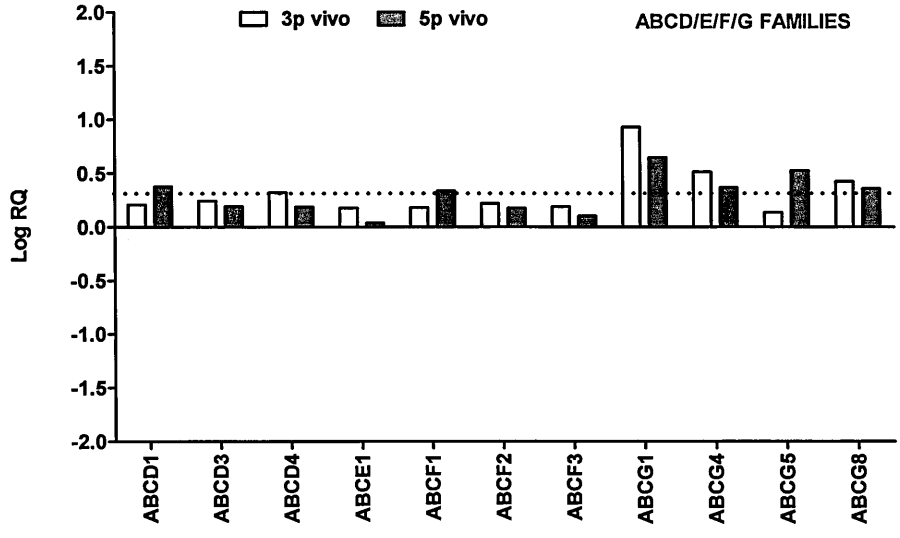


Figure 4.11: Ascites #2 and #3: correlation between selected subpopulations and expression of ABC transporter.

A) Real Time analysis of sorted CXCR4 positive and negative cells of ascites #2 and B) ascites #3 at different *in vivo* passages. C) Real Time analysis of sorted CD133 positive and negative cells of ascites #2 at different *in vivo* passages.

Negative cells were used as an internal calibrator. Fold change of positive subpopulation is indicated as Log RQ (relative quantification). Only genes with a fold change ≥ 2 were plotted in the graph and considered as statistically significant in terms of gene expression profile.

4.4 Ascites #2 and #3: *in vivo* modulation of CXCR4 and CD133 expression

Another hallmark of the CSC subpopulation is the ability to generate phenotypically heterogeneous cancer cell lineages both *in vitro* and *in vivo*. To verify if CXCR4 or CD133 positive cells had this potential, giving rise to tumour cells that either expressed or did not express these markers, xenografted ascites derived from injection of either positive or negative fractions were characterized by FACS analyses (FIG. 4.12).

Injection of CXCR4 sorted cells gave rise to ascites that contained cancer cells expressing moderate levels of this receptor, which were observed with a higher frequency than in the original sorted ascites. Irrespective of the nature of the injected cells, positive or negative for CXCR4 surface expression, xenograft-derived ascites showed a similar level of CXCR4 expression (range of positive cells: 4.8 -32% and 3.6-49.2% for positive and negative fractions respectively; results are for 2 experiments, 3 mice in each group). Furthermore, samples derived from injection of both CXCR4 positive and negative fractions were enriched in CD133-expressing cells, even if the percentage of positive cells was lower than that of the initial sample (range of positive cells: 0.1-5.9% and 0.7-13.1% for positive and negative fractions respectively; results are for 2 experiments, 3 mice in each group) (FIG. 4.12).

Injection of CD133 positive cells generated ascites that showed moderate expression levels of both CD133 and CXCR4 (range of positive cells: 25.2-

26.9% and 3.3-1.6% for CXCR4 and CD133 expression respectively; results are for 2 experiments, 3 mice in each group) (FIG. 4.12).

On the contrary, CD133 negative cell injection gave rise to ascitic cells showing moderate levels of CXCR4 positivity and a total absence of CD133 expression (range of positive cells: 0.7-12% and 0-0% for CXCR4 and CD133 expression respectively; results are for 2 experiments, 3 mice in each group) (FIG. 4.12). Evaluation of the double positive CXCR4/CD133 subpopulation revealed that this cell subset was also *in vivo* modulated, as the percentage of double positive cells was variable among the samples analyzed, ranging from 2.4% to 8.1%.

