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Transdifferentiation of Stem Cells: A Critical View

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Abstract Recently a large amount of new data on the plasticity of stem cells of various lineages have emerged, providing new perspectives especially for the therapeutic application of adult stem cells. Previously unknown possibilities of cell differentiation beyond the known commitment of a given stem cell have been described using keywords such as "blood to liver," or "bone to brain." Controversies on the likelihood, as well as the biological significance, of these conversions almost immediately arose within this young field of stem cell biology. This chapter will concentrate on these controversies and focus on selected examples demonstrating the technical aspects of stem cell transdifferentiation and the evaluation of the tools used to analyze these events.

Keywords Adult stem cells, Transdifferentiation

Contents

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Abbreviations

BM	Bone marrow
BMC	Bone marrow cells
CAC	Circulating angiogenic cells
eGFP	Enhanced green fluorescent protein
EPC	Endothelial progenitor cells
FISH	Fluorescence in situ hybridization
GFP	Green fluorescent protein
HNF	Hepatocyte nuclear factor
HSC	Hematopoietic stem cells
HUVEC	Human umbilical cord vein cells
MAPC	Multipotent adult progenitor cells
MHC	Myosin heavy chain
MNC	Mononuclear cells
MSC	Mesenchymal stem cells
NOD-SCID	Nonobese diabetic severe combined immunodeficient
NRCM	Neonatal rat cardiomyocytes
USSC	Unrestricted somatic stem cells

1 Introduction

During the past decade, stem cell research has become a rapidly evolving field providing new insights into developmental biology, as well as new hope for therapeutic applications. The most versatile stem cells to date are pluripotent embryonic stem cells (ESC) with the capability of differentiating into the whole panel of somatic cell types derived from all three germ layers, i.e., endoderm, mesoderm and ectoderm. Some cell types which can be generated from adult tissue have now been described to have similar characteristics; these cells include the so called “induced pluripotent” stem (iPS) cells [1, 2] or germ-line derived stem cells [3]. Notably, it is not clear at present whether the adult testis contains rare pluripotent stem cells in vivo. It is considered more likely that isolated unipotent spermatogonial stem cells can be reprogrammed into pluripotent stem cells under certain culture conditions. In contrast to ESC, the natural potential of stem and progenitor cells found in various organs of the adult body appears to be limited and was initially considered restricted to cells related to the respective organs, or at least derived from the same germ layer. This concept was challenged by reports on the plasticity of stem cells of various lineages going beyond these boundaries, an event which is often referred to as “transdifferentiation.”

However, a critical view on the "transdifferentiation of stem cells" should start with a critical view on the term itself. The observation that one cell type can change its phenotype and become another cell type in vivo was described in 1922 by Maccarty et al. to occur in ovarian tumors [4]. This phenomenon was termed "metaplasia" and believed to be mainly a response to physiological or pathological stress.

A classical example of metaplasia is the epithelial–mesenchymal transition (EMT), a highly conserved and fundamental process, mediated by transforming growth factor β (TGF-β) signaling, that governs morphogenesis in embryonic development and may also contribute to cancer metastasis [5]. The most prominent feature of EMT is the complete loss of epithelial traits, such as E-cadherin expression, by the former epithelial cells and the acquisition of mesenchymal characteristics, such as vimentin and fibronectin expression, gaining invasive motility and others [6, 7].

In the adult organism, examples of metaplasia can be found in the eye, with reports dating back as early as 1934 [8]. More recent reports include the conversion of limbal basal epithelial cells into corneal epithelial cells [9], retinal pigmental epithelial (RPE) cells into neural epithelium [10], conjunctival epithelial cells into corneal epithelium [11] and neural retina into lens epithelium [12]. Another form of metaplasia in the eye, the conversion of lens epithelial cells into myofibroblasts [13], reflects a common mechanism of the body in response to injury, i.e., the replacement of functional tissue specific cells by myofibroblasts, e.g., in scar formation. This process is mediated by increased levels of tumor necrosis factor α (TNF-α) and/or TGF-β, and has been described for a large variety of cell types including, but not limited to, fat storing cells in the liver [14], tubular epithelial cells in the kidney [15], keratocytes in the skin [16], fibroblasts in the lung [17], the heart [18], and the prostate [19], as well as Schwann cells in the brain [20].

"Transdifferentiation is a subclass of metaplasia and by definition an irreversible switch of one already differentiated cell to another, resulting in the loss of one phenotype and the gain of another" [21].

Like other sources, this statement by Eberhard and Tosh explicitly defines "transdifferentiation" as a "nonstem cell" transformation. Therefore, under a critical view, the expression "transdifferentiation of adult stem cells" seems to be contradictory in itself. However, in recent years this classical definition has been broadened when it became evident that adult stem cells with a presumed commitment not only underwent differentiation into anticipated progenies, but differentiation also resulted in phenotypes beyond the expected lineage of the respective stem cells. This "plasticity," which has been defined as the ability to undergo transdifferentiation, can be seen, for example, in the differentiation of hematopoietic stem cells into nonblood cells. Subsequently, we will use this broadened definition to investigate the alleged transdifferentiation of stem cells into, or from various tissues, reviewing conflicting reports in this relatively new field of stem cell research with a focus on technical aspects of the given data, the methods used, and their power to prove differentiation events unequivocally.

2 Mechanism of Stem Cell Transdifferentiation

Stem cells were thought to differentiate usually into one or more typical cell types of the very tissue from which the respective stem cell originated. In addition to this lineage-restricted multipotentiality, stem cells, under certain circumstances seem to be able to cross lineage boundaries and differentiate into atypical cell types, or, as Rota et al. expressed it, to "break the law of tissue fidelity" [22]. Theoretically, this transdifferentiation can occur directly, or via the generation of an intermediate cell type. In this case, a *de*-differentiation of the stem cell would be followed by a subsequent differentiation into another cell type [23] (Fig. 1).

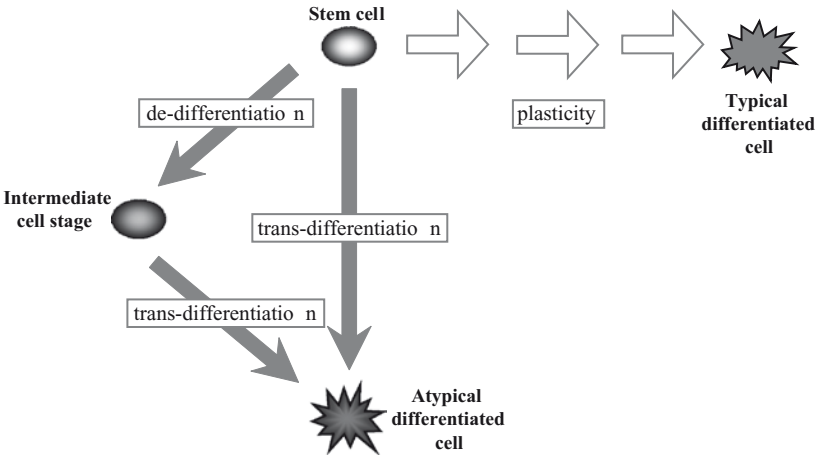

Fig. 1 Mechanism of stem cell transdifferentiation. Modified after Koestenbauer et al. [23]

Early reports on this previously unobserved form of differentiation were fairly surprising: "… But just because we scientists were surprised, it does not mean that the cells themselves were surprised by their broad potential! …" Eisenberg and Eisenberg [24].

3 Examples of Adult Stem Cell Transdifferentiation

3.1 Transdifferentiation into Hepatocytes

3.1.1 Hematopoietic Stem Cells

In vivo, liver progenitor/oval cells differentiate into hepatocytes and biliary epithelial cells, repopulating the liver when the regenerative capacity of hepatocytes is impaired. Bone marrow (BM) derived hematopoietic stem cells (HSC), which, apart from their putative main function in the body, i.e., replenishing blood cells,

have also been associated with organ repair. Transdifferentiation has been proposed as one underlying mechanism. After Petersen et al. identified BM as source of hepatic cells in 1999 [25], Lagasse et al. confirmed a therapeutic effect of HSC transplantation in mice with an inherited liver disease [26] and Theise et al. demonstrated similar effects in humans [27]. This idea was then challenged by contradictory reports by Wagers et al. [28], Dahlke et al. [29] and others [30, 31], introducing cell fusion as an alternative mechanism to transdifferentiation. Closer investigation of the methods used to analyze stem cell transdifferentiation in these respective studies provides insight into some of the contradicting results.

