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# The Dark Side of Cellular Plasticity: Stem Cells in Development and Cancer

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## 1. Introduction

All multicellular organisms require a continuous homeostatic control of cellular proliferation and differentiation in order to maintain the numbers of the different types of cells and the global size of the organism, while at the same time compensating for all the permanent loss of cells due to aging and to the attacks from the environment. In general, this control is achieved by the use of a hierarchical system where a small number of multipotential, slow-dividing, stem cells give rise to more differentiated, actively proliferating intermediate progenitors, which in turn will originate large numbers of cells committed to a specific cellular fate. These cells will then terminally differentiate and integrate functionally into the mature organs or tissues. Along this process there is a gradient of developmental potential, in such a way that, as they mature into a certain fate, the cells lose the capacity of giving rise to other cell types.

This process is tightly controlled by extrinsic environmental signals (either permissive or inductive) and, more importantly, by the intrinsic transcriptional and epigenetic profiles of the developing cells. These profiles change and evolve along with development and are the responsables for establishing both the cellular identity and the susceptibility of the cell to

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alterations that might alter the outcome of cellular differentiation. The nature of these alterations can be environmental (new signals) or, more frequently, internal to the cells (genetic or epigenetic alterations). In this context, it is therefore easy to understand that any deregulation of the transcriptional or epigenetic equilibrium will lead to an unwanted final outcome, like it is the case in tumors, where the cellular identity is reprogrammed by oncogenic alterations to give rise to a new pathological lineage. This aberrant deviation of the normal developmental program is only possible if the initial cell suffering the oncogenic insults possesses enough plasticity so as to be reprogrammed by them. In this chapter we describe the nature of physiological plasticity, its biological necessity for normal development and its underlying molecular and cellular mechanisms, to put them afterwards into the context of tumor development. In order to do this, and before discussing the concepts in depth, we need to first define the terminology used and to be aware of its historical origin within the discipline of developmental biology.

Physiological plasticity is here defined as the capacity of cells (stem or differentiated) to adopt the biological properties (gene expression profile, phenotype, etc.) of other differentiated types of cells (that may belong to the same or different lineages). Competence (potency) would therefore be a specific manifestation of plasticity, defined as the ability of undifferentiated cells (stem cells and progenitors) to give rise to their different descendant lineages during normal development (i.e. not pathologically- or experimentally-induced). We group both concepts under the same umbrella (plasticity) since it is increasingly clear that the same mechanisms involved in stem cell competence during normal development are involved in the plasticity of more differentiated types of cells, not only in pathological conditions like tumorigenesis, but also in experimentally-induced fate-changing processes. In the last years, many advances have been made in our understanding of the biology of cellular plasticity (Graf and Enver, 2009; Gurdon and Melton, 2008; Hochedlinger and Jaenisch, 2006; Vicente-Duenas et al., 2009a). However, the molecular bases of stem cell competence (i.e. plasticity) maintenance or entry into the differentiation programs are not yet completely understood (Niakan et al., 2010).

Competence (potency) as we have defined it above is only one of the main properties that define stem cells. The other is their self-renewal capacity, determined by their ability to undergo the asymmetric cell divisions that allow them to maintain themselves in an unchanged state and, at the same time, to generate daughter cells that enter into the differentiation/proliferation cascade (Ward and Dirks, 2007). In this way, when the stem cell divides asymmetrically, it gives rise to a new identical stem cell and a multipotential progenitor/precursor that will originate all the variety of differentiated cells. When the division is symmetrical, two identical daughter cells are created that either retain the same stem properties of the mother cell or start the differentiation program, losing the self-renewal capability and their stem cell properties.

What we have outlined are the main features of normal development of stem-cell based tissues in physiological conditions. However, these processes can be deregulated by many different mechanisms, both experimentally in the laboratory and in numerous pathologies, like cancer or developmental abnormalities. In all these cases, cellular reprogramming is the cause, but the consequences can be very different depending on the triggering mechanisms and the plasticity of the initiating cell. As we will discuss in the next section, our understanding of the biology of development has increased enormously in the last half century, and many different processes have been described in diverse organisms in different laboratories. This has also created a great deal of confusion in the scientific nomenclature,

and many of the terms commonly used have different meanings for different authors in different fields (for example, researchers working in different model organisms), in some cases more restrictive, in some more wide-ranging. In this chapter, besides the terms that we have already defined, we will use the following terminology (Figure 1):

- “Dedifferentiation”: the mechanism by which the normal developmental program is reverted in such a way that differentiated cells give rise to more plastic, earlier progenitors.
- “Transdifferentiation” designs the direct conversion (reprogramming) of a differentiated cell type into another different mature cell, without the need of dedifferentiating to earlier developmental stages; it usually involves the passage through cellular intermediates that are non-physiological and share markers that are normally mutually exclusive, corresponding to the initiating and the final cell. As we will discuss later, induced pluripotency would be a particular case of transdifferentiation, rather than being a dedifferentiation process, due to the existence of those non-physiological intermediates.
- “Commitment”: the point of no return in physiological development, where the cell irreversibly enters a specific differentiation program. For a stem cell, it implies the loss of self-renewal.
- “Epigenetic”: the inheritance of patterns of gene expression, without affecting the genetic code itself. In other words, the inheritance that is not codified in the DNA sequence. From the molecular point of view, it designs all the chromatin modifications that establish (and determine the propagation of) the different possible patterns of gene expression of a given, unique genome.
- “Reprogramming”: from the cellular point of view, the natural or experimentally-induced alteration of the differentiation program of a given cell. From the molecular point of view, all the molecular changes (i.e., epigenetic) that take place in a cell that is changing its identity. Dedifferentiation and transdifferentiation are types of reprogramming, usually experimentally-induced. Oncogenesis is also a form of reprogramming, in this case one that spontaneously happens in nature.
- “Cancer Stem Cells” (CSCs): the cells responsible for the maintenance, propagation, metastasis and relapse of tumors. They possess self-renewal and differentiation capabilities and can give rise to all the cellular types that compose the tumor mass. Also named cancer-maintaining-cells.
- “Cancer cell-of-origin”: the normal cell that first suffers the oncogenic hit and initiates the tumoral process. It is usually the one giving rise to the CSC. It can be either a differentiated cell or a stem/progenitor cell.

## 2. Historical perspective

Since the beginning of human history, men have looked for the ideal of eternal youth, and the myths about regeneration of diseased organs (or even resurrection) are among the oldest of mankind (Odelberg, 2004). The Egyptian god Osiris had his body resurrected and recomposed after having been torn into pieces and thrown in the Nile. The Hydra from the Greek mythology could regenerate its multiple heads when they were severed, and only by burning the stumps could Hercules defeat the creature. Also, as a punishment for revealing the secret of fire to the humans, Prometheus was chained to the mountain where an eagle ate his entrails, which would regenerate every new day. All these imaginary creatures have a reflection in the

natural world, and this was also observed in very ancient times, and already Aristotle (384-322 BC) reported that lizards regenerated their tails. But only in the Age of Enlightenment will this aspect of the natural world become the matter of scientific study. In 1712, Réaumur reports the regeneration of the limbs and claws of crayfish (Reaumur, 1712); in 1744, Trembley discovers that the two halves of the Hydra polyp can regenerate a whole new organism (hence its name) (Trembley, 1744); in 1769, Spallanzani describes how tadpoles can regenerate their tails and salamanders can regrow amputated limbs, tails and jaws (Spallanzani, 1769). During most of the 19<sup>th</sup> and first half of the 20<sup>th</sup> centuries, research was mainly focused in the description of these processes from the morphological point of view (Birnbaum and Sanchez Alvarado, 2008; Odelberg, 2004). Nevertheless, the detailed analyses performed already showed that, in order for the regenerative process to take place, the cells that are normally forming part of the organs are not enough, and a special type of cells are required: the progenitor cells. The origin of the latter was at that time unclear; in some cases, like for the regeneration of skin, blood, muscles or bones, progenitors are shown to exist in the tissues in small numbers, and to become activated as a consequence of the lesions. In other cases, the progenitors seem to arise from differentiated cells that change their developmental program and become dedifferentiated. The best example of this mechanism is observed in urodeles, a group of very primitive vertebrates (salamanders, newts, axolotls). In them, once the lesion has occurred, cells from the normal tissues form a pool of proliferative progenitors known as the regenerative blastema (Bodemer and Everett, 1959; Chalkley, 1954; Hay and Fischman, 1961). These cells will in turn give rise to all the tissues in the new limb/tail. This extraordinary example of cellular plasticity has been almost completely lost in more evolved vertebrates. Amphibians also provided the first animal model of experimentally-induced reprogramming when, in 1952, Briggs and King managed to generate frog tadpoles by transplanting the nucleus of cells from the blastula into *Xenopus* oocytes, reverting cellular differentiation (Briggs and King, 1952). Afterwards, Gurdon showed that also differentiated cells could be reprogrammed by using nuclei from intestinal epithelia cells as donors (Gurdon, 1962). These milestone findings clearly indicated that the genetic potential of cells did not diminish during differentiation, and that there were no genetic changes occurring during development. The final proof that this principle extends also to mammals was the cloning of Dolly the sheep by Wilmut and colleagues in 1997 (Wilmut et al., 1997). This was the definitive proof that the changes that happen during differentiation are fully reversible, demonstrating that the fate restrictions that occur during normal development are the result of epigenetic modifications. These studies also showed that there were factors in the oocyte cytoplasm capable of reverting the epigenetic program and inducing a reprogramming that led to the appearance of a totipotent phenotype.

