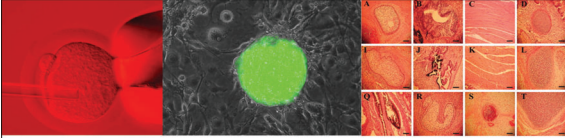# Pluripotency and differentiation in embryos and stem cells

Pavia, 17-18 January 2008

Introduction

Each year many scientific meetings are held on stem cells to appraise the state of knowledge on their potency, differentiation and applications. So why did we hold another meeting? Because we thought one aspect was not adequately addressed in the others. When thinking of how our body is derived from a single fertilized egg, it is self-evident that the embryo is the 'mother' of all stem cells. This fact is probably overlooked because it is so remote (decades back in our lives!) and because embryonic stem cells do not exist as such in the embryo. However, this also tends to be ignored on purpose in many stem cell meetings because working on (human) embryos brings up substantial ethical concerns that bear on the scientific undertaking like nothing else. The origin of stem cells has become even more of a sensitive issue since the discovery in 2006 that embryonic stem (ES) cell-like cells can be generated in a Petri dish straight from somatic cells by retrovirus-mediated transfer of selected genes. These new cells have been named 'induced pluripotent stem' (iPS) cells and have been obtained without any egg or embryo consumption (Takahashi and Yamanaka, 2006). This leads to the first topic of our meeting: natural and induced pluripotency. Many stem cells, irrespective of their origin (adult, fetal, embryonic, induced), almost invariably grow *in vitro* as multicellular structures referred to as 'colonies' to secure their potency. From an ontogenic perspective, the first 'colony' is arguably the morula-stage embryo with its 8-16 cells. Despite the fact that access to embryos is inherently difficult, more about cell fate control appears to be known in embryos than in stem cells. This leads to the second topic of our meeting: mechanisms of cell fate control. Stem cells can be propagated for many cell doublings and can give rise to a wide range of cell types found in the body. This makes them excellent candidates for cell- and tissue-replacement therapies. However, there is a general sense that the more potent stem cells are, the more likely they are to deviate from the normal physiological path of their differentiation, and that they may give rise to tumours. This leads to the third and last topic of our meeting: adult and cancer stem cells.

The Pavia meeting took place on January 17th and 18th 2008 inside the beautiful, frescoed halls of Collegio Ghislieri and Collegio Borromeo, two University colleges in Pavia, with a tradition of excellence with roots in the Renaissance. A small but prominent group of scientists lectured at this meeting – in alphabetical order: James Adjaye (Germany), Anne Grete Byskov (Denmark), Jose Cibelli (USA), Ruggero De Maria (Italy), Stephen Minger (UK), Maurilio Sampaolesi (Belgium), Hans Schöler (Germany), Giuseppe Testa (Italy), Catherine Verfaillie (Belgium) and Magdalena Zernicka-Goetz (UK). All the speakers were invited to contribute to this report.

Natural and induced pluripotency

Stem cells are indispensable to the organism as they safeguard tissue homeostasis through a fine balance of self-renewal and differentiation. Stem cells occur in very small numbers in adult tissues and in higher numbers in the fetus and its annexes. Thus,

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Abbreviations used in this paper: CSC, cancer stem cell; ES, embryonic stem cell; iPS, induced pluripotent stem cell; MAPC, multipotent adult progenitor cell; MEF, mouse embryonic fibroblast.

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they can be derived from whole embryos or parts thereof. There is probably no sounder example of self-renewal and differentiation than the properties of ES cells. These cells, first derived from mouse embryos (Martin, 1981; Evans and Kaufman, 1981) and later obtained in humans (Thomson *et al.,* 1998), can give rise to all the tissue types of the adult body, as was conclusively demonstrated in mice (Nagy *et al.,* 1993). The prevalent definition of mouse ES cells as pluripotent underscores their ability to form all tissues except the trophectoderm (TE) after culture under current protocols. In analogy, human ES cells (hESCs) are also considered pluripotent.