Petersen et al. recognized the bone marrow as a potential source of hepatic oval cells using cross-sex or cross-strain BM and whole liver transplantation in rats to trace the origin of the repopulating liver cells [25]. Following liver injury a proportion of the regenerated hepatic cells were shown to be donor-derived as identified by markers for Y-chromosome, dipeptidyl peptidase IV (DPP IV) enzyme, and L21-6 antigen. Immunohistochemical staining of hepatocyte-specific cytokeratins and fluorescence in situ hybridization (FISH) for X- and Y-chromosomes identified hepatocyte engraftment. This was observed both in human females receiving male BM transplants and in male recipients of orthotopic female liver transplants [27]. In this study, peak values were observed in one of the liver transplant recipients with recurrent hepatitis C. Therefore, this setting resembled an injury approach in an animal model like that of Lagasse et al. who demonstrated that mice with an inherited liver disease (corresponding to human tyrosinaemia type 1) could be cured by HSC transplantation leading to the reconstitution of functioning mature hepatocytes [26].

In follow-up studies, more sophisticated methods have been used to investigate the controversial fate of hematopoietic stem cells in the liver. Using chimeric animals, as well as green fluorescent protein (GFP)-positive:GFP-negative parabiotic mice, Wagers et al. showed that single HSC robustly reconstituted the BM, as well as peripheral blood leukocytes in these animals, but did not contribute appreciably to nonhematopoietic tissues, including brain, kidney, gut, liver, and muscle. It was concluded that transdifferentiation of circulating HSC and/or their progeny is an extremely rare event, if it occurs at all [28]. Wang et al. performed serial transplantation of BM-derived hepatocytes [31]. Southern blot analysis and cytogenetic analysis of hepatocytes transplanted from female donor mice into male recipients provided evidence of fusion between donor and host cells rather than liver-specific (trans-) differentiation of hematopoietic stem cells. Reviewing the role of various stem cell populations, including hematopoietic stem cells in liver regeneration, Dahlke et al. claimed that closer scrutiny of the data published by Lagasse et al. [26] also reveals that cell fusion rather than transdifferentiation appears to be responsible for liver regeneration in their model [29].

Further studies investigating whether BM-derived liver progenitor/oval cells can repopulate the liver were unable to confirm the early data by Theise et al. and Lagasse et al. One possible explanation for this discrepancy might be the time point of analysis. Menthena et al. transplanted lethally irradiated female DPP IV-negative mutant F344 rats with wild-type male F344 BM cells [30]. Initially, donor-derived cells were detected in all liver sections of recipient rats after the application of

different liver injury protocols. However, most of the donor-derived clusters disappeared over time and very few oval cells (less than 1%) and none of the small hepatocytic clusters showed double labeling for the donor-derived DPP IV and hepatocyte markers. Consequently, the authors conclude that the sources of oval cells and small hepatocytes in the injured liver are endogenous liver progenitors which do not arise through transdifferentiation from BM cells.

In a comprehensive review of the available data, Thorgeirsson et al. suggested that one or more types of hematopoietic cells may rarely acquire the hepatocyte phenotype in the liver (frequency ~10^{-4}). However, the nature of the hematopoietic cells involved and the mechanisms responsible for acquisition of a hepatocyte phenotype are still controversial. HSC do not appear to be direct precursors of hepatocytes; instead hepatocytes that carry a BM tag can be generated by fusion of hepatocytes with cells of the macrophage–monocyte lineage [32], which have been reported to be highly fusogenic [33]. Thorgeirsson et al. concluded that hematopoietic cells contribute little to hepatocyte formation under either physiological or pathological conditions, but may provide cytokines and growth factors that promote hepatocyte functions by paracrine mechanisms.

Thus, an important question was raised by Thorgeirsson et al., that is which specific type of hematopoietic stem cell may be able to support liver regeneration. Meanwhile, different subsets of HSC were analyzed with respect to their hepatic differentiation capacity [34–36], again yielding contradictory findings. The discussion around stem cell identity and definition of a pure population involves another issue, namely the request for the use of single cells as the ultimate test for multipotentiality of a given stem cell. In 2001 Krause et al. demonstrated multiorgan, multilineage engraftment by a single BM-derived stem cell using an elegant model of serial stem cell transplantation in mice [37]. Injection of single, selected BM stem cells generated a variable proportion of epithelial cells in various organs such as the lung, gastrointestinal tract, skin, and liver. Notably in the liver, only BM-derived cholangiocytes were detected, and no bone marrow derived hepatocytes [37].

According to Krause et al., the different engraftment frequencies in different organs observed in their study may be due to (1) the degree of tissue damage induced by the transplant, (2) the residual tissue-specific stem cell capacity within the organ, and/or (3) the normal rate of cell turnover in each organ [37]. These factors, however, might also explain some of the differences in the outcome of other studies, i.e., the formation of BM-derived liver cells in the presence of tissue injury [25, 26] while no or low numbers of such cells were detected in the absence of injury [27].

Recently, another interesting explanation of apparent transdifferentiation events in the liver was proposed. In a comparative study, Brulport et al. transplanted four different types of human extrahepatic precursor cells (cord blood derived, monocytes, BM, and pancreatic) into the livers of NOD/SCID mice. Initial results argued in favor of hepatic differentiation of the transplanted cells as they stained positive for human albumin and glycogen, given that the cells were negative for both markers before transplantation. However, cells with human nuclei (detected by in situ hybridization with human DNA-specific alu probes) did not show a hepatocyte-like morphology. In addition, they did not express cytochrome P450 3A4, a key marker

of functional hepatocytes, suggesting that the engrafted human cells represented a mixed cell type potentially resulting from partial transdifferentiation. Surprisingly, a human albumin-positive cell type with hepatocyte-like morphology was found to contain a mouse, but not a human nucleus, therefore challenging the existence of human cell transdifferentiation. Although unproven, Brulport et al. suggest horizontal gene transfer as a likely mechanism, especially because small fragments of human nuclei were observed in mouse cells that originated from deteriorating transplanted cells. In conclusion, Brulport et al. provided evidence not favoring transdifferentiation, but rather suggesting a complex situation including partial differentiation of cord blood-derived donor cells and possibly horizontal gene transfer.

3.1.2 Mesenchymal Stem Cells

In addition to hematopoietic stem cells, the BM contains mesenchymal stem cells (MSC), another type of stem cell extensively studied for organ regeneration. MSC are typically enriched via isolation of the plastic adherent, fibroblast-like cell fraction. Despite their functional heterogeneity, MSC populations obtained from various tissues commonly express a number of surface receptors including CD29, CD44, CD49a–f, CD51, CD73, CD105, CD106, CD166, and Stro1 and lack expression of definitive hematopoietic lineage markers including CD11b, CD14, and CD45 [38]. Mesenchymal stem cells were also detected in the peripheral blood, most likely mobilized from the BM [39].

While their differentiation into adipocytes, chondrocytes and osteocytes as described by Prockop et al. [40] has become the gold standard for proving MSC differentiation capacity, reports on MSC (trans-)differentiation into other lineages such as hepatocytes are highly controversial. Contribution of MSC to the liver has been described in baboons by Devine et al. who infused MSC retrovirally tagged with enhanced GFP (eGFP) in adult animals following lethal total body irradiation [41]. The resulting data, 9–21 months later, suggested that MSC could contribute to the liver and possess the capacity to proliferate in a hepatic environment. In vitro differentiation into hepatocyte-like phenotypes has also been described for MSC derived from several species including mice [42], rats [43] and humans [44].

One problem concerning reports on the potential contribution of BM-derived cells to liver regeneration, is the lack of a comparable definition of the cell type used. Most of the early studies investigated whole BM preparations, while others defined certain subpopulations, such as recycling stem (RS-) cells [45, 46] or “human bone-marrow derived multipotent stem cells” (hBMSC) [47]. For example, Verfaillie’s laboratory was able to demonstrate that postnatal BM-derived multipotent adult progenitor cells (MAPC) can differentiate into hepatocyte-like cells in vitro [48]. While MAPC copurify from the BM with MSC, they are considered a distinct population with a different phenotype. Human and rodent MAPC represent a CD44-negative, CD45-negative, HLA class I- and II-negative, as well as a cKit-negative subset of cells. When cultured on Matrigel with FGF-4 and HGF, they differentiated into epithelioid cells that expressed hepatocyte nuclear factor 3β

(HNF-3β), GATA4, cytokeratin 19 (CK19), transthyretin, and α-fetoprotein by day 7, and expressed CK18, HNF-4, and HNF-1α on days 14–28 [48]. Another in vitro study by Khurana et al. characterized the potential subpopulation of BM cells (BMC) involved in the repair of injured liver tissue to be a distinct subset of lineage (Lin)-negative BMC coexpressing CXCR4 and oncostatin M receptor β (OSMRβ), with/without stem cell antigen-1 (sca-1) [49].