The search for the reprogramming factors followed a parallel route. In 1987 it was shown that ectopic expression of the *Antennapedia* homeotic gene lead to changes in the body plan of *Drosophila*, that got extra legs instead of antennae (Schneuwly et al., 1987). Later it was found that the ectopic expression of *eyeless* controlled the full gene cascade responsible of eye development and could lead to the formation of ectopic eyes in *Drosophila* legs (Gehring, 1996). In mammals, the first master regulatory transcription factor identified was MyoD, which could transdifferentiate fibroblasts into the myogenic lineage (Davis et al., 1987). Other examples of these reprogramming events dependent on single factors are the transdifferentiation of mouse B cells into macrophages by C/EBP $\alpha$  (Xie et al., 2004) or the dedifferentiation of committed B cells by the loss of Pax5 (Cobaleda and Busslinger, 2008; Cobaleda et al., 2007a; Nutt et al., 1999). All these data proved that the alteration of the transcriptional profile by just one factor could cause stable fate changes, and provided the



rationale for the search of the factors capable of reprogramming to full pluripotency that led, in 2006, to the identification by Takahashi and Yamanaka of the four transcription factors capable of inducing pluripotency in terminally differentiated cells (Takahashi and Yamanaka, 2006), as we will describe with more detail in the following sections.

On the other side, cancer has also been known since the origins of mankind. The first references are the Edwin Smith and Ebers papyri from the 3000 BC and 1500 BC, respectively (Hajdu, 2004). The Edwin Smith papyrus contains the first description of breast cancer, with the conclusion that there is no treatment for the disease. Cancer was not so prevalent in ancient times, since life span was much shorter, but it was already clearly identified. Hippocrates (460–375 BC) noted that growing tumors occurred mostly in adults and they reminded him of a moving crab, which led to the terms *carcinosis* and *cancer*. Celsus (25 BC–AD 50) also compared cancer with a crab, because it adheres to surrounding structures like if it had claws; he introduced the first classification for breast carcinoma and recommended surgical therapy. However, he already noted that tumors could only be cured if removed at early stages because, even after excision and correct healing of the scar, breast carcinomas could recur with swelling in the armpit and cause death by spreading into the body. Galen (131–AD 200) already advised surgery by cutting into healthy tissue around the border of the tumor (Hajdu, 2004). If we make a 2000-year leap to our days, it seems disappointingly surprising how little those old critical findings have been overcome by modern medicine. Indeed, for solid tumors, still today clean surgical margins and lack of lymph node invasion are the most important prognostic markers, and only if tumors are resected completely before spreading (something that it is anyway impossible to ascertain with current technologies) can curation be guaranteed. Much more is what we have learnt in the last thirty years about the molecular biology of the disease. In 1979 it was shown that the phenotype of transformed cells could be transferred to normal fibroblasts by DNA transfection (Shih et al., 1979). In 1982 the molecular cloning of the first human oncogene was reported simultaneously by several groups (Goldfarb et al., 1982; Lane et al., 1982; Parada et al., 1982; Santos et al., 1982), to be soon identified as the RAS gene. Since then, many genes have been described as oncogenes or tumor suppressors, and the molecular basis of their transforming activities have been described to great detail. A comprehensive study of this topic falls out of the scope of this chapter, but there are some aspects that must be taken into account for further posterior discussion. One of them is that, for many types of tumors, specific mutations have been described to be tightly associated to the tumor phenotype, especially in the case of mesenchymal tumors caused by chromosomal aberrations (Cobaleda et al., 1998; Sanchez-Garcia, 1997). This association already suggested that the oncogenic aberrations might be acting as new specification factors that determine the tumor appearance and/or phenotype. In 2000, Hanahan and Weinberg summarized the main features that needed to be disrupted in normal cellular behavior in order for allow a tumor to appear and progress (Hanahan and Weinberg, 2000). These main aspects are related with the survival and proliferation of cancer cells. However, much less attention has been paid to the aspects related to the differentiation. In fact, if cellular fate was carved into stone, cancer would be impossible, since no new lineages could be generated other than the normal, physiologic ones. Here is where the normal mechanisms regulating cellular identity and plasticity play an essential role in allowing cancers to arise and hopefully, as we will discuss, they might be the key to its eradication.

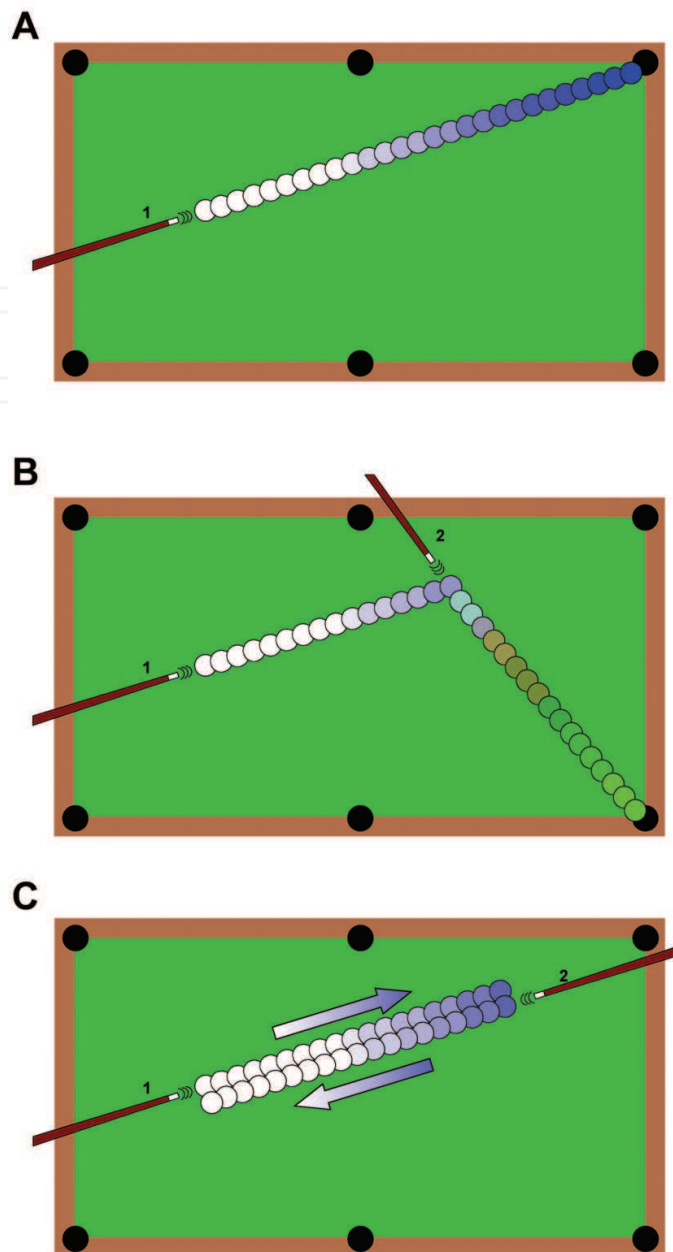
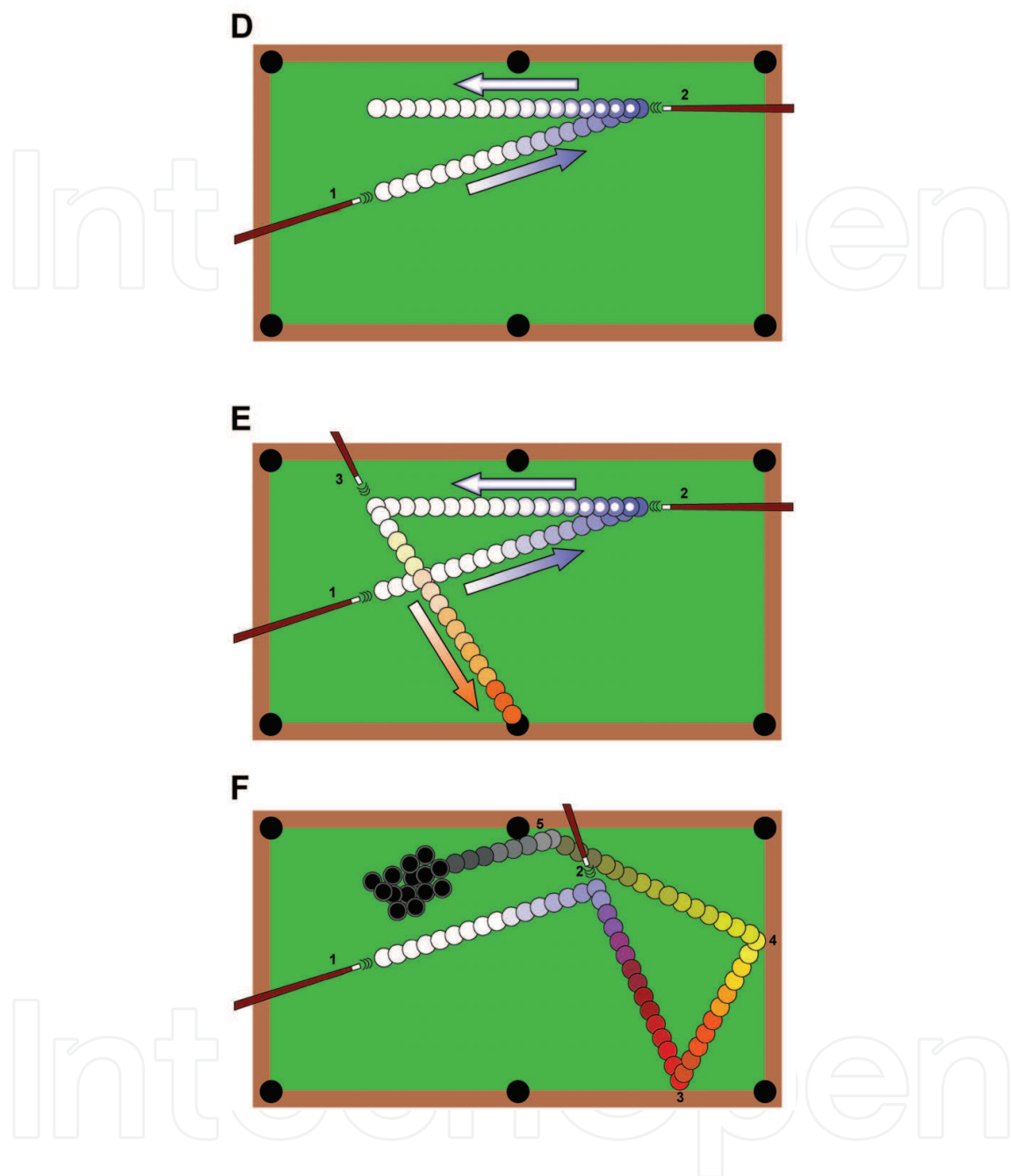