These results suggest that cancer cells derived from injections of either CXCR4 positive or negative fractions give rise to phenotypically heterogeneous ascitic cells with a highly plastic phenotype. Moreover these data highlight the possibilities that, while CD133 expression is not regulated by extrinsic factors but seems to be dependent on intrinsic cellular mechanisms, CXCR4 expression could be affected and modulated by both cellular and microenvironmental stimuli.

% positivity in growing tumours (RANGE)	SORTING MARKER			
	CXCR4 (2 exp)		CD133 (2 exp)	
	positive	negative	positive	negative
CXCR4	4.8 - 32	3.6 - 49.2	25.2 - 26.9	0.7 - 12
CD133	0.1 - 5.9	0.7 - 13.1	3.3 - 1.6	0 - 0
CXCR4/CD133	7.04	8.01	0.1 - 2.4	0 -0

Figure 4.12: modulation of selected marker expression.

After sorting for CXCR4 or CD133 the sorted subpopulations were *in vivo* re-injected and xenograft-derived ascites were phenotypically characterized by FACS analysis. The Table summarizes the result of FACS analyses after *in vivo* injection of sorted subpopulations. The percentage of CXCR4, CD133 and double positive cells are reported.

4.5 Discussion

4.5.1 Establishing ovarian cancer sphere cell lines

Is a great challenge to identify the most appropriate source of tumour cells for the isolation of ovarian CSCs. While it would be desirable to use freshly collected samples from primary tumours or ascites, both direct sorting of these samples and/or establishing primary *in vitro* cultures have proven to be difficult tasks (see Chapter 2 'Material and Method'). Our results showing that ovarian cancer cell lines were difficult to derive from primary ovarian surgical specimens are consistent with previously published results. In the first published report supporting the existence of ovarian TICs, ascitic fluid cells from patients with advanced stage ovarian carcinomas were used and the growth potential of these cells was tested using *in vitro* colony formation assays (308).

Starting from a large cohort of ovarian cancer samples (primary tumours and ascites), we tried to select and to expand *in vitro* ovarian cancer cells able to grow in suspension as spheroids and to obtain a tumour sphere cell line for further characterization. After mechanical or enzymatic disaggregation, the growth as spheroids was possible from only a few cases and then for only a few *in vitro* passages. These results suggest that the *in vitro* conditions are critical for the expansion of spheres from ovarian cancer, but as yet they have not been precisely defined. We hypothesize that the sphere medium we used, designed for mammospheres, could induce the differentiation of ovarian tumour cells, as we observed for the

A431 cancer cell line (see 'Chapter 3'). Moreover, the attempt to create a more specific microenvironment for ovarian cancer cells by the addition of particular stimulating molecules, such as LPA (361) (359) and bFGF (362) (363) did not enhance sphere formation and proliferation. It cannot be excluded that a more appropriate mixture of growth factors and other molecules essential for ovarian cancer cell proliferation could be the key to a more conducive environment for primary sphere cultures from ovarian surgical samples.

However, the recent findings that spheres are not composed entirely of stem cells, as supported by recent papers investigating heterogeneity in mammospheres and melanospheres (372) possibly lessens the belief that the sphere formation assay is the definitive methodology for identifying cancer stem cells.

4.5.2 Identification of ovarian CSCs by marker expression

Besides anchorage independent *in vitro* growth, the expression of particular cell surface markers is another feature that has been used to identify and isolate CSCs from a variety of malignancies (364) (290) (365) (366).

Markers useful for the identification and characterization of normal stem cells in one organ are frequently exclusive of that tissue and may be not shared with stem cells of another organ (367) (368). This is likely to be applicable to cancer stem cells and the knowledge of markers of normal epithelial cells of the ovary could be of great help. Unfortunately, the difficulty in raising cultures of OSE cells and their pluripotency (299) (369),

have limited the acquisition of information in this field. Thus, we still have to rely on markers already identified as associated with CSCs in tumours other than ovarian cancer.

The search for TICs in ovarian cancer has resulted in observations that epithelial ovarian CSCs may be isolated on the basis of the expression of CD44/CD117 or CD133 markers, all associated with CSCs in other malignancies (310) (311) (312). Another potential CSC marker in EOC is the chemokine receptor CXCR4, expressed by CSCs in various solid tumours, described as associated with a subset of ovarian tumour cells, (228) (229) (230) (207).