Another problem became evident by the identification of cell fusion as the underlying mechanism for some of the earlier observations on MSC transdifferentiation, very similar to what has been outlined above for hematopoietic stem cells. Alvarez-Diego et al. described the cell fusion between MSC and resident liver cells detected by means of sophisticated genetic labeling [50]. For this study, mice expressing Cre recombinase ubiquitously under the control of a hybrid cytomegalovirus (CMV) enhancer β-actin promoter were used, and the conditional Cre reporter mouse line R26R. In this line, the LacZ reporter is exclusively expressed after the excision of a loxP-flanked (floxed) stop cassette by Cre mediated recombination, resulting in expression of the LacZ in fused cells.

Nevertheless, cell fusion not only accounts for misleading data on stem cell transdifferentiation, but can also have a therapeutic effect. Vassilopoulos et al. reported that transplanted BM regenerates liver by cell fusion in a model of tyrosinaemia type I [51]. Transplanted mice regained normal liver function and formed regenerating liver nodules with normal histology. Their hepatocytes expressed both donor and host genes, consistent with polyploid genome formation by fusion of host and donor cells.

Partial transdifferentiation was also observed, resulting in a chimeric phenotype with the expression of several lineage markers, but missing other markers fundamental to a bona fide functional cell type of a particular tissue. Lysy et al. demonstrated the persistence of a chimerical phenotype after hepatocyte differentiation of human BM-derived MSC, with the MSC partially preserving their mesenchymal phenotype [52]. Only after transplantation of MSC-derived hepatocyte-like cells into the liver of SCID mice did these cells lose their chimeric phenotype, but they conserved their hepatocyte-lineage markers, indicating that a hepatic environment in vivo is necessary for full maturation into functional hepatocytes.

To date, there is still not a common understanding of the processes occurring after transplantation of mesenchymal stem cells into the hepatic environment; thus further research will be needed to clarify the mechanism, in addition to the biological significance of MSC contribution to the liver.

3.2 Transdifferentiation into Myocytes

In contrast to studies on the hepatic differentiation of HSC, mostly investigating liver repopulation by circulating cells in vivo, studies on the conversion of HSC into different muscle cell types largely focused on stem cell transplantation via transmural injection directly into skeletal or heart muscle tissue in vivo.

In 1998, Ferrari et al. reported that BM cells can contribute to myogenesis in response to physiological stimuli [53]. However, according to Ferrari et al., the origin of the BM-derived myogenic cells, as well as their physiological role in the homeostasis of muscle tissue, could not be defined. Further studies concentrated on the identification of the myogenic cell type within the BM. In 2003, using a lineage tracing strategy, Corbel et al. showed that the progeny of a single HSC can both reconstitute the hematopoietic system and contribute to muscle regeneration [54]. Other reports identified immature myeloid cells as the predominant source of myogenic differentiation in vivo. Doyonnas et al. used fluorescence-activated cell sorter (FACS)-based protocols to test distinct hematopoietic fractions and showed that only fractions containing c-kit-positive immature myelomonocytic precursors were capable of contributing to muscle fibers after intramuscular injection [55]. In a similar approach, Abedi et al. transplanted animals with different populations of BMC from GFP transgenic mice, and the presence of GFP-positive muscle fibers were evaluated in cardiotoxin-injured *tibialis anterior* muscles [56]. GFP-positive muscle fibers were found mostly in animals that received either CD45-negative, Lin-negative, c-Kit-positive, Sca-1-positive or Flk-2-positive populations of BMC, suggesting that HSC rather than mesenchymal cells or more differentiated hematopoietic cells are responsible for the formation of GFP-positive muscle fibers. According to Adebi et al. and in contrast to Doyonnas et al., a CD11b-positive population of BMC was also associated with the emergence of GFP-positive skeletal muscle fibers.

While the contribution of HSC to skeletal muscle regeneration was confirmed by several groups, the exact phenotype and developmental stage of contributing cells, as well as the exact mechanism remains to be elucidated. Particularly, the question as to what extent cell fusion might play a role in this setting has not been answered. In contrast, the probability of adult stem cell contribution to cardiac muscle is still the subject of an ongoing debate.

3.2.1 Whole Bone Marrow and Hematopoietic Stem Cells

Initial reports on the possibility of BM-derived stem cells to regenerate cardiac myocytes after myocardial infarction in vivo were published by the group of Piero Anversa [57, 58] and others [59–61] while Eisenberg et al. proposed cardiac differentiation in vitro [62].

In 2001, Orlic et al. investigated whether ischemia damaged myocardium could be restored by transplanting BMC into infarcted mice [58]. Shortly after coronary ligation, Lin-negative/c-kit-positive cells were injected in the heart muscle wall bordering the infarct. This study claimed that donor cell-derived, newly formed myocardium occupied 68% of the infarcted portion of the ventricle 9 days after transplantation. In a similar study from the same group, a sex-mismatched mouse model with male eGFP-positive donor animals demonstrated that the engrafted cells were positive for eGFP, Y chromosome, and several myocyte-specific proteins including cardiac myosin and the transcription factors GATA-4, MEF2, and

Csx/Nkx2.5 [57]. The authors concluded that locally delivered BMC can generate de novo myocardium, ameliorating the outcome of coronary artery disease by improving several hemodynamic parameters [57, 58].

Coculture experiments with adult mouse BM cells and embryonic heart tissue seemed to confirm that hematopoietic progenitor cells are able both to integrate into cardiac tissue and to differentiate into cardiomyocytes [62]. Remarkably, Eisenberg et al. reported that macrophages cocultured with cardiac explants were also able to integrate into contractile heart tissue and undergo cardiac differentiation. Another cell population from the BM, the so-called Side Population (SP) cells, or highly purified CD34-negative/c-kit-positive/sca-1-positive cells, have also been reported to differentiate into cardiac lineages and improve cardiac function after transplantation into infarcted myocardium [60, 61]. According to Agbulut et al. BM-derived cells that can contribute to cardiac differentiation are present in total unpurified BM, but not in the sca-1-positive hematopoietic progenitor cell population [59]. However, the very small number of transdifferentiated cells (5.6 ± 2.3 cells per 3×10^{-2} mm^3 of mouse heart tissue at 7 days after transplantation of 6×10^6 cells) raised concern regarding their functional efficiency.

These early reports on transdifferentiation were challenged by contradictory data. Nygren et al. reported that BM-derived hematopoietic cells generate cardiomyocytes at a low frequency through cell fusion, but not transdifferentiation [63]. While they were able to confirm earlier reports on efficient engrafting of unfractionated BMC and a purified population of hematopoietic stem and progenitor cells to the injured myocardium, they also found this engraftment to be transient. In addition, all engrafted cells expressed the pan-hematopoietic marker CD45, coexpressed myeloid blood lineage markers (Gr-1/Mac-1) failed to express cardiac-specific markers. In contrast, BM-derived cardiomyocytes were observed outside the infarcted myocardium at a low frequency and were derived exclusively through cell fusion.

These results are in line with the observations of Murry et al., who used both cardiomyocyte-restricted and ubiquitously expressed reporter transgenes to track the fate of HSC transplants into normal and injured adult mouse hearts [64]. Their results indicated that HSC do not readily acquire a cardiac phenotype raising a cautionary note for clinical studies of infarct repair. The notion that hematopoietic cells may engraft to the myocardium without transdifferentiation into cardiomyocytes was further corroborated by Balsam et al. by showing that HSC adopt mature hematopoietic fates in ischemic myocardium [65]. Cells were isolated from transgenic mice constitutively expressing GFP driven by the chicken β-actin promoter and injected directly into ischemic myocardium of wild-type mice. Abundant GFP-positive cells were detected in the myocardium after 10 days, but by 30 days few cells were detectable. These GFP-positive cells did not express cardiac tissue-specific markers; rather, most of the donor cells expressed the hematopoietic marker CD45 and myeloid marker Gr-1, suggesting that even in the microenvironment of the injured heart, HSC adopt only hematopoietic fates. In contrast to widely publicized reports of HSC plasticity, Weissman et al. failed to reproduce transdifferentiation of HSC to lineages comprising skeletal muscle, heart, brain or gut.

They concluded that rare cell fusion events and incomplete purifications of HSC contaminated with tissue-committed stem cells were likely explanations for the other published results [66].