Fig. 1. The road from developmental plasticity to cancer. Development is here conceptualized as a pool ball rolling towards different directions depending on the strokes it has received. For simplicity, the pool table is flat and horizontal, but in reality the shape of the “developmental terrain” also is an essential contribution to fate determination (see text). A) In normal development, fate is established once the initial impulse has been provided by internal transcription factors or external signals, and then the cell develops “lineally” towards this fate. B) Transdifferentiation. The introduction of a new driving force (cue n° 2, for example a transcription factor) redirects the cell towards a new fate, pushing it out of its normal route. C) Dedifferentiation. An inversion of the normal process of development, following the same differentiation intermediates that were followed in the first instance, but in a reversed order. Here, an opposite driving force is depicted (cue n° 2) but this reversion could also be due to a lack of initial impulse (i.e., lack of an essential driving transcription factor).



D) Induction of pluripotency. Again, an external force (Yamanaka factors, for example) counteracts programmed development and sends the cell back to a progenitor condition, but in this case going through non-physiological cellular intermediates. E) Reprogramming. After pluripotency has been induced as depicted in the previous panels, the cells can be redirected towards new fates with the help of external or internal stimuli (cue n° 3). F) Tumorigenesis. An oncogenic hit (cue n° 2), hitting the right cellular intermediate with the right strength and angle sends the cell down to a new developmental program that will lead to the development of a tumour. According to this view, many of the second hits in tumorigenesis (n° 3, 4, 5) are already implicit given the first hit and the nature of the cell.



### 3. Molecular bases of plasticity

As we have mentioned before, differentiation has been traditionally considered as an irreversible process. It was more than 50 years ago when Conrad Waddington conceptualized the irreversibility of cellular differentiation as marbles falling down a slope (Waddington, 1957). This conceptual and very graphical image has been afterwards widely used to visually depict the meaning of transdifferentiation, dedifferentiation or pluripotent reprogramming (Hochedlinger and Plath, 2009), all of them “uphill” processes that must overcome natural barriers to take place. Interestingly enough, this conceptual view has been given a new meaning by the studies of the gene regulatory networks (GRNs) that control differentiation; from the mathematical analysis of the interactions among all the genes that are expressed in a cell in a certain moment, a geometric description of the developmental potential is obtained. In this way, a “landscape” of developmental probabilities is generated (Enver et al., 2009; Huang, 2009; Huang et al., 2009) in which “valleys” represent the different cellular fates, connected through “slopes” or “channels”, that are the differentiation routes. It is important to realize that, in this conceptualization, the landscape is in fact defined by the gene expression pattern of the cell itself, not something external to it. In this landscape, pluripotency would be a “basin of attraction” situated at the top of a peak. Pluripotency therefore behaves like a mathematical attractor, a metastable state maintained by small variations in the levels of expression of transcriptional and epigenetic regulators. The cells would slide towards the most stable configuration through the slopes, and those primed to differentiate would be located at the edge of the “attractor basin”. Therefore, the stemness of a cellular population is a metastable equilibrium defined by the gene interactions at the level of each individual cell and, consequently, each cell has a different intrinsic developmental potential. So, the stem cell condition is not static, but rather is a continuum that moves within certain boundaries. For example, in the case of the established stem cell marker *Sca-1* it has been shown that, in a clonal population of progenitor cells, there is a Gaussian distribution of its levels of expression (Chang et al., 2008). But these cells are not confined to a specific level of expression, as cells at both ends of the levels of expression can, with time, recapitulate the whole population with the complete range of expression levels. Furthermore, these sub-compartments present different transcriptomes that confer them distinct intrinsic developmental tendencies towards diverse lineages. These results indicate that each individual cell is an intermediate in a continuum of fluctuating transcriptomes. This range of variation is at the basis of the stochastic choice of lineage (Chang et al., 2008). The study of a different marker, *Stella*, in this case in embryonic stem (ES) cells, has provided similar findings (Hayashi et al., 2008). *Stella* is a marker of stem cell identity that shows a mixed expression in ES cells, demonstrating that they are not uniform, but rather represent a metastable state between intracellular mass- and epiblast-like states while retaining pluripotency. This equilibrium can be shifted in response to several factors, like for example epigenetic regulators (Hayashi et al., 2008).

The heterogeneous expression of phenotypic markers can be extended to the much more significant level of the transcription factors. Phenotypic heterogeneity is a known characteristic of progenitors at the population level, and it has been long known that they present a promiscuous activation of lineage-associated genes (Hu et al., 1997). Also the genes that are associated with the maintenance and specification of the pluripotent state vary in the population. In this context, recent results (Kalmar et al., 2009) show that *Nanog* levels experience random fluctuations within the ES cell population, giving rise to two

different compartments: one stable, with high levels of *Nanog* expression, and another much more unstable, with low levels of *Nanog*, and much more prone to differentiate and lose pluripotentiality (Kalmar et al., 2009). With the examples that we have provided, we can see that pluripotency is a state of dynamic heterogeneity of a population, and it is at the same type maintained and driven towards differentiation by fluctuations in the levels of expression of transcriptional and epigenetic regulators. The cells that are in the centre of the attractor “basin” are less prone to differentiate than the ones approaching the “edge” of the “basin”. The latter are already primed to differentiate, so that commitment is a spontaneous but rare phenomenon, unless it is elicited by external signals that disrupt the metastable equilibrium (Enver et al., 2009; Huang, 2009). This dynamic view explains the duality between the simultaneous plasticity and heterogeneity of multipotent populations, and also how the balance between instructed and stochastic cell fate decisions takes place.