Immunohistochemical analyses of cryostat-cut sections from primary ovarian cancer specimens from patients at an advanced stage revealed that CD44 and CD133 positivity on tumour cells was heterogeneous, frequently limited to small groups of cells, often present at the edge of the tumour invasive front. Analysis of CXCR4 expression has not been informative in cryostat-cut sections, probably due to the epitope characteristics of the antibody selected for this analysis. Few formalin-fixed paraffin embedded histological sections were characterized for CXCR4 expression and again the reactivity was highly heterogeneous and frequently confined to small groups of tumour cells.

We also evaluated the expression of CD44, CD133 and CXCR4 molecules in cell preparations from ovarian solid tumours and ascites. To identify the origin of the isolated cells, we used the EpCAM marker that characterizes all tumours of epithelial type until they progress and metastasize acquiring

a mesenchymal phenotype, and whose reactivity was homogeneous on the examined cryostat-cut sections or histological sections from ovarian cancer. EpCAM was present at a high level with a high percentage of cells obtained from solid samples expressing the marker indicating that they are carcinoma cells. In the case of cell preparations from ascites, while the expression level of EpCAM on positive cells was high, the percentage of positive cells was variable, indicating that cells recovered from ascites are more heterogeneous and tumour cell preparations could be contaminated by other cell types. This was confirmed by the presence of monocytes and macrophages in the ascites, identified by CD45 expression analysis; however, subpopulations negative for both EpCAM and CD45 are present in some ascitic samples. We suggest that they are normal cells of mesothelial origin, often demonstrated to be present in ascites (370). However, we cannot exclude that some of the EpCAM negative/CD45 negative cells are tumour cells.

CD44, CD133 and CXCR4 markers had a variable degree of expression on cells from both solid tumours and ascites, and all were present on a subset of ovarian cancer cells ranging from 3 to 27%. These results demonstrated that in a wide range of EOC primary tumours and ascites, cells do express CD133, CD44 and CXCR4 but they are generally restricted to a subpopulation of ovarian cancer cells, potentially suggesting their association to ovarian CSCs. However, the limited number of cells recovered in the majority of cases precluded the sorting of cells expressing these markers and their further characterization for stemness markers.

4.5.3 Isolation of tumorigenic subpopulations by xenotransplantation

Operationally, CSCs isolated from various type of cancer, would be more tumorigenic than the bulk population when xenografted into immunocompromised mice such as athymic nude mice, lacking B lymphocytes, or in severe combined immunodeficient mice (SCID), lacking both T and B lymphocytes. In our *in vivo* assays, we used both mouse models and we found that ovarian cancer initiation was weakly supported after intraperitoneally injection in Nu/Nu mice (CD1 outbred and BalbC inbred). Tumour and ascitic growth was much more reproducible in the SCID mice. Injection of ovarian cancer cells isolated from ascites was able to propagate ascites much more efficiently if injected intraperitoneally instead of subcutaneously. The difficulties observed in solid tumour growth after subcutaneous implantation may be due to the differences between the native environment of ovarian cancer cells in patients and the environment into which these cells are transplanted in mice, which can reduce the engraftment of cells with tumorigenic potential. On the other hand, intraperitoneal injection, mimicking the native environment of ovarian cancer development, has to be considered the best *in vivo* assay for the evaluation of ovarian CSC tumorigenicity.

In spite of this, we were only able to establish transplantable tumour cell lines by intraperitoneal injection of ascitic cells from three cases of ovarian carcinoma ascites. Despite diversity in terms of histopathology, response to

treatment and clinical features, the examined samples shared same common properties related to a stemness profile.

4.5.4 ALDH activity as a stemness marker in EOC

In conjunction with other markers, ALDH activity has emerged as a potential screen to characterize highly clonogenic and undifferentiated multipotential stem/progenitor cells both in normal and neoplastic tissue (138) (325).