In contrast to the negative findings concerning the transdifferentiation capacity of HSC into cardiomyocytes, the Anversa group published further data in favor of this phenomenon. In 2005, Kajstura et al. reported that BMC differentiated into cardiac cell lineages after infarction, independent of cell fusion [67]. In this publication, using the same mouse model as described in the reports of Orlic et al., transdifferentiation into cardiac myocytes was demonstrated by immunohistology followed by morphological measurements of infarcted and regenerated areas in addition to Y-chromosome FISH analysis. Finding no evidence of angiogenesis or myocyte proliferation in remote parts of the heart, the authors excluded a paracrine effect of injected BMC in myocardial recovery. Kajstura et al. attribute the obvious discrepancy between their findings and others to (1) technical differences in experimental protocols, (2) identity of the applied donor cell(s), and (3) details in tissue preparation and immunocytochemical analysis of the myocardium. However, Kajstura et al. did not provide data on long-term engraftment beyond 10 days. In addition, the fact that Kajstura et al. did not observe angiogenesis or proliferation after cell transplantation, does not unequivocally exclude paracrine effects, e.g., on cardiomyocyte survival.

How animated the controversy on the subject has become by now can be estimated from the following statement of Kajstura et al.: “The assumption made by Balsam et al. [65] and Murry et al. [64] that the technical approach that they have used in the identification and measurement of myocardial structures is superior to that used in our laboratory does not reflect any scientific reality but the emotional disbelief that bone marrow cells can adopt myocardial cell lineages and repair the injured heart.”[67]

Nevertheless, it should be noted that some of the criticism concerning methods and conclusions described by the Anversa group might be justified. For example, Kajstura et al. report the difficulty of cell transplantation into the infarcted myocardium with a 50% probability of correct injection. To control for this, rhodamine particles were added to the cell suspension used for transplantation. It was stated that “the unsuccessfully injected mice (no rhodamine particles) were considered the most appropriate control animals for the successfully treated mice” [67]. This practice obviously neglects general (nonspecific) effects of cell transplantation into the myocardium, in particular local inflammatory processes that can be expected after the usually injection-related death of transplanted cells. In addition, the improvement in heart function after stem cell transplantation reported by Orlic et al. [57] leaves room for discussion as acquisition of functional data in small animals is extremely difficult and should be interpreted with caution.

The accuracy of Y-chromosome FISH analysis may be another issue. Kajstura et al. reported that this method underestimated the frequency of positive cells by nearly 50%. Other studies reported visualization of 62% of nuclei in a male mouse due to partial nuclear sampling as the plane of each section does not always cut through the Y-chromosome [37]. Thus, FISH data can show significant variations.

On the other hand, it has been argued that at least in the human system, data is available which demonstrate the existence of male cells in a female's heart, totally unrelated to any cell transplantation, which in turn might lead to false-positive results. This phenomenon is attributable to the persistence of fetal cell microchimerism following the birth of male children, a fact that should be considered when using sex-mismatched transplantation models [68, 69].

Thus, the phenomenon of cardiac transdifferentiation of HSC is still controversially discussed and should be addressed diligently and with an open mind in the future.

3.2.2 Mesenchymal Stem Cells

Most of the early reports on cardiac differentiation of MSC focused on the effect of 5′-azacytidine on MSC marker expression in vitro [70–72] and on the outcome of subsequent MSC transplantation into the infarcted myocardium [73–75] with contradictory results. Although some studies claimed improvement of heart function after stem cell transplantation [75, 76], different explanations have been proposed including transdifferentiation [75], scar formation [77], improved revascularization [78] and/or cell fusion [50]. In contrast, and even though their influence on cardiac function has not been evaluated yet, calcification and/or ossification after MSC transplantation into the infarcted myocardium as demonstrated by Breitbach et al. show that these cells can also adapt fates with potentially deleterious effects in the engrafted tissue [79].

Wakitani et al. were among the first to describe a myogenic differentiation of BM-derived mesenchymal stem cells after treatment with the DNA demethylating compound 5′-azacytidine [72]. Rat BM-derived MSC were exposed to 5′-azacytidine for 24 h resulting in long, multinucleated myotubes with spontaneous contractions. Later studies using immortalized murine MSC, demonstrated not only the formation of myogenic structures, but the resulting cells displayed spontaneous beating, as well as the expression of several cardiac marker proteins, specific characteristics of cardiac myocytes [71]. Likewise, cardiac differentiation of murine MSC was described after cocultivation with rat cardiomyocytes [80]. However, it should be noted that the expression of certain cardiac marker genes alone, does not provide evidence for cardiac transdifferentiation. Other evidence, including the absence of markers from other lineages should be demonstrated, in addition to functionality of the resulting cell type.

Importantly, the DNA demethylating agent 5′-azacytidine does not induce specific genes, but effects global gene expression, suggesting that partial "reprogramming" rather than transdifferentiation of the MSC may occur. Recently, 5′-azacytidine has been used to enhance the reprogramming efficiency of mouse and human somatic stem cells by ectopic expression of transcription factors, thus generating induced pluripotent stem (iPS) cells, by approximately tenfold [81].

Further studies aiming at the differentiation of MSC isolated from rat bone marrow yielded contradictory results. In contrast to Wakitani et al., other studies were not able to generate spontaneously contracting cells after 5′-azacytidin or 5′-

aza-2-deoxycytidin treatment of MSC. Furthermore, the resulting cells did not express cardiac marker proteins such as cardiac myosin heavy chain, connexin 43 or troponin [70]. Experiments in rats [74] and pigs [75] using marrow stromal cells showed an improved heart function after transplantation of 5′-azacytidine-treated cells in an infarct model, as well as induced angiogenesis in the scar. However, improvement of cardiac function was also observed after transplantation of untreated BM stromal cells [73], as well as the formation of fibrotic scar tissue [77]. In the following years similar findings have been described after transplantation of human BM-derived cells [47, 76, 82].

Moreover, the mechanism of tissue engraftment and improvement of cardiac function is controversial. On one hand, cellular effects could play a decisive role if the applied cells led to an improvement by differentiation into functional cardiomyocytes. On the other hand, there are also reports on the fusion of transplanted stem cells with cardiomyocytes [50, 83], which may account for false-positive data on transdifferentiation. Nevertheless, fusion may also have a therapeutic effect as described for liver damage above [51]. Importantly, injected stem cells may exert paracrine effects potentially influencing the survival and/or proliferation of endogenous myocardial cells thereby reducing scar formation. Additionally, paracrine effects could result in stabilization of the infarcted area leading to an improvement of cardiac function. As the expression and secretion of cytokines, i.e., FGF, VEGF and angiopoetin, are upregulated in MSC under hypoxic conditions [84], enhanced vascularization by these cytokines is also plausible. In fact, the differentiation of MSC into endothelial phenotypes [78], as well as induction of cardiac nerve sprouting after MSC injection in a pig model of myocardial infarction [85] have been described.

Therefore, neither improvement of cardiac function nor homing of the transplanted cells to the myocardium as such, can provide clear evidence for the transdifferentiation of MSC into cardiomyocytes. For that reason, there is a clear need to investigate the cellular events following transplantation in order to analyze further cell fate, i.e., engraftment and transdifferentiation. Müller-Ehmsen et al. showed effective engraftment, but poor mid-term persistence of mononuclear (MNC) and mesenchymal BMC in acute and chronic rat myocardial infarction in a sex-mismatch setting [86]. The percentage of intramyocardially transplanted MNC or BMC in the heart decreased rapidly, independent from the donor cell type, donor cell number, and the application time (0–7 days post myocardial infarction). Besides the heart, transplanted cells were found predominantly in the lung and more rarely in liver and kidney. In other organs, donor cells were either absent or detected few in number.

Although Rota et al. worked with a similar animal model using transgenic mice for transplantation of BMC to the myocardium in a sex-mismatch setting, they obtained completely different results. According to their comprehensive study using sophisticated methods for donor cell detection and phenotype analysis, it was found that BMC adopt a cardiomyogenic fate in vivo [22]. Rota et al. reported that BMC engraft, both survive and grow within the spared myocardium following infarction by forming junctional complexes with resident myocytes. BMC and endogenous cardiomyocytes expressed connexin 43 and N-cadherin at their interface, as determined by immunofluorescence staining using primary antibodies directly labeled by

quantum dots to enable discrimination from autofluorescence. BMC subsequently transdifferentiated into cardiomyogenic and vascular phenotypes. This process seemed to occur independently of cell fusion (only diploid DNA and a maximum of two sex chromosomes were detected within the cells) and ameliorated structurally and functionally the outcome of the heart after infarction [22].

Most of the data presented in this study relate to rather early time-points after transplantation (up to 48 h) and some of the data on long-term engraftment have been challenged by other studies. Rota et al. using two-photon laser scanning fluorescence microscopy (TPLSM) demonstrated that some donor-derived cells develop electrical stimulation-evoked rhod-2 transients in synchrony with host cardiomyocytes 30 days following transplantation [22]. However, Scherschel et al. claim that control experiments demonstrating sufficient in situ *z*-axis spatial resolution to discriminate between signals originating in donor and host cells under the experimental conditions employed were lacking [87]. Based on previous reports [88, 89], they conclude that it is highly possible that the rhod-2 transients observed in donor-derived cells in the study arose as a consequence of fluorescence contamination from juxtaposed host cardiomyocytes, and do not represent intrinsic cardiomyogenic activity in the donor cell.