Over the last decade, increasing enthusiasm, as well as ethical concerns about the potential of hESCs for therapeutic uses, have divided the public opinion. On the one hand, hES cells may be differentiated into tissues that could be used in the therapy of injured or diseased organs. On the other hand, undifferentiated ES cells persisting in cell grafts have issued a warning, as the undifferentiated cells typically cause tumours (teratomas) in engrafted animals (Nussbaum *et al.,* 2007; Shih *et al.,* 2007). However, sometimes the differentiated ES cells seemed instead to be the cause of the tumours (Roy *et al.,* 2006). Tumour formation is enabled by a host of ES cell properties, including their immunologic profile (Saric *et al.,* 2008). Regardless of what ES cells can or cannot do, the production of hES cells requires the deployment of human embryos, which is held by many as unacceptable because it prevents a potential human being from coming into existence. In this respect, cloned embryos might be less objectionable given their very limited ability for development *in utero.*

Despite the complications inherent to hES cells, a large group of scientists are still on this rocky road, not only to pursue clinical prospects but also to gain knowledge on a model system that holds important lessons on pluripotency. It is no news to these scientists that the generation and maintenance of hES cells is particularly laborious and tricky, thereby making the knowledge harder to come by. In this light, it is remarkable that the first laboratory to achieve iPS cells from human fibroblasts lacked a published record of hESC work (Takahashi and Yamanaka, 2007). While this argues that anybody can handle hESCs, perhaps it is more likely that the research went through a preliminary phase of work, which has not been accounted. If so, the quest for refined and standardized protocols for the derivation and maintenance of hES cells remains on the agenda. In order to help achieve these protocols, the *International Stem Cell Initiative* (2007) has so far characterized 59 hESC lines from 17 laboratories and 11 countries. Stephen Minger, a member of the Initiative, explained at this meeting that there is a core set of features that appear to be common to all the lines examined. These include the expression of characteristic cell surface proteins, pluripotency genes including *Oct4*, *Nanog* and *Sox2* and the ability to differentiate into cell types representative of all three germ layers. However, certain differences are still unexplained. For example, Minger displayed data indicating that individual hES cell lines have different propensities to generate cells from each germ layer, even when comparing hES cell lines generated in the same laboratory under identical derivation and culture conditions (Pringle and Minger, unpublished data). Anne Grete Byskov showed that even within one colony of hESCs, there are regional differences in the expression of defining markers (Laursen *et al.,* 2007). This observation emphasizes the fact that even ES cells derived from one source are heterogeneous under current conditions. Byskov then examined a property of ES cells that has been known *in vivo* for a long time, but could not be reproduced *in vitro* until 2003: the ability to form germ cells (Hübner *et al.,* 2003). Consistent with this ability, in sexually undifferentiated (female) gonads, Byskov described a stem cell activity whereby oocytes appear to mature, undergo parthenogenesis and produce a new generation of 'stem cells' that again can differentiate into oocytes. Whether these 'stem cells' are primordial germ cells is under investigation. The obvious remark is that altogether, based on the data of Minger and Byskov, various factors contribute to the differences between and within (human) ES cell lines. These factors include but are not limited to differences among the precursors in the inner cell mass (ICM) (Lauss *et al.,* 2005; Furusawa *et al.,* 2004), genetic background and culture conditions.