#### 4. Loss of plasticity during normal development

As we have already mentioned, through the normal developmental processes that allow stem and primitive progenitor cells to become differentiated, and as a result of physiological plasticity, the identity of the cells change and new fates are adopted. These events occur in a progressive manner, in such a way that several distinct cell intermediates are generated with more restricted potential until the final mature, specialized cell types are generated and functionally integrated into the tissues and organs. Each lineage is characterized by a defined gene expression profile, resulting of the action of transcription factors and epigenetic modifications in a certain cellular environment. We have described how the stem cell state is that of a metastable equilibrium that can be disrupted towards differentiation either by random intracellular noise variation or by the induction by extracellular signals. Once the stem cells start the differentiation process, they begin to make reciprocally excluding lineage choices controlled by cross-antagonism between competing transcription factors, in such a way that different transcription factors, controlling different subsets of genes associated with specific lineages, are also controlling their activities in a reciprocal manner, maintaining an equilibrium that can easily be skewed towards one or the other side by external signals (Loose et al., 2007; Swiers et al., 2006). With the advent of flow cytometry and its capacity to separate cells according to defined combinations of surface markers, the study of the development of the hematopoietic system has provided enormous insight into the molecular and cellular mechanisms of lineage commitment. Indeed, their peculiar characteristics have allowed the isolation and purification of many distinct differentiation intermediates, making developmental haematopoiesis the ideal field of research to explore the mechanisms of lineage commitment and plasticity. From there, the developmental models identified have been extrapolated to other experimental systems, usually with great success. The above-described cross-antagonism model can therefore also be found in the development of the haematopoietic system. For example, the interaction between the transcription factors GATA-1 and PU.1 in myeloid progenitors, where they reciprocally inhibit each other and therefore create a binary decision situation for the progenitor that must choose between erythroid/megakaryocyte or myeloid-monocytic fates (Enver et al., 2009; Laiosa et al., 2006). This equilibrium creates a third intermediate condition defined by the balance between the expressions of both factors, which would correspond to a bipotent progenitor condition. This model has also been found to apply in other systems, like the early fate choice of pancreatic progenitors between endocrine and acinar cell lineages, in this

case under the control of cross-repressive interactions between the transcription factors Nkx6 and Ptf1a (Schaffer et al., 2010). So, in non-committed progenitors there are basal levels of parallel expression of opposed transcription factors; this explains the occurrence of multilineage gene priming, initially described in haematopoietic stem and progenitor cells (Enver et al., 2009; Hu et al., 1997). However, either in *in vitro* or *in vivo* settings many different developmental intermediates have been described by different groups, and there is still a lot of controversy about the exact steps that are really followed in normal development, because all experimental systems are imperfect and, like it happens to particles in Heisenberg's uncertainty principle, the mere isolation of the cells already affects their developmental potential, and the conditions under which this potential is studied are also to a certain degree dictating the possible outcomes. Nevertheless, it is generally accepted that there is a hierarchical loss of developmental potential in a gradual progression through many serial differentiation options in such a way that, at any point, a progenitor would only have to choose between two mutually exclusive options (Brown et al., 2007; Ceredig et al., 2009). Afterwards, and to mature towards terminally differentiated cells, the progenitors will have to interact with the suitable extrinsic signals (like the cytokines, for example) that would for that reason carry out a more permissive than instructive function. Although this process is mainly governed by transcription factors, epigenetic modifications occur in a progressive manner that modify the chromatin in different ways and help in stabilizing expression patterns and their transmission to daughter cells. These epigenetic memory systems involve mainly chromatin regulators of the Trithorax and Polycomb group proteins, and are in charge of maintaining cell-type-specific expression patterns in many developmental systems (Ringrose and Paro, 2004, 2007). For many years these epigenetic marks were considered irreversible (in parallel with differentiation), but the most recent findings are revealing that they are much more dynamic than initially thought and that they contribute greatly to the competence of progenitors. Along these lines, the so-called bivalent chromatin regions have been found in embryonic stem (ES) cells, that correspond to genome sections simultaneously marked by H3K27me3 (a repressive mark) and H3K4me3 (an activating one), and it has been proposed that these domains work by controlling developmental genes in these cells while keeping them poised for activation or deactivation, suggesting a chromatin-based mechanism for pluripotency maintenance (Bernstein et al., 2006; Mikkelsen et al., 2007; Sharov and Ko, 2007). The resolution of the bivalent domains into either a permanent 'on' or 'off' state is closely related to the commitment of the cell. Initially it was thought to be restricted only to progenitors and only related with genes that had to be kept silent and then activated. However, it seems that bivalent domains also can appear in differentiated cells like T cells (Roh et al., 2006) and seem to provide a way to postpone either the activation or the repression of a functionally distinct group of genes, mainly developmental transcription factors (Pietersen and van Lohuizen, 2008). The fact that epigenetic modifications themselves are much more flexible than previously thought fits very well with the increasing examples of plasticity during development. Indeed, a rigid model based on irreversible molecular modifications of the chromatin cannot accommodate all the different processes of differentiation, and it is especially difficult to reconcile with developmental systems in which terminal differentiation steps require an extensive reprogramming of the gene expression profiles with respect to the ones existing in previous partially differentiated cellular intermediates. In these systems in which the so-called mature cells should still maintain a high degree of plasticity (i.e., a certain degree of "stemness") a different molecular mechanism must exist to make such quick reprogramming possible.

As a way of an example to illustrate the above-mentioned points, and how developmental plasticity plays a role in both normal and pathological differentiation we are going to describe the development of a system that has been very well characterized: B cells in the hematopoietic system. In the adult, the generation of mature B cells begins with the hematopoietic stem cells (HSCs) in the bone marrow (BM). HSCs will be gradually restricted towards the B lymphocyte lineage through several stages of differentiation. Initially they give rise to multipotent progenitors (MPPs), which have lost the self-renewal capacity but retain multilineage differentiation potential. After that, they generate lymphoid-primed multipotent progenitors (LMPPs) that already lack erythroid and megakaryocyte potential (Adolfsson et al., 2005). LMPPs give rise to early lymphocyte progenitors (ELPs) characterised by the activation of recombination-activating genes (Igarashi et al., 2002); these will afterwards differentiate into common lymphoid progenitors (CLPs) with potential already restricted to B, T and NK pathways (Hardy et al., 2007; Kondo et al., 1997). The expression of the transcription factor Pax5 determines definitive commitment to the B cell lineage at the pro-B cell developmental stage (see below). Rearrangements of immunoglobulin heavy and light chain genes lead to the generation of immature B cells in the bone marrow, expressing a functional B cell receptor (BCR) in their surface (IgM) (Jung et al., 2006). These immature B cells leave the bone marrow and travel to the peripheral lymphoid organs where they become mature B cells (Hardy and Hayakawa, 2001). However, mature B cells in the periphery are not in fact, regardless of their name, the last differentiation stage of their lineage, because they are in fact waiting for an external signal (the antigen recognition) to experience the terminal differentiation process that will result in the generation of antibody-producing plasma cells. So, in response to T cell-dependent antigens, a dedicated structure, the germinal centre (GC) is formed, where B cells undergo several cycles of proliferation, somatic hypermutation, immunoglobulin class switching and selection. Positively selected GC B cells can then either become terminally differentiated plasma cells or memory cells (Klein and Dalla-Favera, 2008). However, the gene expression program of plasma cells is very different to the one of B cells and, in fact, for many genes it shows similarities with the expression profile of progenitors (Delogu et al., 2006; Shaffer et al., 2002; Shapiro-Shelef and Calame, 2005). So this is an example of a case where the terminal differentiation involves a complete reprogramming of the transcriptional profile of the previous developmental stage. Clearly, in a system like this plasticity must be guaranteed in the late differentiation stages to allow for the last reprogramming step to occur, even if a progressive limitation of developmental options takes place together with differentiation. This last step of terminal differentiation to plasma cells would not be possible if the epigenetic marking of activated and repressed genes that have been established during lineage specification was irreversible. Therefore, a mechanism must exist for the maintenance of B cell identity that allows this identity to be lost for terminal differentiation. In order to understand the molecular basis for this process we must first describe the mechanisms that establish and maintain B cell characteristics.

In uncommitted hematopoietic progenitors, as we have described, plasticity (competence) is based on their capacity to maintain a promiscuous level of basal expression of lineage-specific genes in the process of multilineage priming (Akashi et al., 2003; Hu et al., 1997). This promiscuous gene expression pattern allows the progenitors to respond to environmental signals that, in combination with the right transcription factors, will lead them into the different specific lineages. In the case of B cells, this signalling is provided by IL7, in combination with the transcription factors E2A, EBF1 and PAX5 (Cobaleda and



Busslinger, 2008; Cobaleda et al., 2007b; Miller et al., 2002; Nutt and Kee, 2007). Although the precise roles of these transcription factors in these very early stages is still the subject of active investigation, it seems that E2A and EBF1 are in charge of activating the expression of B lymphoid genes at the beginning of B cell development. However, the real commitment to the lineage is controlled by PAX5. PAX5 is a transcription factor whose expression within the haematopoietic system is restricted to B cells. Due to its protein structure it has the dual capacity of acting either as a transcriptional activator or as a repressor, depending on the interacting partners (Czerny et al., 1993; Dorfler and Busslinger, 1996; Eberhard and Busslinger, 1999; Eberhard et al., 2000). Induced by Ebf, Pax5 commits cells to the B cell lineage and maintains B cell identity by concurrently repressing B-lineage-inappropriate genes and activating B-cell specific genes (Delogu et al., 2006; Schebesta et al., 2007). Once Pax5 expression has been initiated, progenitors lose their potential and are only able to differentiate along a unidirectional path towards mature B cells. In *Pax5* knockout mice (Nutt et al., 1999; Urbanek et al., 1994) B cell development cannot progress beyond the pro-B cell stage. However, since they are not yet committed, *Pax5*<sup>-/-</sup> proB cells behave as multipotent progenitors, because they express multilineage genes (that would have been otherwise repressed by Pax5 in normal conditions), and this allows them to be programmed into most of the haematopoietic lineages under the right conditions. All these developmental options are shut down by the reintroduction of Pax5, which actively represses all non-B cell genes (Nutt et al., 1999).