Ghodsizad et al. detected neither transdifferentiation nor fusion of cord blood derived mesenchymal cells after transplantation into the acutely ischemic lateral wall of the left ventricle [90]. They applied an alternative somatic cell type, human cord-blood derived unrestricted somatic stem cells (USSC), in a porcine model of acute myocardial infarction. Although a remarkable improvement of cardiac function was demonstrated using transesophageal echocardiography, sex- and species-specific FISH/immunostaining failed to detect engrafted donor cells 8 weeks postinfarction. Since differentiation, apoptosis, and macrophage mobilization at the infarct site were excluded as underlying mechanisms, paracrine effects are most likely to account for the observed functional effects of the USSC treatment. One possible reason for the failure of long-term engraftment might originate from the fact that a xenogeneic model was used for this study. As immunodeficient pigs are unavailable to date, to mimic the setting of small animal experiments in SCID mice, an immunosuppressive regimen has to be used in this setting. However, it is important to note that an effective immunosuppression in the human-to-pig xenotransplantation setting is difficult to achieve and a rapid rejection of the xenograft might have occurred despite the medication.

In summary, the outcome following stem cell transplantation into the infarcted heart seems to depend strongly on the donor cell type(s) and particularly on the animal model used in the respective study.

3.2.3 Endothelial Progenitor Cells

The blood is also a source for another progenitor cell type that has been tested for heart repair. Circulating endothelial progenitor cells (EPC) and endothelial cells have been proposed for transdifferentiation into cardiomyocytes [91, 92]. However,

these reports have been challenged by others that attributed these findings either to cell fusion [93], inappropriate viral labeling of transplanted donor cells [94], or concerns regarding donor cell detection and stringency of data analysis [95]. In addition, serious doubts on the cell type identity of EPC have been raised [96–98].

The identification of human EPC disproved the assumption that a postnatal vascularization depended exclusively on the proliferation and migration of terminally differentiated endothelial cells. EPC were identified based on their expression of CD34 and flk-1, as well as their adherence to tissue culture plastic surfaces. In contrast to leukocytes, they are CD45-negative and express further endothelial marker proteins, e.g., Tie-2 and CD117 [99].

After transplantation of labeled EPC into ischemic tissue of mice and rabbits, the cells were incorporated into neovascularized areas of capillaries and smaller arteries [99]. Thus, in the adult organism EPC may ameliorate reduced perfusion as in myocardial infarction and lead to improved cardiac function [100].

First reports on a cardiac transdifferentiation of endothelial cells were published by Condorelli et al. in 2001. Endothelial cells of various origins were labeled first using adenoviral or lentiviral vectors and subsequently cocultivated with neonatal rat cardiomyocytes or transplanted into ischemic areas of an infarcted mouse heart. In up to 10% of the labeled cells, the expression of cardiac marker proteins was detected by immunofluorescence staining. Such double staining as an indicator of transdifferentiation of endothelial cells was observed only after direct cell–cell contact of endothelial cells and cardiomyocytes [92]. However, these results might potentially be due to the transfer of viral vectors from one cell type to another as was proposed by Blomer et al. [94] and others [101, 102].

In contrast to Condorelli et al., Welikson et al. reported in 2006 that human umbilical vein endothelial cells (HUVEC) fuse with cardiomyocytes, but do not activate cardiac gene expression [93]. Analysis with a Cre/lox recombination assay indicated that virtually all HUVEC containing cardiac markers had indeed fused with cardiomyocytes.

A similar controversy exists on the cardiac differentiation potential of endothelial progenitor cells. In 2003, cardiac differentiation of circulating human endothelial progenitor cells after cocultivation with neonatal rat cardiomyocytes was described by Badorff et al. [91]. Within these cultures, an increase in cell size was demonstrated for the 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (DiI)–labeled EPC and immunofluorescence staining determined that approximately 10% of these labeled cells expressed cardiac marker proteins. Notably, double staining was observed only after direct cell–cell contact. Dye transfer between EPC and cardiomyocytes demonstrated the formation of gap junctions between the two cell types. Control experiments were carried out using fixed cardiomyocytes to exclude the possibility of cell fusion as an underlying reason for the double labeling.

To date, cardiac differentiation of EPC as described by Badorff et al. has not been confirmed by other groups and the phenotype of the cells used in the study is controversial. Different studies defined EPC as VEGFR2-positive/CD133-positive/CD34-positive subpopulations of MNC [103], or as CD34-positive/VEGFR2-positive [104] or CD133-positive/VEGFR2-positive cells originating from the BM

and mobilizing as the need arises [105]. However, CD34-negative cell populations have also been identified which have differentiated into EPC and endothelial cells [106]. While the cells described by Badorff et al. were assumed to have an endothelial phenotype due to the uptake of acetylated low density lipoprotein (LDL) and binding of the lectin Ulex europaeus agglutinin-1 (UEA-1), more recent data suggest that these cells represent almost exclusively monocytes/macrophages [95, 98]. Only a small percentage of the cells express endothelial markers; therefore, they have been termed "circulating angiogenic cells" (CAC) [96]. The CD14-positive/CD34-negative cell population within the expanded EPC might exert a proangiogenic effect by releasing paracrine factors [107]. In addition, CD14-positive cells release cytokines that may be important signals for wound healing [108, 109]. Recently, it has been confirmed that blood-derived monocytes [98], as well as other immune cells [97] can mimic EPC due to LDL uptake and lectin binding abilities in addition to colony forming capacities.

In contrast to Badorff et al., a study by our group performing coculture experiments with DiI-labeled huEPC and neonatal rat cardiomyocytes (NRCM) did not support transdifferentiation of huEPC into functionally active cardiomyocytes. Gruh et al. analyzed the cocultivated cells by means of flow cytometry, 3D confocal laser microscopy, species-specific RT-PCR for the expression of human cardiac marker genes, and electron microscopy [95]. Although FACS analysis and conventional wide-field fluorescence microscopy suggested the existence of DiI-positive human cardiomyocytes in cocultures, we obtained no convincing evidence of cardiac differentiation of huEPC. Rather, DiI-positive cardiomyocytes were identified as necrotic NRCM or NRCM-derived vesicles with high levels of autofluorescence, or alternatively, as NRCM lying on top of or below labeled huEPC or huEPC fragments. Accordingly, no expression of human Nkx2.5, GATA-4, or cardiac troponin I was detected. Although it cannot be excluded that slightly different culture conditions may have prevented transdifferentiation in our own experiments, our data highlight technical limitations of FACS analysis and conventional 2D immunofluorescence, as well as confocal microscopy for the analysis of stem cell differentiation in coculture settings.

3.3 Transdifferentiation into Neuronal Cells

3.3.1 Hematopoietic Stem Cells

First reports on the contribution of HSC to the brain described the differentiation into microglia and macroglia in adult mice [110], and were later confirmed in several studies [111, 112]. In contrast, the contribution of HSC to other cell types in the brain is controversial and initial reports on neuronal differentiation of HSC [113–115] could not be confirmed by others [116, 117]. These discrepancies have led to a discussion on the validity of different approaches used for cell tracing in transplantation experiments [118].

To test the ability of adult HSC to contribute to the central nervous system, Eglitis et al. transplanted adult female mice with donor BMC genetically marked with either a retroviral tag or by using male donor cells [110]. Using in situ hybridization histochemistry, a continuing influx of BM-derived hematopoietic cells into the brain was detected. These cells were widely distributed throughout regions in the brain, including the cortex, hippocampus, thalamus, brain stem, and cerebellum. When in situ hybridization histochemistry was combined with immunohistochemical staining using lineage-specific markers, some BM-derived cells were positive for the microglial marker F4/80. Other BM-derived cells expressed the astroglial marker glial fibrillary acidic protein (GFAP). From these results, Eglitis et al. concluded that some microglia and astroglia arise from a precursor that is a normal constituent of adult BM. This idea became widely accepted [111, 112]; however it was followed by controversial discussions regarding the contribution of HSC to other cell types in the brain.

In 2000, Brazelton et al. reported the expression of neuronal phenotypes from BM-derived cells, following intravascular delivery of genetically marked adult mouse BM into lethally irradiated adult mice. These cells persisted in the brain for at least 6 months after transplantation, as assessed by flow cytometry and showed typical neuronal gene expression profiles (NeuN, 200-kilodalton neurofilament, and class III beta-tubulin) demonstrated by confocal microscopy [113].