In the field of ovarian CSCs, three different examples have been reported. Irrespective of any association to CSCs, ALDH positivity correlated negatively with progression-free survival of EOC patients (326) (325) (142). We have evaluated the ALDH activity in one of the selected samples (ascites #2) and in its xenograft-derived ascites. A low percentage of ALDH positive cells (1.9%) characterized the original ascites, but ALDH activity significantly increased after three and nine *in vivo* passages (73.3% and 78.4% respectively). It could be speculated that, in the case reported here, *in vivo* transplantation might have selected those cancer cells endowed with high detoxifying ability due to the expression of the ALDH enzyme. Due to its high expression (>70% of tumour cells) here, as also observed for the A431 cancer cell line, it is unlikely that ALDH activity could be considered as a good indicator of a putative ovarian CSC. However, it is possible that ALDH positivity could be an independent prognostic factor not linked to a stemness phenotype in this type of cancer. To support this hypothesis, we evaluated the possible presence of double positive $CXCR4^{bright}$ /Aldefluor+ cells (data not shown). Preliminary

observations indicate that CXCR4 and ALDH mark two different subpopulations of ovarian cancer cells, further supporting the hypothesis that ALDH function is not an intrinsic property of an ovarian CSC.

4.5.5 Phenotypic characterization of xenotransplants from selected ascites for CD44, CD133 and CXCR4

The analyses of serial *in vivo* transplants, particularly detailed in the case of ascites #2, revealed heterogeneity in term of marker expression. Indeed, we detected a stable *in vivo* selection of cancer cells that expressed high levels of CD44, while heterogeneous levels of CD133 and CXCR4 were observed within ascites grown in animals injected with the same tumour population and within different *in vivo* passages.

Since the literature has identified CD44/CD117-expressing cells as a CSC subpopulation in EOC (311), we evaluated CD117 expression, observing that at least 1% of ovarian cancer cells were positive for this marker. However, we have not yet characterized the subpopulation of CD177 positive cells; future plans will be to evaluate the potential role of this molecule as an ovarian CSC marker in our *in vivo* model.

The *in vivo* increased expression of CD44, independently of tumour growth as a solid mass or as ascites, suggests that this molecule, whose expression is under epigenetic control (371) (372) (373), is exceedingly sensitive to some, as yet unidentified, factor(s) present in the mouse microenvironment, thus precluding its further analysis as a CSC/TIC marker in *in vivo* models.

Since CD133 expression was found to be associated with the ovarian CSC phenotype (374) (311) (312), we evaluated the possibility that CD133 and CXCR4 could recognize the same subpopulation of ovarian cancer cells with CSC properties. The existence of a double $CXCR4^{bright}/CD133^{bright}$ positive subpopulation was not easily detectable due to the variable degree of expression of both antigens. In double staining experiments and FACS analyses, a small percentage of ovarian cancer cells seemed to co-express CD133 and CXCR4, but we did not identify a clear overlap between the two tested markers. Moreover, morphological parameter analysis revealed that these two molecules are expressed by diverse subpopulations of ascitic cells with distinct side- and forward-scatter properties (SSC and FSC respectively), which did not overlap with each other. CXCR4 positive cells corresponded to the subpopulation with lower SSC and FSC parameters, which is a typical characteristic of normal and cancer stem cells. Finally, epifluorescence analyses confirmed that only a small number of ovarian cancer cells expressed either CXCR4 or CD133.

These data argue for the possibility that CD133 and CXCR4 recognize distinct subsets of ovarian CSCs with different morphological and functional properties. Although CSCs seem to be a special compartment of cancer cells, a recent theory suggests that CSC themselves are still a heterogeneous population with different biological properties and that multiple populations with CSC characteristics can co-exist in the same tumour (47) (375). From this point of view, CD133 and CXCR4 could specify

two different and co-existing subsets of ovarian cancer cells with particular features, possibly related to diverse CSC characteristics (49).

4.5.6 Characterization of CD133 or CXCR4 sorted ascitic cells

Ascitic cells recovered from EOC xenografts were sorted for CD133 or CXCR4, and positive and negative cell fractions were characterized.

Enrichment in mRNA levels of stemness genes, such as *NANOG*, *NESTIN* and *OCT4*, was observed with a good reproducibility in CXCR4 positive cells, whereas it was more variable in CD133 positive cells. Furthermore, the expression levels of ABC transporters which can efflux drugs as well as physiological substrates (e.g. lipids), suggested that these genes can be reversibly turned on and off in CXCR4 positive cells, while in CD133 positive cells their up-modulation was a much more stable and evident feature, suggesting a difference in terms of chemoresistance potential between CXCR4 and CD133 subpopulations.