Typically hES cells are grown on feeder cells, either 'xenogenic' (e.g. mouse embryonic fibroblasts, MEFs) or 'allogenic' (human somatic cells). James Adjaye showed that the ability of conditioned media made from MEFs to support hESC self-renewal and pluripotency depends primarily on one growth factor, FGF2. This factor stimulates MEFs to secrete self-renewal supporting factors (e.g. TGFβ1, GREM1 and INHBA), which in turn repress differentiation-inducing activities such as BMP4 (Greber *et al.,* 2007). Adjaye also presented data on the complexity of the OCT4-regulated transcriptional networks in stem cells based on ChIP-on-chip, a technique that combines chromatin immunoprecipitation (ChIP) with microarray technology (chip). From the presented data it appears that there are distinct, evolutionarily conserved (human to opossum) genetic modules containing OCT4-binding motifs. For example, one of the highly conserved OCT4-binding sites investigated so far is within the *NANOG* promoter, where OCT4 forms a dimer with SOX2 thus facilitating binding to the OCT4-SOX2-binding site. This arrangement is referred to as 'module 1'. 'Module 2' consists of the OCT4-binding motif without the SOX2-binding motif but contains either characterized or uncharacterized motifs as deduced from the TRANSFAC database of transcription factor binding sites (Jung, Lehrach and Adjaye, unpublished). An example of a gene falling within 'module 2' is *GADD45G*. This gene is negatively regulated by OCT4 and is implicated in a host of growth-regulatory mechanisms such as DNA replication and repair, apoptosis, G2/M checkpoint control and differentiation (Babaie *et al.,* 2007).

Whether derived from a zygote or from a cloned embryo, ES cells might not prove to be the ideal option for regenerative medicine anyway, because of the highly controversial consumption of potentially viable human embryos. Alternatives are being pursued. For instance, Adjaye announced to our audience the first achievement of iPS cells in Europe (Die Zeit, 17 Januar 2008) and

Fig. 1 (Right). Meeting photos. Middle three photos: *Giuseppe Testa, Ruggero De Maria and Hans Schöler.* Group photo (left to right): *James Adjaye, Catherine Verfaillie, Hans Schöler, Raul Fernandez-Donoso, Silvia Garagna, Nicola Crosetto, Michele Boiani, Gianna Milano, Juan Arechaga, Carlo Redi, Stephen Minger, Magdalena Zernicka-Goetz, Giuseppe Testa, Anne Byskov and Jose Cibelli.* Lower three photos: *Maurilio Sampaolesi, Carlo Redi and Stephen Minger, and Catherine Verfaillie.*

Collegio Borromeo

Collegio Ghislieri

presented initial results of his fibroblast reprogramming work using defined factors (OCT4, SOX2, NANOG and LIN28). Besides the new iPS approach, alternative approaches could be the generation of hESCs by parthenogenesis and by interspecies somatic cell nuclear transfer (iSCNT). These procedures are currently being pursued by Jose Cibelli and his group (Behyan *et al.,* 2007). During parthenogenesis, an egg is 'tricked' by chemical means to behave as if it had been fertilized (Cibelli *et al.,* 2002). This process has been shown to give rise to non-totipotent embryos that are unable to develop into a full organism, while still being able to give rise to ES cells (Allen *et al.,* 1994). This partial potency is in line with the definition of ES cells as pluripotent. However, embryos generated by parthenogenesis can form TE – the main exclusion criterion for totipotency. Alternatively, Cibelli explained that a human somatic nucleus transplanted into a non-human primate oocyte, activated by parthenogenesis, and cultured to the blastocyst stage could generate immunotype-matched human ES cells without consumption of any human oocytes or embryos. Despite the potential benefits, iSCNT is a touchy issue for regulatory authorities. Controversial results from human-to-rabbit nuclear transfer experiments (Chen *et al.,* 2003) suggest that the final product is not a surrogate, but truly human. It came therefore as a positive surprise that a short-term research license for cross-species nuclear transfer was granted to Minger by the UK's Human Fertilization and Embryology Authority (HFEA), while he was on his way to this meeting. The goal of this work is to create cloned human embryonic stem cell lines from individuals with known genetic mutations that result in a number of common neurodegenerative conditions, including Alzheimer's and Parkinson's diseases, as well as motor neuron disease and spinal muscular atrophy (Minger, 2007).