But the role of Pax5 is not over once commitment has taken place; quite the opposite, it is continuously required to maintain B cell identity and function all the way through the life of the B cell (Cobaleda et al., 2007b). Actually, deletion of *Pax5* at different B cell developmental stages by using a conditional *Pax5* allele has shown that its loss leads to the loss of B cell identity and commitment. In proB cells, loss of *Pax5* causes committed B cells to recover the capacity to differentiate into macrophages and T cells, proving that Pax5 is required not only to initiate the B cell program, but also to maintain it in early B cell development (Mikkola et al., 2002). Deletion of *Pax5* at later stages of B cell development results in the loss of mature B cells, inefficient lymphoblast formation, and reduced IgG formation. Most B cell membrane antigens are downregulated, and the transcription of B cell-specific genes is decreased, whereas the expression of non-B cell-specific genes is activated (Horcher et al., 2001; Schebesta et al., 2007). Thus, mature B cells radically change their gene expression pattern in response to *Pax5* inactivation. These effects can be easily understood when considering that Pax5 activates at least 170 genes that are essential for B cell signalling, adhesion, migration, antigen presentation, and germinal-centre B cell formation (Schebesta et al., 2007), indicating that Pax5 controls in a direct manner both B cell development and function. In the absence of Pax5, all this network collapses and the cells lose their B cell identity. The loss of B-cell specific genes upon *Pax5* deletion goes together with the loss of Pax5-dependent repression of non-B cell genes. Derepression of these genes (around 110 genes controlling functions such as receptor signalling, cell adhesion, migration, transcriptional control, and cellular metabolism (Delogu et al., 2006)) unveils a new plasticity for peripheral *Pax5*-deleted mature B cells: they can dedifferentiate *in vivo* back to early uncommitted multipotent progenitors in the bone marrow, which can afterwards give rise to other haematopoietic cell types like macrophages or T cells (Cobaleda et al., 2007a).

This Pax5-dependent plasticity has a biological reason and is directly related with the physiology of B cells. As we already mentioned, the final function of mature B cells is to



become plasma cells. For this terminal differentiation to take place, *Pax5* must be downregulated, to permit the closing down of all the B cell transcriptional program (Delogu et al., 2006; Schebesta et al., 2007; Shapiro-Shelef and Calame, 2005) and allow the transition to the plasma cell stage. The process starts with the binding of the membrane BCR to its cognate specific antigen. This activates a signalling cascade that leads to the upregulation of *Blimp1*, the master regulator of the plasma cell transcriptional program and identity (Kallies and Nutt, 2007; Martins and Calame, 2008). Mature B cells and plasma cells have very different gene expression programs, which are controlled in a mutually exclusive manner by *Pax5* and *Blimp1*, respectively. In fact, *Pax5* is directly repressed by *Blimp1*, as a way of eliminating B cell identity and allowing for plasma cell differentiation to proceed (Lin et al., 2002). The expression of many *Pax5*-activated genes is either absent or considerably reduced upon *Pax5* loss in plasma cells, and *Pax5*-repressed genes are reexpressed in plasma cells (Delogu et al., 2006). Many of the genes that are expressed in plasma cells are also expressed in uncommitted lymphoid progenitors (Delogu et al., 2006). But, since these genes are not compatible with B cell development or function they must be silenced to maintain B cell identity. However, as they will be required for terminal differentiation into plasma cells, they cannot be irreversibly repressed in B cells by stable epigenetic modifications. The molecular mechanism underlying this versatility is based on the function of *Pax5*: first, it preserves B cell identity, and afterwards it allows for a simple mechanism (repression of *Pax5*) of eliminating this identity when reprogramming becomes necessary to generate a plasma cell. This is the reason why mature B cells retain such a high degree of plasticity dependent on a single gene.

This mechanism that we have outlined for B-cell differentiation is present in other systems and can explain the existence of plasticity in many other developmental models. For instance, in the process of melanocyte differentiation from adult melanocyte stem cells, the transcription factor *Pax3* initiates a melanogenic program and, simultaneously, prevents downstream terminal differentiation (Lang et al., 2005). *Pax3*-expressing melanoblasts are therefore committed, but remain undifferentiated until *Pax3*-mediated repression is relieved. Hence, also in this example a transcription factor can simultaneously determine cell fate and maintain an undifferentiated state, leaving a cell poised to differentiate in response to external stimuli. This molecular mechanism implies a high degree of cellular plasticity, since the elimination of the factor(s) responsible allows the cells to readily differentiate to other lineages. Perhaps the most striking example of this plasticity is the reprogramming of adult mouse ovaries into testes induced by the removal of transcription factor *Foxl2* (Uhlenhaut et al., 2009). In a fascinating result, the deletion of this single, organ-identity-maintaining gene leads to the full conversion of all the female ovary tissues into their male ontological equivalents, showing that cellular (and even organ) plasticity can be much less hidden than we think, and that cell (and organ) identity can be maintained by just a single gene (Uhlenhaut et al., 2009).

## 5. Experimental control of plasticity: reprogramming

In the previous sections we have described the different levels of physiological plasticity that can be found during normal development, and shown that they are in fact necessary for differentiation to occur. However, we have also seen that this plasticity is usually not manifested spontaneously, but is rather something latent in the cells that we can only reveal in an artificial way. As a general rule, the ultimate cellular identity of any particular

differentiation pathway is stable and typically corresponds to a very specialized cellular type with a highly specific physiological function. Therefore, on paper, plasticity, from the point of view of normal development, is a property that should in principle be limited to stem cells and progenitors (i.e. cells that require this competence for their function). This could be called the physiological plasticity, that is, the normal competence of progenitors that we have previously discussed. All other types of cells should remain stable and maintain their identity. Indeed, most reprogramming cases occur either “on purpose” in the lab (experimental reprogramming for regenerative medicine) or in an “accidental” manner in nature (reprogramming in tumorigenesis, see below). However, this notion of stability was seriously challenged by the results for Yamanaka’s group showing that, and least in an experimental setting in the laboratory, reprogramming specialized cells to pluripotency only required the action of four factors (or even less): the 4 transcription factors from Yamanaka: Oct4, Sox2, c-Myc and Klf4 (Takahashi and Yamanaka, 2006). This finding showed in a definitive manner that there is a latent developmental potential retained in the cell, and what are the factors required to unleash it. The knowledge of reprogramming as a reality was already present, as we have mentioned before, in the results from the seminal nuclear reprogramming from the 1950-60s (Briggs and King, 1952; Gurdon, 1962). However, even though it was since then obvious that a cell nucleus could be converted from the program of a differentiated cell into that of a pluripotent progenitor just by being transferred into the right cytoplasmic environment, it was difficult to imagine that only a few of factors were really required to make the entire process possible. We have also seen that the gain and/or loss of single, essential, factors can alter the whole developmental program of a cell.

In the laboratory, there are several experimental approaches to achieve cellular reprogramming that might lead to pluripotency. On one side, there is nuclear transfer, where the whole nucleus is taken away from one cell and transferred into a new one, a previously enucleated oocyte whose cytoplasm contains all the factors required to impose an multipotential state. Although this method does not involve the acquisition of genetic changes, obviously the whole nuclear environment is changed, with all the possible consequences that this may have (Byrne et al., 2007; Gurdon and Melton, 2008; Hochedlinger and Jaenisch, 2006). Another possibility for reprogramming is cellular fusion, which allows the nuclei of a cell to act over that of another cell and therefore, under the appropriate circumstances, alter fate (Yamanaka and Blau, 2010). Exogenous expression of transcription factors was one of the first ways of demonstrating how reprogramming could take place (see Section 2), in this case without reverting cells back to a pluripotent stage (Zhou and Melton, 2008a). Some examples include transdifferentiation of adult pancreatic exocrine cells to  $\beta$  cells after expression of the transcription factors Ngn3, Pdx1 and Mafa (Zhou et al., 2008; Zhou and Melton, 2008a, b), the conversion of fibroblasts into myogenic cells by the myogenic factor MyoD (Davis et al., 1987) and the transdifferentiation of committed B lymphocytes to macrophages by expression of C/EBP $\alpha$  (Xie et al., 2004). The identification of the right cocktail of factors led to the reprogramming to pluripotency (induced-pluripotency stem cells, iPSCs) by the introduction of stem cell-specific genes into a differentiated cell (Maherali et al., 2007; Okita et al., 2007; Takahashi and Yamanaka, 2006; Wernig et al., 2007). This can be done by introducing genetic changes in the treated cells or in a less invasive, transient way, using specific drugs or transient vectors (Abujarour and Ding, 2009; Mikkelsen et al., 2008; Stadtfeld et al., 2008a; Stadtfeld et al., 2008b).