ABCF2 has been found a useful prognostic marker in cervical and ovarian clear cell carcinoma, but its expression was unrelated to prognosis and clinical factors in endometrial cancer (376) (377). Our data revealed that *ABCF2* expression levels were up-regulated in both CXCR4 and CD133 positive cells, being particularly marked in the CXCR4 positive subset. We may speculate that ABCF2 could represent a prognostic factor not only in ovarian clear cell carcinoma but also in ovarian serous adenocarcinoma, but more detailed analyses in a large and well annotated series of cases are needed before drawing any conclusion on this subject.

ABCG1, *ABCG2* and *ABCB1* (*MDR1*) have been reported to be up-regulated in TICs of numerous tumour types (127) (360) (128). ABCB1 was also demonstrated to be a good prognostic parameter in women with ovarian cancer (378). Our analyses showed that ABCB1 and ABCG1 were strongly up-modulated in CD133 positive cells, supporting our previous suggestion regarding CD133 chemoresistance potential.

In summary, we did not found any correlation between CD133 positivity and the ovarian cancer stemness phenotype. However, there was an association between the expression of resistance-related genes such as the ABC family transporters and CD133. Because CD133 expression is linked to a resistant phenotype, detection of CD133 positive cells may be useful to predict the efficacy of specific cytotoxic therapy.

On the other hand, CXCR4 positive cells were associated with a more undifferentiated expression profile, but did not appear to be correlated with resistance-related factors (i.e. ABC transporters). Thus, CXCR4 positive cells may identify those tumour cells which maintain a partially un-differentiated state (high levels of stem cell marker expression) and possibly responsible for tumour invasion and metastasis.

4.5.7 Tumorigenic potential of CD133- or CXCR4- sorted ascitic cells

The importance of tumorigenicity in defining CSCs has been debated because the CSCs represent "*per se*" a heterogeneous subpopulation of cancer cells. In all published studies in the field of ovarian CSCs, the tumours resulting from the putative CSC population contained both tumour

and non-tumour initiating cell populations, demonstrating the multipotential ability of the selected subpopulation.

We found that cells isolated from xenograft-derived ascites and sorted for CXCR4 and CD133 positive cells were not enriched in cells with higher tumorigenic capacity. These results fitted well with previous studies, where CD133 positive and negative fractions did not differ in their ability to form tumours in mice (260) (268). As noted for CD133, both CXCR4 positive and negative cells were able to generate ascites in immunocompromised mice. The tumorigenic ability of both CXCR4 and CD133 positive and negative cells seems to indicate that both markers, rather than distinguishing cells at different levels of a hierarchy, are reversibly expressed by tumorigenic ovarian cancer cells.

Cancer cells derived from injections of either CXCR4 positive or negative fractions gave rise to heterogeneous ascitic cells with a plastic phenotype; CXCR4 negative cells were able to generate ascites positive for this marker. The observed modulation of CXCR4 expression suggests that this marker could effectively identify the plastic and heterogeneous subpopulation of CSCs, but at the same time highlights the difficulty to use this molecule as a potential ovarian CSC therapeutic target. On the contrary, CD133 negative cells generated ascitic cells that maintained the CD133 negative phenotype.

Overall, these data highlight the potential involvement of different mechanisms regulating the expression of these two markers, being that of CD133 inherently dependent on cellular mechanisms, and that of CXCR4

under the control of both cellular and microenvironmental stimuli, such as CXCL12/SDF1, the natural ligand of CXCR4 (241). Further molecular analyses are needed to verify the existence of the hypothesized different expression regulators of CD133 and CXCR4 in EOC CSCs.

4.5.8 EOC CSCs existence: tentative conclusions and working hypothesis for future analyses

From our *in vivo* data, we would estimate that at least $5x10^5$ ovarian cancer cells are needed to reliably propagate ascites and tumours when injected in a specific mouse model and in a specific mouse-body location. Although we were not able to identify markers that distinguish tumorigenic from non-tumorigenic ovarian cancer cells, our data do not exclude the possibility that ovarian cancer follows the cancer stem cell model. Altogether, our results are in keeping with the recent notion that the heterogeneous phenotypic and molecular traits of human cancer could be a function of their CSC content (379) and of the CSC plasticity; in fact, marker modulation could be explained in terms of the ability of CSCs to change their features depending on the surrounding environment (6)

In conclusion, if we consider our results and recent developments of the CSC model (368) (50), we believe that epithelial ovarian cancer may arise from mutated stem or progenitor cells and that the observed heterogeneity of the CSC population is caused by clonal evolution or partial differentiation of the ovarian TICs. During cancer progression, it is possible that a range of variable factors, such as oxygen concentration, immune status and therapeutic treatments, causes the emergence of new CSC clones through

genetic and epigenetic alterations. These CSC clones may have acquired survival advantages during tumour progression and may co-exist, compete or cooperate with each other. (368) (51). Translating this assumption to our data, CD133 and CXCR4 could identify two different clones of the ovarian CSC subpopulation with distinct properties, related to TIC features (FIG. 4.13).