Mechanisms of cell fate control

For a few years, ES cells have been derived outside of the blastocyst stage. Indeed, ES cells have been derived from isolated blastomeres of pre-blastocyst stage embryos (Klimanskaya *et al.,* 2007). This leads one to ask if early developmental stages also have the heterogeneity that Minger and Byskov illustrated for hESCs. Answering this question implies understanding whether mammalian embryos have completely emancipated themselves from the patterns and polarities that play such an important role in the development of lower vertebrate taxa, e.g. Amphibia. It has been clear since 1965 that "any rigid 'pre-patterning' must be considered unlikely, at least in the mouse" (quote of Beatrice Mintz by Anne McLaren, 1976). Despite this considered opinion, which does not rule out developmental bias (in the sense that bias is not the same as pre-patterning), the prevalent view of mammalian development has been taken to the extreme of saying that regulative processes make the blastomeres homogeneous. Indeed, exposing bias in cell fate has been a challenge because the regulative processes in the embryo would often mask its existence. Magdalena Zernicka-Goetz and her group have indeed shown that early mouse blastomeres are not all alike in their epigenetic nuclear makeup and are not equivalent in their developmental potential (Torres-Padilla *et al.,* 2007). By tracking the cleavage pattern, and thereby examining how the spatial organisation of an early embryo is partitioned into individual cells of the 4-cell stage embryo, her group was able to show that the four blastomeres differ in the extent of histone H3 methylation at Arginine 26 and undergo skewed allocation towards ICM or TE. Deploying a very elegant set of micro-manipulation experiments, Zernicka-Goetz and colleagues have further demonstrated that if 4-cell mouse embryos are reconstituted with blastomeres of the same cleavage subtype, such chimaeras diverge in developmental outcomes (Piotrowska-Nitsche *et al.,* 2005). Most recently, by tracing all cells in embryos in 3D during the first 3 days of development, Zernicka-Goetz's group revealed that the skewed allocation of individual blastomeres to either ICM or TE is due to them undertaking different patterns of symmetric versus asymmetric divisions from the 8-cell stage onwards (Bischoff *et al.,* 2008). Thus, it appears that although the development of mammalian embryos is highly regulative, cell fate decisions do not occur at random, but that there is a developmental bias which can be exposed under appropriate conditions. It may be possible to magnify such bias (and thereby make it more amenable to study) by expressing certain factors in a given blastomere. Hans Schöler and colleagues showed that a reduction of the *caudal type homeo box 2* (*Cdx2*) gene product in the mouse blastocyst by RNA interference alters cell fate and affects ES cell derivability (Wu and Schöler, unpublished data). This finding revives the concept originally explored in the so-called 'altered nuclear transfer' (ANT; Hurlbut, 2005) approach, namely the possibility of using donor nuclei mutated for one or more genes that are essential for implantation *in utero*. Practically, a genetic mutation (ANT) or the transcriptional manipulation of *Cdx2* ('knock down' of its mRNA) would pose a roadblock to placenta formation, thereby hindering the possibility of cloning the animal while still allowing ES cell derivation. This embryo would be 'locked' into a pluripotent but not totipotent state, assuming once again that ES cells are pluripotent but not totipotent. In this way, some of the ethical concerns related to the generation of human ES cells might be relieved, although the wisdom of preferring these embryos over the thousands discarded every year from IVF programs should be pondered.