Another possibility of exploiting physiological plasticity for experimentally-induced reprogramming is to eliminate the specific transcription factors (usually master regulators)

responsible for maintaining the identity and function of the differentiated cell and for keeping its epigenetic state. This, as we have seen, leads to a lineage reprogramming into new cell types like in the case of the conversion of mature B cells into T cells (Cobaleda and Busslinger, 2008; Cobaleda et al., 2007a).

Of all these methods, nuclear transfer is empirical, but all the other ones require a precise knowledge of the transcriptional and epigenetic machineries that control the identities of the starting cellular material and the final desired product. It is very clear now that, together with the specific activation or repression of transcription factors (usually master regulators of specific lineages), the epigenetic modifications are an indispensable part of the process, since they are the ones that define the “flexibility” of the cell to be reprogrammed. As we have mentioned before, in general, differentiated cells correspond to a highly specialized compartment with no plasticity. According to this fact, it has been recently described that in the haematopoietic system the HSCs are 300 times more prone to reprogramming than B or T cells (Eminli et al., 2009).

Since the differentiated state is the more stable one, a certain level of “activation energy” is required to move the cells “uphill” to become again pluripotent. From this point of view of inducing pluripotency, there are two possibilities (Yamanaka, 2009): i) either only some cells in the population can be reprogrammed, because they are the ones that are responsive to the reprogramming factors (elite model), or ii) all the cells are equally susceptible to reprogramming (stochastic model). The latest evidences indicate that the second possibility happens to be true and that, given the appropriate combination of stimuli (in this case, the reprogramming factors), any cell can be reprogrammed to change fate (Hanna et al., 2009), and that the process can be accelerated either by interfering with the DNA damage checkpoint (see below) or by increasing the expression of some of the reprogramming factors, like Nanog (Hanna et al., 2009). The global inefficiency of the reprogramming process, even in the most favourable conditions, clearly suggests that, independently of the initial number of cells that are actually responsive to the reprogramming factors, very few of them can finally achieve full reprogramming. It has been shown that factor-induced reprogramming is a gradual process with several more or less defined cellular intermediates (Stadtfield et al., 2008a). Some of these non-physiological reprogramming intermediates (remember our definition of transdifferentiation) can be isolated as cell lines stuck at some point of the conversion process (Mikkelsen et al., 2008). The study of these incompletely reprogrammed intermediates shows that they have re-activated stem cell renewal and maintenance genes, but those genes in charge of pluripotency are still repressed. Also, the cells have not been able of completely repressing the expression of lineage-specific transcription factors. On top of that, these cells have failed in completing epigenetic remodelling and still retain persistent DNA hypermethylation marks (Mikkelsen et al., 2008).

## 6. Cancer: the dark side of plasticity

We have shown that plasticity is an essential feature of development. However, as all aspects of normal physiology, it also represents a “weakness” that can give rise to the origin of diseases. As we have mentioned, cancer is a differentiation disease, and tumorigenesis represent the outcome of a deviation of the normal process of differentiation in which a new lineage, the tumour, is created, with new properties and characteristics, but still similar in some ways to normal lineages. In other words, cancer could be considered as a particular case of “wrong” reprogramming.

In the last decade great advances have been made in our understanding of the cellular origin of cancer. Many of these findings have been driven by the postulation and final coming of age of the theory of the cancer stem cells (CSCs). It is beyond of the scope of this chapter to detail all the aspects and implications of this theory, which have been previously discussed to great extent (Cobaleda et al., 2008; Cobaleda and Sanchez-Garcia, 2009; Lobo et al., 2007; Reya et al., 2001; Sanchez-Garcia et al., 2007; Vicente-Duenas et al., 2009a), so here we will limit our discussion to the aspects related to cellular plasticity and differentiation. The CSC theory proposes that tumours are heterogeneous tissues, maintained by tissue-specific stem cells, in a manner very similar to any other stem cell-based tissue in the organism. Therefore in any tumour, different types of cells coexist: some of them are differentiated cells, lacking the possibility of propagating cancer, and that normally constitute the main mass of the tumour. However, there is also a variable, but generally small, percentage of cancer stem cells (CSCs), which are defined by the fact that they are the only ones that possess the capacity of replenishing the tumour mass and of transplanting the cancer (Castellanos et al., 2010; Greaves, 2010; Hermann et al., 2010; Lane and Gilliland, 2010; Sanchez-Garcia, 2010; Shackleton, 2010; Vicente-Duenas et al., 2010). Therefore, if cancer is a stem-cell driven tissue, it becomes crucial to identify the first cell suffering the oncogenic alteration(s) i.e., the normal cell that will give rise to the cancer stem cell, and the mechanisms that are behind this fate reprogramming. This first cell, as previously defined, would be the cancer cell-of-origin. What is clear is that the initiating cell's intrinsic plasticity must allow the cell to be reprogrammed into the new tumoral type(s). So cellular plasticity and the responsiveness of the cell to the reprogramming effects of the oncogene are therefore critical factors in the tumorigenesis process, and this implies that specific cancer-inducing alterations happen in particular cells (stem or differentiated, see below), and that it is the reciprocal interaction between the cellular plasticity and the differentiating capabilities of the oncogenic event(s) what determines the final resultant tumor phenotype.

From the point of view of the nature of the oncogenic alteration(s) and its potential reprogramming capabilities, traditionally in the field of cancer research it was assumed that more than one hit was required to switch from a normal healthy cell into a tumoral one, implying that many different aspects of cellular biology must be altered in the progress to final tumorigenesis (Hanahan and Weinberg, 2000). Also in the field of plasticity it was consequently assumed that, to convert a certain cell into a different one, more than one single alteration was required. This was partially supported for a long time by the fact that the only way to achieve full reprogramming to pluripotency was nuclear transplantation, a purely empirical method in which it was impossible to isolate or identify the factors responsible for the stem state. This seemed to suggest that many elements were necessary for reprogramming to occur. In fact, as we have discussed before, for "simple" changes in identity, like it could be a transdifferentiation process, a single, transcription factor could be all that is required to induce the reprogramming, as long as it is the right factor for the right type of cell (Cobaleda et al., 2007a; Davis et al., 1987; Nutt et al., 1999; Xie et al., 2004). This was similar as how a single initial oncogenic lesion may only cause an alteration in proliferation, or a partial block in differentiation. The breakthrough of Takahashi and Yamanaka (Takahashi and Yamanaka, 2006) showed that only 4 transcription factors ("four hits") were necessary for induction of pluripotency. Of note, the 4 transcription factors have been shown to play an oncogenic role in different contexts, and both *c-Myc* and *Klf4* are well-known oncogenes (Chen et al., 2008; Okita et al., 2007; Rowland et al., 2005; Tanaka et al., 2007). This is a clear evidence of the essential mechanistic link between reprogramming



and cancer, and illustrates the fact that there are a certain number of genes/proteins that are strong enough so as to induce the change of expression patterns in a global manner affecting cellular identity. Only strong regulators of the transcriptional and/or epigenetic machineries can reprogram. Therefore, the multistep nature of tumorigenesis can be compared with the series of developmentally unfavoured “uphill” steps required for full reprogramming to pluripotency. All these barriers are biologically designed to protect cells from transformation, that is, to prevent cells from changing their identity. There are many articles and reviews describing the capacity that the different oncogenes have for blocking or interfering with essential cellular functions (Hanahan and Weinberg, 2000). In the case of the reprogramming factors our knowledge is still incomplete, but the answers are gradually arising from the study of partially reprogrammed states and also by introducing the different factors at different times during the process of induction of pluripotency, starting from mouse fibroblasts (Sridharan et al., 2009). This kind of experiments has allowed showing that the different factors have temporal and separable contributions during the reprogramming process. In the initial stages, and previously to the induction of the ES-cell-like gene expression program, silencing of the somatic cell gene expression program takes place, mainly due to the action of c-Myc, although it is not yet clear how this gene mediates repressive effects in this context. Nevertheless, it has previously been shown that histone deacetylase inhibitors like valproic acid (VPA) can partially substitute for c-Myc in the reprogramming process (Huangfu et al., 2008) (see below) collaborating in the repression of the differentiated cells’ gene program. Therefore, it would seem that c-Myc mostly acts before the pluripotency regulators are activated and, consequently, ectopic expression of c-Myc is only required for the first few days of reprogramming (Sridharan et al., 2009). Actually, it seems that c-Myc could be dispensable for reprogramming, but in its absence there is a massive decrease in the efficiency of the process (Nakagawa et al., 2008; Wernig et al., 2008). It seems that the other factors, Oct4, Sox2, and Klf4, need to act together in establishing the pluripotent condition, since they cannot associate with their target genes in cells that are only partially reprogrammed, most probably because the histone methylation pattern does not allow their binding (Sridharan et al., 2009). This correlates with our knowledge about the function of these factors in ES cells, where they bind cooperatively to hundred of genes in overlapping genomic sites (Boyer et al., 2005; Loh et al., 2006), acting in a coordinated manner to maintain the transcriptional program required for pluripotency. However, even though the four Yamanaka factors can be sufficient for reprogramming most cell types, there are cases where they are not enough. One of the most striking examples is precisely that of B cells. In mature B lymphocytes, the four factors cannot achieve full reprogramming, and another molecular manipulation is required: the extinction of Pax5 expression (Hanna et al., 2008). As we have mentioned before, the elimination of Pax5 by itself is all what is required for mature B cells to dedifferentiate to early multipotential progenitors, since Pax5 is the responsible for the initiation and maintenance of B-cell identity and function (Cobaleda et al., 2007a). So the presence of such a strong factor requires its specific elimination in order to achieve reprogramming. These results also connect reprogramming to tumorigenesis, since it had previously been described that the loss of cellular identity induced by the absence of Pax5 led to the development of tumours or an early-B cell progenitor phenotype (Cobaleda et al., 2007a), indicating that the loss of the identity of the initial cell is an essential step in tumorigenesis. In fact, a very similar observation has been made in human patients with the uncommon transdifferentiation of follicular B cell lymphoma (FL) into a myeloid histiocytic/dendritic cell (H/DC) sarcoma