In the same year, Mezey et al. showed that transplanted adult BMC migrated into the brain and differentiated into cells that expressed neuron-specific antigens [114]. Later, the same group also investigated whether HSC contribute to neuronal cells in humans. To this effect, they examined postmortem brain samples from females who had received BM transplants from male donors [115]. Using a combination of neuron-specific antibodies for immunocytochemistry and FISH histochemistry, cells containing Y-chromosomes were detected in several brain regions. Most of these cells were identified as nonneuronal (e.g., endothelial cells); however, neurons in the hippocampus and cerebral cortex were detected. The distribution of the labeled cells was not homogeneous with clusters of Y-chromosome-positive cells, suggesting that single progenitor cells underwent clonal expansion and differentiation. Mezey et al. concluded that adult human BMC can enter the brain and generate neurons in a manner similar to rodent cells.

In contrast to these data, Castro et al. report the failure of BMC to transdifferentiate into neural cells in vivo, both after transplantation of BM-derived side population cells, as well as unfractionated BM [116]. None of the recipients had donor-derived neural-like cells in the brain and cervical spinal cords, regardless of injury. Comments on this report by Mezey et al. point out that this discrepancy might be due to the different methodologies used for cell tracing [118]. While Mezey et al. used immunocytochemistry in combination with FISH histochemistry for Y-chromosome-positive cells in a sex-mismatch model, Castro et al. used genetically labeled donor cells from a Rosa-LacZ mouse strain expressing the LacZ reporter gene under transcriptional control of the Rosa26 promoter. The latter approach, however, depends on uniform ubiquitous transgene expression in the tissues analyzed, as well as on error-prone detection methods [119]. Therefore, it is

not unlikely that the study by Castro et al. underestimated the actual number of donor-derived cells in their model.

Interestingly, another study using a reporter gene approach for labeling of HSC was also not able to detect transdifferentiation of BM-derived cells into neuronal lineages. In 2006, Roybon et al. investigated whether highly purified mouse adult HSC, characterized by lineage marker depletion and expression of the cell surface markers Sca1 and c-Kit (Lin-negativ/Sca1-positive/c-Kit-positive), can be stimulated to adopt a neuronal fate [117]. In this study, transgenic mice expressing GFP under control of the chicken β-actin promoter were used. First, Roybon et al. tried to induce neural differentiation in vitro with protocols that have been successfully used to differentiate either neuronal or embryonic stem cells or multipotent adult progenitor cells from BM into neuronal cells. As a result, up to 50% of the cells expressed the neural progenitor marker nestin. However, electrophysiological recordings on neuron-like cells showed that these cells were incapable of generating action potentials. Therefore, at least in vitro, HSC did not seem to be able to differentiate into functional neuronal cell types. According to Roybon et al., neither cocultivation with neural precursors nor transplantation into the striatum or cerebellum of wild-type mice, resulted in HSC-derived cells with a true neuronal phenotype. Rather, the applied HSC differentiated into macrophage/microglia or died.

One major point of criticism concerning the findings of Castro et al. raised by Mezey et al. was that blue LacZ-positive microglia, which like other monocyte/macrophage cells originate from HSC, were absent from the brains of the transplanted animals. In contrast, Roybon et al. did find GFP-positive microglia after HSC transplantation. Thus, their method seems valid for the detection of transdifferentiated neuronal cells in principle, in return raising doubts on the data presented by Brazelton et al. and Mezey et al. In conclusion, further studies using sophisticated methods are mandatory to unambiguously prove or disprove the contribution of BM-derived HSC to functional neuronal cell types in vivo.

3.3.2 Mesenchymal Stem Cells

It was reported by several groups that stem cells isolated from the BM were capable of differentiation towards neural like cells (reviewed in [120]). Most studies based their conclusions on an evaluation of changes in cell morphology, i.e., the formation of neurite-like structures, and on the detection of neuronal-cell specific marker gene expression, mostly detected by immunohistology. However, other studies demonstrated that neuronal marker expression was already present in undifferentiated MSC [121] and is induced in response to stress [122, 123]. In addition, these studies questioned the validity of morphological analyses of neuronal transdifferentiation in vitro. While some studies attributed the beneficial effects of MSC transplantation to the brain as a result of transdifferentiation [124], immunological effects have also been considered [125].

Early reports on neuronal transdifferentiation of MSC were contradicted, for example by in vitro experiments based on protocols by Woodbury et al. [126], that

used exposure to certain chemicals as a neural differentiation stimulus for MSC. Investigation with time-lapse video recording showed that the formation of neurites is not the result of an outgrowth of dendrite- and axon-like structures, but merely a result of cell shrinkage and retraction of the cell edge in response to stress [122, 123, 127]. In addition, some neural marker proteins have been found to be expressed in undifferentiated MSC [121]. Furthermore, exposure of MSC to stress causes an increase in expression levels of the neural markers neuronal nuclei (NeuN), neuron-specific enolase (NSE) [123], neurofilament 200 (NF200) and tau [122].

In addition, for MAPC from the BM, Raedt et al. reported a baseline expression of neural markers beta III tubulin and NF200. Furthermore, the application of several protocols for neural differentiation did not result in an increase in expression levels as determined using real-time PCR and immunohistochemistry [128].

Nevertheless, in vivo experiments using MSC for transplantation into the brain yielded positive results. In 2006, Arnhold et al. investigated the therapeutic potential of MSC by stereotactic engraftment into the lateral ventricle of adult rats [124]. They reported that human BM stromal cells display certain neural characteristics and integrate into the subventricular compartment after injection into the liquor system and took up a close host graft interaction without any degenerative influence on the host cells. Arnhold et al. reported morphological, as well as immunohistochemical evidence for a transdifferentiation of MSC within the host tissue.

In contrast, Gerdoni et al. obtained different results investigating the therapeutic effect of MSC transplantation to the brain in experimental autoimmune encephalomyelitis [125]. MSC-treated mice showed a significantly milder disease and fewer relapses compared to control mice. This was also accompanied with a decreased number of inflammatory infiltrates, reduced demyelination, and axonal loss. However, no evidence of GFP-labeled neural cells was detected inside the brain parenchyma, thus not supporting the hypothesis of MSC transdifferentiation. In contrast, the analysis of in vivo T- and B-cell responses and antibody titers suggested that the beneficial effect of MSC in experimental autoimmune encephalomyelitis is mainly the result of an interference with the pathogenic autoimmune response.

In fact, it is conceivable that any stem cell transplantation may lead to a reaction that could be characterized as a “proregenerative inflammation.” In this setting, the induced lesion, as well as the transplanted cells can trigger the attraction of immune cells to the site of transplantation and result in a proregenerative cytokine release.

4 Critical Aspects of Differentiation Experiments

4.1 Cell Type Identity

Identifying the stem cell type used in experiments investigating transdifferentiation is critical. For a comparative analysis of stem cell plasticity, especially when

being performed by different groups, an unambiguous definition of the cell's phenotype is crucial. However, besides inconsistencies in the protocols for isolation and cultivation of the described cells, the rapidly expanding knowledge on stem cell populations and subpopulations complicates an objective comparison of the existing data. While early reports investigated the fate of "adult BMC" [114] or "BM stromal cells" [73], others used different subpopulations. These were classified either by the expression of single marker proteins like "CD34-positive BMC"[35], "purified BM Sca-1-positive cells" [59], or differentiation potential as for "multipotent adult progenitor cells (MAPC)" [106] or "human BM-derived multipotent stem cells" (hBMSC) [47]. To address inconsistencies, Horwitz et al. suggested a clarification of the nomenclature for MSC in an International Society for Cellular Therapy position statement [129]. Herein, the authors propose that the plastic-adherent cells currently described as mesenchymal stem cells be termed multipotent mesenchymal stromal cells, while the term mesenchymal stem cells should be reserved for a subset of these (or other) cells that demonstrate stem cell activity by clearly stated criteria. These include demonstrations of long-term survival with self-renewal capacity and tissue-repopulation with multilineage differentiation. For both cell populations, the acronym MSC may be used, however, investigators should unequivocally define the acronym in their work.

The analysis of transdifferentiation processes is especially complicated in the case of mixed populations or when investigating in vivo migration and homing to sites of injury. Besides engraftment of a single cell type potentially leading to the regeneration of damaged tissue, synergistic effects might play a key role. This might be conceivable following transplantation of BMC with different cell subtypes exerting proangiogenic, antiapoptotic and/or antiinflammatory effects. For example, the expression and secretion of cytokines like FGF, VEGF and angiopoetin in MSC [84], could potentially modulate the transdifferentiation capacity of other cell types. The complications resulting from the use of mixed cell populations can be circumvented by the clonal transplantation of single cells as performed, for example, by Krause et al. [37]. However, this approach has certain limitations and may be difficult to perform for many cell types, as in vivo cell survival and proliferation capacity following a single cell transplantation are usually low.