Cell fate flexibility in response to genetic and environmental cues may not be an exclusive prerogative of early embryonic cells. A property termed 'plasticity' has been controversially attributed to adult stem cells (a central topic in the next section), in relation to their ability to generate cell types of unrelated organs (Wagers *et al.,* 2004; Prockop, 2003). Unlike ES cells, adult stem cells do exist *in vivo* in most but not all body organs and tissues. Some think that during development, as potency becomes gradually restricted, somatic stem cells arise as remnants of more potent cells that remain trapped and dormant in adult tissues. For instance, one might hypothesize that adult stem cells arose from primordial germ cells that migrated aberrantly to tissues outside the gonads during development. Others think that adult stem cells arise *de novo* in the tissue by other mechanisms, for instance by cell fusion. Compared with ES cells, tissue-specific stem cells have less self-renewal ability and, although they can differentiate into multiple lineages, they are considered multipotent and not pluripotent. Manipulation of adult stem cells' plasticity would have enormous potentials for regenerative medicine. Indeed, because stem cells can be derived in larger numbers and relatively easily from certain tissues (the best example probably being the hematopoietic compartment), the possibility of manipulating their fate in a patient-customized manner would provide a basis for syngeneic (homologous) tissue regeneration. However, it is diffi-

cult to study plasticity when the 'plastic' cells are rare and hard to identify (this is even more true in the blood where the identity of the 'true' hematopoietic stem cells is still a matter of conjecture). Studying histone lysine demethylation, Giuseppe Testa addressed ways to make plasticity more amenable to study. Using mouse macrophages challenged with pro-inflammatory cytokines, Natoli and Testa addressed the role of histone lysine demethylation in cell fate transitions. In particular they showed that the histone 3 lysine 27 (H3K27) de-methylase Jmjd3 promotes extensive epigenetic rearrangements resulting in cell identity changes in the inflammatory response (De Santa *et al.,* 2007). Studying a different biological problem, namely the neural commitment of mouse ES cells, Testa and colleagues found that Jmjd3 enables this process by regulating key drivers and markers of neurogenesis, indicating that H3K27 demethylation is apparently a common mechanism involved in cell fate transitions (Testa, unpublished data). It will be very interesting to test this response in other cell types. In a different model system, Catherine Verfaillie and co-workers have gathered evidence that cells derived from bone marrow under specific culture conditions to isolate Multipotent Adult Progenitor Cells (MAPCs) express a number but definitely not all transcription factors known to endow ESC with pluripotency. Like ES cells, some of the MAPC lines can participate in normal development after injection in the blastocyst cavity of mice, but unlike ES cells they have the advantage of not giving rise to teratomas *in vivo* (Jiang *et al.,* 2002a,b). A molecular portrait of MAPCs is currently being pursued. Certain signalling cascades, such as the TGFβ, Wnt and Notch pathways, are responsible for controlling the plasticity of the MAPCs. At the transcriptome level, MAPCs differ from classical mesenchymal stem cells (Ulloa-Montoya *et al.,* 2007). Whether MAPCs truly exist in the living organism or whether they are artifacts due to reprogramming in response to the very specific culture conditions used to isolate them, is not known yet. However, this question may be regarded as secondary to the actual benefits arising from the applications of MAPCs.

Adult and cancer stem cells

The increasing interest of the public and scientific community towards stem cells originates from their promise to regenerate tissues or organs damaged by traumatic agents, impaired by auto-immune processes or genetic disease, or ablated by invasive oncological/surgical procedures. Currently, the best example of therapeutic deployment of adult stem cells is certainly the use of hematopoietic stem cells for bone marrow transplantation. In the past few years, several groups including that of Maurilio Sampaolesi have reported a successful use of other types of adult stem cells for the therapy of certain diseases, such as myocardial necrosis and muscular dystrophy. Bone marrow-derived stem cells and mesoangioblasts are incorporated into regenerating skeletal muscle fibers when transplanted into dystrophic mice (Pelacho *et al.,* 2007; Cossu and Sampaolesi, 2007; Torrente *et al.,* 2007) and dogs (Sampaolesi *et al.,* 2006).