(Feldman et al., 2008). The FL and H/DC tumors of each patient are clonally related, since they contain the same immunoglobulin rearrangements and an identical IgH-BCL2 translocation breakpoint. It has been suggested that the translocation-induced overexpression of BCL2 leads to a prolonged survival of FL B that can facilitate their loss of B-lineage identity and subsequent reprogramming into H/DC tumor cells (Feldman et al., 2008). There are more examples corroborating the fact that loss of cell identity is essential for tumoral reprogramming. For example, in human Hodgkin lymphomas the inactivation of the B cell factor E2A by overexpression of its specific antagonists activated B cell factor 1 (ABF-1) and inhibitor of differentiation 2 (Id2) leads to the loss of B cell markers and expression of lineage-inappropriate genes that characterizes the tumour pathognomonic Reed-Sternberg cells (Mathas et al., 2006). Another aspect worth mentioning is the fact that, in contrast to mature B cells, earlier B cell developmental stages could be reprogrammed to pluripotency just with the four Yamanaka factors (Hanna et al., 2008), again underscoring the idea that the degree of differentiation of the target cell impacts directly in the reprogramming efficiency.

An essential component of both the reprogramming process and tumoral progression are epigenetic changes. It is clear that cancer does not only depend on genetic mutations, but also on epigenetic changes that establish a new pattern of heritability, providing a cellular memory by which the new tumoral cellular identity can be maintained, and that these alterations constitute an essential part of cancer initiation and progression (Ting et al., 2006). The role of epigenetic alterations in tumour origin and progression is well known and it has been comprehensively reviewed elsewhere (Esteller, 2007, 2008; Esteller and Herman, 2002). All epigenetic marks become altered in tumours, leading to changes in gene expression. These changes have been very well described to affect many specific genes in charge of controlling cellular functions, which therefore become altered in cancer. But these changes are in fact global and affect the whole cellular identity. The tumour-related epigenetic alterations can either be independent from the initiating oncogenic mutation and simply due to tumour progression, or they can be directly linked to the first oncogenic event, like it happens in the case of chromosomal translocations that affect histone-modification genes (Esteller, 2008). In the case of reprogramming to pluripotency, something similar happens, since epigenetic modifications are an intrinsic part of the process and they need to take place in a global manner, not just by the specific regulation of some individual genes that is mainly accomplished by the transcription factors. This explains why the efficiency of reprogramming increases greatly in the presence of chemicals interfering with epigenetic marks in an unspecific (i.e., not locus-restricted) manner. For example, treatment with 5-azacytidine (AZA), a DNA methyltransferase inhibitor, induces a rapid transition to fully reprogrammed iPSCs (Huangfu et al., 2008; Mikkelsen et al., 2008), and the use of valproic acid (VPA), a histone deacetylase (HDAC) inhibitor, greatly improves the induction to pluripotency (Huangfu et al., 2008). Treatment with the inhibitor of the G9a methyltransferase named BIX-01294 increases the efficiency of reprogramming using just two factors, Oct4 and Klf4, to levels similar to the ones achieved when using the four factors (Shi et al., 2008). G9a methyltransferase is essential for the extinction of the pluripotency program upon exit to differentiation because, by means of its histone methylation activity, it blocks target-gene reactivation in the absence of transcriptional repressors, and this leads to the silencing of embryonic genes like Oct4 (Feldman et al., 2006). Also, simultaneously, G9a promotes DNA methylation, and therefore prevents the reprogramming to the undifferentiated state (Epsztejn-Litman et al., 2008; Feldman et al., 2006). All these facts

support the idea that global epigenetic changes affecting a large and unknown number of genes are a critical selective component of the reprogramming process, and that the addition of chemicals that facilitate these molecular changes helps the process by lowering the activation energy barrier for this “uphill” process. A very important practical consequence of these findings is the fact that epigenetic therapies are already in use or in very advanced clinical trials against cancer. Their mechanisms of action are based on the assumption that, by globally affecting epigenetic patterns of tumoral cells, they can restore the normal levels of expression of genes that are required for the normal control of cellular proliferation and/or differentiation. Like for any other chemotherapy, the effects are systemic, but it is likely to affect primarily the tumoral cells and leave non-proliferative cells relatively unaffected. Since 2004, AZA is FDA-approved as the first drug of the new class of demethylating agents for the treatment of myelodysplastic syndromes (Kaminskas et al., 2005), and there are many other clinical trials evaluating the effects of AZA in other cancer types (Sacchi et al., 1999). Something similar happens with HDAC inhibitors (Dey, 2006; Lane and Chabner, 2009). All these findings emphasize once more the nature of cancer as a pathological case of “wrong” reprogramming, as a differentiation disease.

As we have seen, both the changes in the epigenetic patterns and the gain or loss of transcriptional regulators are essential components of the tumour generation and of the experimentally-induced reprogramming processes. It is clear that these alterations, although based in mechanisms normally existing in the cells, are undesirable for normal cellular development and functioning, so the cells have evolved a series of safety mechanisms to avoid these alterations or their effects and maintain their identity and function. In the context of cancer there have been many studies in the last decades describing how all these safety mechanisms are bent, broken or bypassed to allow tumour generation and progression (Hanahan and Weinberg, 2000). The most recent results in the less advanced field of reprogramming seem to indicate that, also in this experimentally-induced “progression to pluripotency” (in analogy to tumoral progression) exactly as it happens in tumour progression, the elimination of the DNA damage control checkpoint tremendously increases the efficiency of the reprogramming process. Thus, the inactivation of the p53-p21 axis by different approaches allows a much higher percentage of the starting cells to successfully complete the process to full pluripotency (Banito et al., 2009; Hong et al., 2009; Kawamura et al., 2009; Krizhanovsky and Lowe, 2009; Li et al., 2009; Marion et al., 2009; Utikal et al., 2009; Zhao et al., 2008). However, this enhanced efficiency is achieved at the price of a much higher genetic instability, and the iPSCs generated in this way carry many genetic aberrations of different types. This is corresponding to the facts that we have previously mentioned showing that reprogramming is an “uphill”, developmentally unfavourable process that imposes a great stress to the cells and that most of the cells therefore, in normal conditions, fail to complete (Mikkelsen et al., 2008). These results do not only further support the idea of cancer as a disease of cellular differentiation but, furthermore, indicate that indeed, the aberrant transcription factors, deregulated signalling molecules and epigenetic regulators are the main dynamic forces behind the tumoral process, and that many of the other alterations (for example, loss of p53) play just a permissive role for tumoral progression.

We have until now examined the processes of reprogramming and tumorigenesis mainly from a molecular point of view. The inclusion of epigenetics in our description encompasses to a certain degree cellular identity, since the epigenetic pattern of chromatin modifications can be broadly assimilated to cellular identity. However, in the next final paragraphs we are