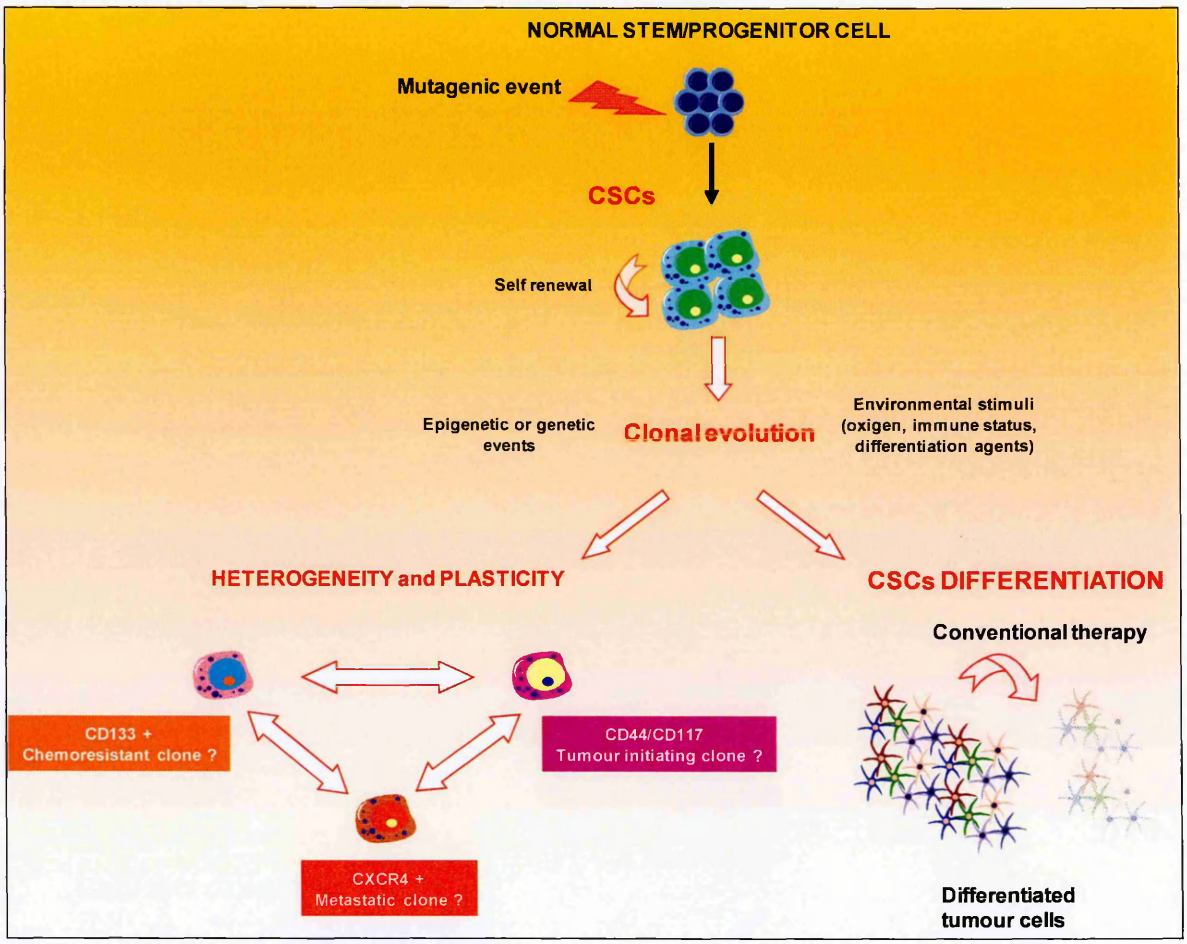


Figure 4.13: Proposed model for ovarian CSC clonal evolution and heterogeneity

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