The recent identification of different types of multi-potent stem cells, some of which are suitable for protocols of clinical cell therapy, has disclosed new perspectives in the treatment of genetic diseases, including muscular dystrophy. Sampaolesi showed that a recently identified population of stem cells – the

mesoangioblasts (Tonlorenzi *et al.,* 2007) – can confer functional improvement upon intra-arterial injection in a mouse model of muscular dystrophy (Sampaolesi *et al.,* 2003). In particular, the delivery of mesoangioblasts ameliorates function *in vivo* and determines a recovery of force of contraction of skinned dystrophic muscle fibres *in vitro*. Moreover, by detecting dystrophin in the same individual fibres used for force determination, it was possible to show that muscle fibres which recover dystrophin expression also recover force, whereas muscle fibres which do not recover dystrophin expression do not recover force. The latter finding has established the clearest link so far between the recovery of expression of a missing protein and functional recovery in dystrophic animals. More recently, transplantation of wild-type canine mesoangioblasts gave promising results in the Golden Retriever dystrophic dog (Sampaolesi *et al.,* 2006, 2007), the most reliable animal model that shows a form of muscular dystrophy very similar to and even more severe lesions than Duchenne muscular dystrophy. After the mouse and dog, mesoangioblasts have been identified in humans and shown to reconstitute dystrophin-expressing fibers in mdx/SCID mice (Dellavalle *et al.,* 2007). Based on these results, Dellavalle and colleagues proposed a pilot clinical trial based on intra-arterial transplantation of normal mesoangioblasts under a regime of immune suppression. Efficacy and possible adverse effects are up for evaluation to assess whether this approach may represent a first step towards an efficacious cell therapy for muscular dystrophy (Cossu and Sampaolesi, 2007).

Although stem cells could be a valuable therapeutic tool, they may also be the target of certain pathogenic insults. Perhaps the best proof of this concept has come over the past 5-10 years from the identification of cancer-initiating cells dubbed 'cancer stem cells' (CSC). Since the times of Virchow (*"omnia cellula e cellula"* that is, all cells come from other cells), the cancer research community has been looking for the cellular origin of human cancers, but it is only now that a possible answer is taking shape. Knowing which cells in a tumour-mass give rise to it and sustain its growth is clearly fundamental, since only the eradication of those cells can result in complete remission. In line with recent reports from other groups (reviewed in Dirks, 2006), Ruggero De Maria and colleagues have demonstrated that a very small subpopulation of human CD133+ (a.k.a. prominin in mice) cells within colorectal adenocarcinomas and all the major forms of lung tumours behave as cancer-initiating cells (Ricci-Vitiani *et al.,* 2007; Eramo *et al.,* 2008). These cells proliferate in culture in an undifferentiated state, sustain tumour growth after xeno-transplantation into NOD/SCID mice, and give rise in mice to cancers phenotypically indistinguishable from the original human tumours. Interestingly, De Maria showed that cancer-initiating cells in glioblastoma, colorectal and lung tumours are much more resistant than their more differentiated progeny to the conventional chemotherapeutic drugs currently used in the clinical setting (Eramo *et al.,* 2006; Eramo *et al.,* 2008; De Maria, unpublished observations). The chemoresistance of the tumorigenic cell populations may explain why in many instances cancers progress during therapy or relapse afterwards, and emphasizes the need of CSC-targeted drugs for the cure of human cancers. The mechanisms responsible for such chemoresistance remain to be elucidated. Normal and cancer stem cells often express ABC transporters that confer drug efflux capacity, easily measured by

the flow-cytometric detection of the so-called 'side population' in assays evaluating the efflux of fluorescent dyes such as Hoechst 33342. However, De Maria demonstrated that, at least in the case of glioblastoma, CSCs can be resistant to chemotherapeutic drugs regardless of the activity of ABC transporters (Eramo *et al.,* 2006). It is likely that the comparative characterization of the survival pathways active in normal and cancer stem cells will be a considerable contribution to devise novel and effective cancer therapies.