going to discuss the tumoral reprogramming phenomenon from a more classical cellular point of view. In the field of cancer research it has conventionally been assumed that the phenotype of the tumoral cell was a mirror of the one of the normal cell from which it arose. Most tumour cells present the characteristics of differentiated cell types (more or less aberrant). Therefore, for every type of tumour, the cell of origin had to be found in its closest relative in the normal tissue. However, the solidification of the cancer stem cell (CSC) theory has led to a re-thinking of these concepts (Cobaleda and Sanchez-Garcia, 2009; Vicente-Duenas et al., 2009b). First, since tumours are postulated to be stem cell-based tissues, not all the tumoral cells are equally capable of regenerating the tumour, but only those cells with CSC properties. Most of the cells lack this capacity, although there can be great variations in the percentage of CSCs within a tumour. This has important repercussions for our understanding of tumour origin. If tumours are maintained by aberrant cells with the characteristics of stem cells, then where do these cells come from? The cancer cell-of-origin would therefore be a normal cell that has undergone reprogramming by the oncogenic events to give rise to a CSC, a new pathological cell with stem cell properties. One possibility is that the oncogenic mutations take place in a normal stem cell that, in this way, becomes reprogrammed to originate the new pathological tissue. This has been long known to be the case for chronic myelogenous leukaemia (CML), where the causing chromosomal translocation t(9;22) is present in most lineages of differentiated haematopoietic cells, thus indicating that an early progenitor is the cell of origin (Melo and Barnes, 2007). Recent advances in modelling human diseases in the mouse have allowed us to prove this fact experimentally; indeed, restricting the oncogenic alteration to the stem cell compartment in the mouse is all that is required to generate a full CML with the whole variety of differentiated cells (Perez-Caro et al., 2009; Vicente-Duenas et al., 2009b). Also for intestinal cancers it has been proven in mice that they have their origin in the crypt stem cells, by activating the Wnt signalling pathway specifically in the stem cell compartment. This leads to the generation of adenomas where a differentiation hierarchy is maintained. On the contrary, if the oncogenic lesions are targeted to the non-stem, more differentiated intestinal epithelial cells, only small, short-lived microadenomas appear (Barker et al., 2008; Zhu et al., 2008). In other tissular context, targeting astrocytoma-associated oncogenic lesions to the nervous system progenitors results in tumour development, whereas targeting them to the zone containing just differentiated cells only gives rise to local astrogliosis (Alcantara Llaguno et al., 2009). In all these and other similar cases (Dirks, 2008; Joseph et al., 2008; Zheng et al., 2008) it is therefore clear that the initiating event must take place in a stem cell, even if, afterwards, the macroscopic tumour is composed by differentiated cells. This indicates a pathological direct reprogramming mediated by the oncogenic lesions.

The other alternative is that of the cancer cell-of-origin being a differentiated cell type. In this case the cells must be reprogrammed not only towards a new fate, but also to regain stem cell characteristics in a process of tumoral reprogramming to pluripotency. For this to occur, two aspects have to come together: first, the oncogenic alteration must be capable of conferring the stem properties and, second, the cell must have a degree of plasticity that allows the reprogramming mediated for this specific alteration to take place. It has been shown that some oncogenes, like MOZ-TIF2 (Huntly et al., 2004), MLL-AF9 (Krivtsov et al., 2006; Somervaille and Cleary, 2006), MLL-ENL (Cozzio et al., 2003), MLL-GAS (So et al., 2003) or PML-RAR $\alpha$  (Guibal et al., 2009; Wojiski et al., 2009) can generate CSCs when they are introduced into target cells that were already committed. Some of these genes, like MLL-AF9, have been shown to be able of activating a stem cell-like self-renewal program in

already committed progenitors (Krivtsov et al., 2006). A somewhat comparable situation happens with c-Myc, which can induce some parts of the transcriptional program of an embryonic stem cell in differentiated epithelial cells, thus giving rise to epithelial CSCs (Wong et al., 2008). Other oncogenes, like BCR-ABLp190, are however unable of conferring self-renewal properties (Huntly et al., 2004). In these cases, self-renewal must be provided by the target cell or by additional alterations, so that the oncogene does not immediately generates a CSC, but rather originates a precancerous cell that can afterwards give rise to a *true* CSC (Chen et al., 2007). In any case, the exact cellular origin of the initiating lesions is very difficult to determine, especially since, in many cases, the functional impact of the lesion, the clonal expansion, can become apparent only by the generation of cells that can be either upstream or downstream of the initiating cell, at least in terms of phenotypic markers. For example, in several childhood B acute lymphoblastic leukaemias (ALL) the initiating translocations originate prenatally *in utero* and act in partially committed cells as a first-hit capable of conferring this preleukaemic cell with aberrant self-renewal and survival properties (Hong et al., 2008). In AML1-ETO leukaemias, the translocation can still be detected in patients in remission, indicating that the cells can remain latent and some of their descendants can become tumorigenic with time (Miyamoto et al., 2000). In children's B-ALLs, the CSC properties can be found in blasts of more than one different developmental stage, which can also interconvert among themselves (le Viseur et al., 2008). This obviously makes the determination of the nature of the cancer-cell of origin even more difficult. Also in ALLs, the comparison of relapsed patient samples with the samples obtained from the same patients at their diagnosis by means of genomic analysis has shown that both initial and relapsed tumours share the same ancestral clone (Mullighan et al., 2008) that had diverted in different manners during the different stages of the disease. So, the nature of the CSC evolves over time with disease progression, treatment and relapse, in such a way that the properties of the CSC population in a certain moment do not necessarily reflect the nature of the initial cancer cell-of-origin (Barabe et al., 2007).

In the context of reprogramming to pluripotency, the initiating factors are not necessary anymore once the cells are already iPSCs and the process has been completed, that is to say, when the new identity has been fixed and the cell is already in a new pluripotent "attractor basin". If cancer stem cells arose through a reprogramming-like mechanism then, as a logical consequence, maybe the oncogenes initiating tumour formation might be dispensable for the posterior stages of tumour development (Krizhanovsky and Lowe, 2009). This fact correlates well with the examples of the subsistence of a pre-cancerous lesion in a stable population of cells that are already aberrant, but need secondary hits to initiate the openly tumoral differentiation program. In this way, the initiating lesion would have an active function in the reprogramming process, but afterwards it would become just a passenger mutation, or even perform a different function in tumour development that could very well be independent from its initial reprogramming activity. This could clarify the lack of success of some current targeted therapies, like the anti-BCR-ABL kinase drug imatinib which, although successfully eliminates differentiated tumour cells, fails to kill the BCR-ABL<sup>+</sup> CSCs (Barnes and Melo, 2006; Graham et al., 2002; Perez-Caro et al., 2009; Vicente-Duenas et al., 2009b). From a mathematical modeling point of view and consistent with the gene regulatory network (GRN) approaches, the oncogenic mutations alter one of the nodes and therefore change the architecture of the network, thus leading to a change in the landscape topography and giving rise to new abnormal attractors (new "valleys") where cancer stem cells are trapped (Huang et al., 2009). This modeling also fits with the above-



discussed postulate that a cell can stay in the new attractor even after the stimulus that triggered the transition has already disappeared, implying that the transient expression of an oncogene can be enough to trigger a lasting malignant phenotype that can become independent for its maintenance on the originating mutation (Huang et al., 2009).

## 7. Future prospects

Cancer is the second cause of mortality in the developed countries and its incidence is quickly rising in the Third World too. Current treatments for cancer are still focused in the idea of tumours as diseases in which the normal processes of proliferation are altered and consequently, therapies are targeted against proliferating cells. All these treatments are therefore unspecific and highly toxic, particularly for the non-cancerous cells in the organism with highly proliferation rates (epithelia, hair...). The most recent research advances have shown that cancer must be considered to a great degree as a disease of differentiation in which a new tissue, the tumour, emerges from cells that, following an oncogenic event, acquire new pathological fates. So it follows that cancer is a disease that, at least in its initial stages, is closely linked to reprogramming. Therefore, the research in reprogramming is intimately tied to that in cancer.

Considering cancer as a reprogramming disease gives us a new point of view over the disease in our search for new therapeutic strategies. Differentiation therapies are already in use for some very specific cases of cancer (e.g., differentiation of PML-RAR $\alpha$ -positive acute promyelocytic leukaemias with the use of retinoic acid). Reprogramming to pluripotency also gets stuck at in the "uphill" way to pluripotency (Mikkelsen et al., 2008) and it is very probable that tumoral cells are very similar to these partially reprogrammed intermediates, whose study should help us to learn how to force tumour cells out of their blocked condition. This is in fact what is planned to achieve with the use of the newest epigenetic drugs that are already approved or close to approval for treatment of specific tumours. Along the way we are also progressively learning more about the molecular mechanisms that govern epigenetic marks, and this knowledge about the epigenetic control of self-renewal, differentiation and maintenance of identity should help us to obtain more specifically targeted epigenetic therapies (Jones, 2007).

Our increasing knowledge and control over the mechanisms programming cellular identity should make us able of developing strategies to reprogram cancer cells in different ways. It has already been shown that it is possible to use nuclear transplantation approaches to reprogram melanoma cells (Hochedlinger et al., 2004) embryonal carcinomas (Blelloch et al., 2004) and even to clone mouse embryos from brain tumours (Li et al., 2003). All these findings indicate that it can be perfectly feasible to reprogram tumour cells. Hopefully in a near future we will possess the scientific and technological knowledge so as to be able of modifying tumoral cell fate at will to reprogram them either by forcing them to differentiate and disappear or to become susceptible to new therapies.

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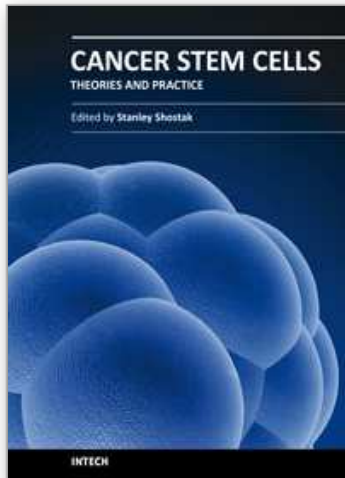
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