One of the most prominent examples of a controversial cell type identification is the ongoing debate regarding "endothelial progenitor cells" (EPC) or "circulating angiogenic progenitor cells" (CAC). In recent years, difficulties in discriminating between EPC and cells of monocytic/macrophage origin became more and more evident [91, 96, 99, 103]. It was demonstrated that blood derived monocytes [98], as well as immune cells [97] can mimic EPC; thus questioning the validity of earlier reports.

Obviously, not only the potential cell source for transdifferentiation can be controversial, but surely also the cell type resulting from this event. The question being: which criteria need to be met by the resulting cell to be considered a hepatocyte, cardiomyocyte or neuronal cell? When Lysy et al. investigated the hepatic differentiation of MSC, the resulting cells displayed expression of several hepatocyte markers such as albumin, alpha-fetoprotein, cytokeratin 18, representing at

least “hepatocyte-like” cells [52]. However, it was also demonstrated that these cells partially retained mesenchymal markers, suggesting that the cells were not “fully” differentiated. Consequently, it seems to be crucial to define the conditions that have to be fulfilled by cells to be considered a fully differentiated and most of all functional cell type.

4.2 Cell Labeling

As studies on transdifferentiation frequently involve more than one cell type, e.g., in cocultivation approaches or transplantation settings, an optimal cell labeling method has to be applied for an interpretable read-out of the experiment.

The first possibility for cell labeling is with fluorescent dyes that bind to cellular components covalently or noncovalently. For example, in a study investigating the cardiac differentiation potential of endothelial progenitor cells, cells were labeled through the uptake of DiI-LDL prior to cocultivation with neonatal rat cardiomyocytes [91]. This approach has several drawbacks: (1) dyes are diluted upon further cell division, (2) once labeled, dead cells will retain the label and (3) fluorescent cell debris can be taken up by other cells, e.g., macrophages, or stick to other cells leading to false positive results.

Some of these problems can be overcome using genetic labeling, most commonly with reporter genes such as LacZ and GFP. These reporter genes have been used in combination with ubiquitous promoters, for example to investigate the capacity of BMC to transdifferentiate into neural cells after transplantation to the brain. As described above, Castro et al. used genetically labeled donor cells from a Rosa-LacZ mouse strain expressing the LacZ reporter gene under transcriptional control of the Rosa26 promoter [116], while Roybon et al. used cells expressing GFP under control of the chicken β-actin promoter [117]. Both studies did not provide evidence for transdifferentiation events, in contrast to Mezey et al., who used immunocytochemistry in combination with FISH histochemistry for Y-chromosome-positive cells in a sex-mismatch model [115]. It is known, that a reporter gene assay depends on uniform ubiquitous transgene expression in the analyzed cells; therefore, it is crucial that the transcriptional activity of a given promoter is on a similar level in both undifferentiated and differentiated (stem) cells. As was demonstrated for murine embryonic stem cells, promoter activity may vary significantly throughout the process of differentiation [130]. This issue should be considered a possible explanation for discrepancies in the outcome of adult stem cell differentiation experiments using genetic labeling.

In addition to the mere labeling of cells by ubiquitous reporter gene expression, conditional genetic labeling techniques have added greatly to the knowledge on stem cell transdifferentiation. Tissue specific promoters can be used to switch on reporter gene expression only in case of differentiation towards a certain cell type.

Rota et al. used the reporters eGFP and a c-myc-tagged nuclear-targeted-Akt transgene, both driven by the cardiac-specific α-myosin-heavy-chain (α-MHC) promoter, to investigate the cardiomyogenic fate of BMC [22]. However, for this approach, the cell specificity of the promoter has to be carefully analyzed, as leaky or unspecific expression in other cell types may occur, especially in case of higher copy numbers of the transgenes within the cells [131]. Recently, sophisticated genetic labeling has been used for the detection of cell fusion. As described above, Alvarez-Dolado et al. used a conditional Cre/lox recombination, enabling detection of fused cells by X-gal staining for LacZ expression [50].

Another important issue is how the transgene is transferred to the cells. When transgenic cell lines or animals are not available, the gene transfer has to be performed directly before the experiment, by using either standard transfection methods or viral vectors. Both with plasmid transfection and nonintegrating viral vectors, e.g., adenoviruses, the problem of signal dilution can occur in dividing cells. In contrast, integrating viruses like lentiviral vectors, have turned out to be an efficient method for stable gene transfer for both in vitro and in vivo studies. However, we have identified an important weakness of this method in cases where cells need immediate transplantation after preparation, e.g., to prevent cell death, differentiation or dedifferentiation [94]. Although these cells are usually washed several times following viral transduction, there may be the risk of viral vector shuttle via transplanted cells resulting in undesired in vivo transduction of recipient cells. We explored a potential viral shuttle via ex vivo lentivirally transduced cardiomyocytes in vitro, following transplantation into the brain and peripheral muscle. By this, we demonstrated that even after extensive washing, infectious viral vector particles can be detected in cell suspensions. As a result, the lentiviral vector particles stably transduced resident cells of the recipient central nervous system and muscle in vivo.

This phenomenon can also be seen using other cell types, as was confirmed by further studies demonstrating that retroviral particles adhere nonspecifically, or "hitchhike," to the surface of T-cells [132]. After transplantation, secondary transduction has been observed due to the adherence of vector particles to hematopoietic target cells [102] or endothelial cells [101]. In some cases, for example in a study by Condorelli et al., these findings might be one of the possible reasons for the discrepancies among studies investigating stem cell differentiation in transplantation models and cocultivation systems [92].

4.3 Imaging Techniques

Transplantation models and cocultivation systems suffer from another difficulty, as the identification of transdifferentiated cells can be complex. Methods based on immunohistology have to be carefully evaluated with respect to the specificity of the obtained signals, inclusion of all necessary controls and exclusion of staining artifacts. As described above, the detection of the LacZ

transgene relies on error-prone detection methods, including the risk of unspecific staining after prolonged incubation [119]. Detection of GFP or using immunofluorescence approaches can also be impaired by high levels of tissue or cell-specific background fluorescence [133]. Importantly, high levels of autofluorescence can frequently be observed in necrotic or apoptotic cells leading to false interpretations, in particular in transplantation or coculture-based transdifferentiation experiments. Laflamme et al. reported that apart from the normal autofluorescence in striated heart muscle, this fluorescence increases after myocardial injury due to accumulated lipofuscin, blood-derived pigments and other intrinsic fluorochromes such as flavins and reduced nicotinamide adenine dinucleotide (NADH) [134]. While early reports using GFP-labeled cells for transplantation might have overlooked this fact, recent publications used GFP-specific antibodies and/or validation of the emission spectrum to unequivocally identify GFP-expressing cells [135]. Increasing levels of autofluorescence in the course of a coculture experiment, as has been demonstrated by our group using flow cytometry analyses as shown in Fig. 2 [95], can potentially lead to misinterpretation of the obtained data as might be the case in

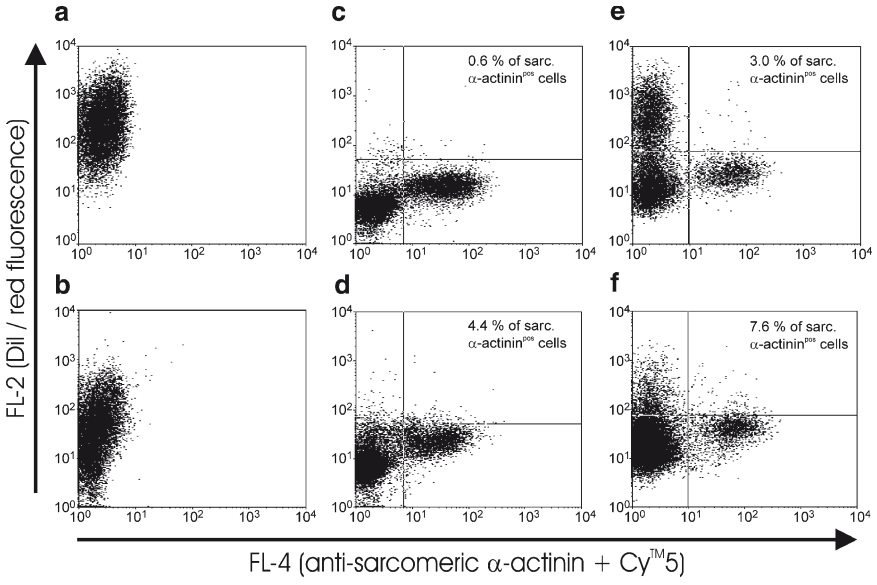

Fig. 2 Conventional flow cytometry analysis is not suitable to identify DiI-positive human cardiomyocytes within cocultures of human endothelial cell progenitors (huEPC) and neonatal rat cardiomyocytes (NRCM). Analyses of cocultures (**e**,**f**), as well as of monocultures of huEPC (**a**,**b**) and NRCM (**c**,**d**) at day 2 (**a**,**c**,**e**) and day 6 (**b**,**d**,**f**) demonstrate a significant increase in sarcomeric α-actinin-positive cardiomyocytes displaying red DiI-like fluorescence. Modified after Gruh et al. [95]

reports on the alleged cardiac differentiation potential of endothelial progenitor cells [91].