Conclusions and perspectives

Stem cells represent probably the most rapidly evolving field of research, bridging biology and medicine and reaching out to society. This research topic has also raised harsh philosophical, religious and political disputes. Until just a few years ago, cloning was praised as a source for ES cells under the 'therapeutic cloning paradigm'. Now we hardly hear of it, although this method is far from dead (Cibelli, 2007). On the one hand, animal cloning raises deep concerns that one day we may be able to clone people (however, we ought to keep in mind that cloning, after all, is only one of many reproductive technologies that have been developed in the past few decades, and all of them - artificial insemination, embryo transfer, IVF - were unsafe and condemned at first but are now much safer and are a big business). On the other hand, stem cells are depicted by media as the 'holy grail' for the therapy of certain incurable diseases, such as diabetes or neurodegenerative disorders. Thus, it is important to assess where we actually stand and how the stem cell field is likely to evolve.

Human ESCs are probably the best example of a tool endowed with potential therapeutic benefit, nevertheless raising intricate ethical issues. hESCs are in principle able to generate all the tissues found in the adult body, but not to sustain TE formation, unlike the zygote does. If ES cells were named totipotent, then their status could be equated with that of the zygote, leading to restriction or prohibition of their use in several countries. For this reason, hESC have so far been considered pluripotent, thereby escaping regulations aimed to protect the totipotent zygote. In fact, hESCs can form trophectoderm-like cells spontaneously or by exposure to BMP4 (Thomson *et al.,* 1998; Gerami-Naini *et al.,* 2004; Xu *et al.,* 2002) and the mouse ICM precursor of ES cells can regenerate trophectoderm (Pierce *et al.,* 1988). The recent finding that mouse epiblast stem cells (Brons *et al.,* 2007; Tesar *et al.,* 2007) are closer than mouse ES cells to hESCs as measured by the gene expression profile, argues that hESCs might, in fact, be a later stage than previously thought. If so, whether human ES cells are pluri- or totipotent remains to be determined, and the possibility that hESCs are totipotent may not be ruled out. After all, not even the totipotent zygote is actually totipotent on its own, as it needs a genital tract for recipient. Likewise, ES cells need an embryo for recipient.

The source of hESC is another delicate issue, since their generation requires the deployment of human embryos. Currently iPS cells are the most attractive and popular surrogate of hESC, while their generation does not require the consumption of embryos. The iPS cells resemble ES cells in many aspects and have been proposed to be pluripotent. However, one should be wary that such a property is a function of *in vitro* culture conditions (which still need to be optimized for iPS). Another pitfall associ-

ated with iPS is that so far they have only been generated by retroviral transduction of selected genes. Even if the proto-oncogene *c-Myc* is dispensable (Yu *et al.,* 2007), such a procedure cannot be considered safe in light of potential clinical applications, since retroviral-mediated genome integration of the transduced cDNAs can cause insertional mutagenesis and impair, for instance, some onco-suppressor genes. Therefore, alternative means to convert adult somatic cells into iPS without the risk of mutagenesis need to be implemented. Transient transfection of selected cDNAs or micro-injection of certain recombinant proteins could be used to achieve somatic nuclei reprogramming. By contrast, the news that proteins could be introduced via carbon nanotubes into cells and turn them into iPS cells was received with skepticism (Cyranoski and Baker, 2008). Inter-species nuclear transfer would be another possibility. As discussed at this meeting, such a procedure would spare human oocytes and embryos while delivering pluripotent, HLA-matched ES cells usable for regenerative medicine. At present, however, inter-species nuclear transfer is a research tool and, because it involves human donor nuclei, it is still very ethically controversial and banned in most countries.

If pluripotency has to be imposed on somatic cells by artificial means, we should first have a clear knowledge of the rules by which pluripotency is established and maintained during physiological development. Namely, what are the molecular mechanisms that govern toti- and pluripotency in mammalian cells, and is there any potential application of such mechanisms to the purposes of regenerative medicine? Answers to these compelling questions may come, not surprisingly, from the pre-eminent biomedical model system: the mouse. For example, Magdalena Zernicka-Goetz showed that certain epigenetic marks, such as histone H3 methylation at Arginine 26 (H3R26Met), can have profound effects on the fate of single blastomeres in mouse embryos (Torres-Padilla *et al.,* 2007). This may not be an exclusive prerogative of early embryonic cells; for instance, the H3K27 histone de-methylase Jmjd3 was shown in this meeting to enable/ evoke identity changes in mouse macrophages (De Santa *et al.,* 2007) and differentiating ES cells. Thus, once we gain a detailed picture of the epigenetic landscape of toti- and pluripotent cells, we may be able to impose pluripotency on adult somatic nuclei in a more efficient and safer way than we are currently doing (while producing iPS cells, for instance).

The last issue addressed at this meeting was the emerging role of stem cells as the target of certain pathogenic insults. CSCs or cancer-initiating cells are probably the best example of stem cell-like entities essential for the maintenance of a given lesion. The existence of CSCs has been proven in hematopoietic neoplasms (Lapidot *et al.,* 1994; Bonnet and Dick, 1997) and in certain solid tumours, including glioblastoma (Singh *et al.,* 2003), breast adenocarcinomas (Al Hajj *et al.,* 2003), head-and-neck squamous carcinomas (Prince *et al.,* 2007), and colon (Ricci-Vitiani *et al.,* 2007), lung (Eramo *et al.,* 2008) and pancreatic adenocarcinomas (Li *et al.,* 2007). Cancer-initiating cells are predicted to be a common theme to most, if not all, solid neoplasms and their identification and characterization is probably only a matter of short time. In line with this prediction, during this symposium we learned that CSCs also appear to exist in thyroid cancers (De Maria, unpublished observations).

CSCs usually represent a tiny fraction of the overall tumour

burden, but are the only subpopulation capable of recapitulating the phenotypic features of the tumour from which they are derived when serially transplanted into NOD/SCID mice. CSCs have been found to express several markers of stemness, such as the surface antigen CD133, while the expression of pluripotency transcription factors in CSCs still remains as questionable as it has always been (Cantz *et al.,* 2008). Therefore, the true potency of CSCs remains unclear. A major question is whether CSCs are derived from rare, adult somatic stem cells hit by oncogenic insults or whether they are instead the product of an aberrant de-differentiation of somatic progenitors. In the case of glioblastoma, De Maria has shown that about 60% of the patients bear tumours formed by multipotent CSCs able to generate both neural and mesenchymal cells. Interestingly, this observation came from the analysis of subcutaneous glioma stem cell xenografts, which in some instances (25% of the patients) gave rise to mixed tumors comprising areas of chondrocytic and osteocytic tissue (Ricci-Vitiani *et al.,* 2008). These new data are in line with previous findings on melanoma stem cells, which have shown plasticity towards multiple lineages (Fang *et al.,* 2005). Although it is theoretically possible that such multipotency is acquired following a genetic reprogramming of a more differentiated cell during the tumorigenic process, the multipotent frequency in glioma and melanoma stem cells suggest that the tumorigenic hit may occur in a normal stem cell. Whatever the answer, it is clear that if the growth of human cancers is supported by a small population of cancer-initiating cells, then any therapy should aim at killing this population in order to be curative. Unfortunately, this conclusion is a double-edged sword, because, as highlighted during this meeting, CSCs tend to be more resistant to conventional chemo-therapeutic agents and to radiation, as compared to their normal counterparts (De Maria, unpublished data). Therefore, we envisage that two main routes should be followed in developing novel anti-cancer therapeutics: CSC-specific targets need to be discovered and, in parallel, knowledge has to be gained on the mechanisms exploited by CSCs to resist anti-neoplastic drugs. Only in this way it is likely that more selective and effective therapies will become available and hopefully cure malignancies so far doomed for a poor prognosis.

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Note: The meeting was open to the general public with no admission fee being charged. More than 200 people attended.

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