When using conventional two-dimensional image analysis for the evaluation of double or multiple immunostaining, sometimes a genuine colocalization within the same cell is hard to discern from an overlay of signals from two neighboring cells and three-dimensional confocal imaging should be preferred instead [95]. However, even in the case of three-dimensional analysis, data interpretation can be difficult.

In some cases, another dimension has to be included: the monitoring of the cell's fate over time. As described above, time-lapse video recording was able to reveal that morphological changes associated with a presumed neuronal differentiation of mesenchymal stem cells were actually not a result of outgrowing neurites, but of cell shrinkage in response to stress signals [122, 123, 127].

Immunohistology and immunofluorescence can also be error-prone and common problems include a weak signal from otherwise specific antibodies and/or nonuniform staining. For example, in our xenogeneic pig model studies, we have observed this problem when using an antibody detecting a human mitochondrial antigen. We found that this antibody led to nonuniform staining when used to detect different human cell types. On one hand, it conferred strong labeling of cardiomyocytes while on the other, human fibroblasts showed insufficient staining. While this finding is in line with expected differences in metabolic activity and numbers of mitochondria per cell in the two cell types, it prevented the use of this antibody in our study. Alternatively, another antibody directed against human nuclear antigen (HuNu) was used [90].

The high background of unspecific staining can also be a problem and unfortunately, published data often lack critical controls such as images of the appropriate isotype staining control. As long as images show the detection of structural proteins resulting in distinct staining patterns, e.g., cardiomyocyte specific staining of contractile proteins that shows clearly visible cross-striations, this might not be problematic. Otherwise, it is difficult to discern diffuse staining of cytoplasmic proteins from background levels. It is therefore advised to include these controls either in the original publication or as online supporting material. Moving forward, both editors and reviewers should be made aware that these controls would add to the reliability, and thus quality of the published data.

Unfortunately, appropriate isotype controls are not always available. This is true when using rabbit serum for staining. The correct control would be preimmunized serum obtained from the same animal. Thus, experiments using unpurified serum should at least include negative control staining with other, nontarget cells; and the specificity of immunostaining strategies with unpurified antibodies should be interpreted with caution.

4.4 Data Interpretation

The interpretation of transdifferentiation experiments can be difficult when too many conclusions are drawn from too little data. Early reports on the presumed neural differentiation of mesenchymal stem cells provide an important example. In this case, initial reports concentrated on the apparent morphology of the cells, as well as the detection of a limited number of markers [120]. Only later did data disprove the initial reports by demonstrating that some neural marker proteins are already expressed in undifferentiated MSC [121] and that stress causes an increase in the expression level of several neural markers [122, 123].

Similarly, later studies investigating differentiation of transplanted stem cells set out to analyze not only colocalization of donor-derived and tissue-specific markers, but also considered cell fusion as an alternative mechanism. In 2003, Wang et al. demonstrated that cell fusion was the principal source of BM-derived hepatocytes by investigating the ploidy of the presumably transdifferentiated donor cells [31]. Subsequent studies using the same assays, for example a study published by Sato et al., tried to elucidate the cellular components of human BM that potently differentiated into hepatocytes. Sato et al. stated that cell fusion was not likely involved, as both human and rat chromosomes were independently identified by FISH [136]. However, fusion as an underlying mechanism for the detection of double labeled, presumably transdifferentiated cells, cannot be excluded from earlier reports, as this possibility was not explicitly investigated.

Early in vivo data on the transdifferentiation of adult stem cells concentrated on the therapeutic effects following stem cell transplantation. Improved heart function and increased angiogenesis in the scar were observed after transplantation of 5-azacytidine-treated marrow stromal cells in an infarct model [74, 75]. Although some labeled bone marrow-derived cells within the infarct region stained positively for a cardiac marker protein, it remains unclear to what extent transdifferentiation into cardiomyocytes is the reason for the improvement or whether this may be due to other cardio-protective effects as described above [77, 78, 84, 85]. Therefore, functional improvement alone, does not provide evidence of transdifferentiation and leaves room for different interpretations with respect to the impact of individual effects triggered by stem cell transplantation.

4.5 Biological Significance

Most of the experiments investigating stem cell transdifferentiation represent a highly artificial setting with limited biological significance in vivo. This holds true especially for transplantation experiments, whereby stem cells are transferred from one part of the body to another, and sometimes to a rather remote compartment. These settings may not resemble naturally occurring stem cell mobilization and/or recruitment processes; and therefore, might not be ideal to mimic and investigate in vivo regeneration. However, the importance of stem cell transplantation, as a

future method with great clinical significance, should not be overshadowed by the complications of replicating the exact events occurring in nature.

Indeed, the detection of a therapeutic effect does not prove stem cell transdifferentiation per se, even unequivocally confirmed stem cell transdifferentiation into another somatic cell type does not guarantee a therapeutic effect. When Wu et al. investigated whether human BMC could contribute to liver regeneration in vivo, they detected cells from extrahepatic sources that had homed to the tissue, ultimately transdifferentiating into hepatocytes. However, these cells did not increase in number, thus a robust repopulation of the tissue was not observed [137].

It should be noted that, apart from the role of transdifferentiation of stem cells in tissue regeneration following injury, and/or in homeostasis, this process might also have an impact on pathogenesis. For example, it has been proposed recently that BM-derived circulating precursor cells participate in the development of human lung fibrosis and lesion formation, especially in *bronchiolitis obliterans* [138].

In reality, the biological significance of transdifferentiation, with respect to its meaning, is still poorly understood. The question remains to be determined whether transdifferentiation reflects a natural process, i.e., an inherent ability of a given cell to switch its fate under certain conditions, or an artificial change in its expression profile, as might be the case for differentiation processes induced by treatment with 5-azacytidine. As this agent confers the demethylation of DNA leading to a random induction of gene expression, subsequent changes could be interpreted as an artificially-induced reprogramming, a rather hard reset of the cellular differentiation program.

Lastly, the incidence of transdifferentiation and/or cell fusion might also play a role in determining biological significance. To date, only rare events have been described, and although interesting, the findings might be irrelevant for therapeutic purposes in vivo, due to the low frequency of occurrence.

5 Conclusions

New data on the plasticity of stem cells of various lineages have emerged. These data, in addition to the developing new field of adult stem cell differentiation, are not without controversy. Today, most of the reported discrepancies cannot be explained satisfactorily due to several reasons. For example, many studies lack a common starting point, i.e., it remains unclear whether the exact same cell population was analyzed. In addition, the methodology for precise analyses of differentiation events is still rapidly evolving. As a reaction to criticism concerning early and sometimes too enthusiastic reports on the transdifferentiation of stem cells and its envisaged therapeutic potential, sophisticated methods have been developed or adapted, e.g., in the area of cell labeling, imaging and tracing. However, to prove unequivocally stem cell transdifferentiation, there is a clear need to prove the functionality of the resulting cell type. It is not sufficient to show that a given cell *looks*

like a transdifferentiated cell solely due to the expression of specific marker proteins, but to answer the question as to whether it *acts* accordingly.

As the field of stem cell biology progresses, it will be crucial to analyze further not only *if* a certain stem cell type differentiates into a certain phenotype, whether or not expected, but also to investigate in detail *how* this process works. This will include the identification of key factors inducing cell fate switches, and the molecular mechanisms and chronological sequence of the conversion itself. This includes the question of how *we*, as investigators, *force* a given cell to transdifferentiate into a desired cell type, for example by the over-expression of cell-type specific transcription factors, regardless of the in vivo and/or in vitro significance of the particular conversion.

By focusing on these mechanisms, insight into the original question will be addressed: Do stem cells undergo direct differentiation towards a more specialized somatic cell, or, must they be reprogrammed or "de"-differentiated, thereby first changing into a more common ancestor to then be "trans"-differentiated into specialized cells ultimately involving the same pathways as in organ development.

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