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Cell Fusion: biological perspectives and potential for regenerative medicine

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1. ABSTRACT

Cell fusion has emerged as a powerful subject of debate in the last few years. Adult stem cell plasticity and the search for mechanisms to explain this process have led to the "rediscovery" of cell fusion. In nature, cell fusion is a normal process involved in sexual reproduction, tissue formation, and immune response. The recent observation that bone marrow derived cells fuse with several cell types introduces new and provocative questions. In this review, I shall recapitulate what is known about cell fusion and discuss its more controversial aspects. I shall highlight the most exciting open questions; its biological potential; pros and cons; and their implications on stem cell plasticity, regenerative medicine, and development.

2. INTRODUCTION

Cell fusion is a natural process with important biological implications. In fact, our lives started with a cell fusion event between an ovum and spermatozoa. During development, cell fusion is involved in the formation of muscle, bone, and placenta (1-3). The immune system also takes advantage of cell fusion by generating multinucleated giant cells to respond against chronic infections and foreign bodies (4). Finally, some viruses induce cell fusion, which may be in the base of diseases such as AIDS and its pathology in the nervous system (5). Despite the relevance of these processes, cell fusion did not captivate the interest of scientific community. More recently, however, cell fusion studies have been revived, being in the core of the

controversy thanks to their link to stem cell plasticity and its regenerative properties.

3. STEM CELL PLASTICITY

Stem cells (SC) are defined as immature cells with self-renewal properties, being able to generate mature progeny including non-renewing progenitors and terminally differentiated cells (6, 7). Consequently, SC are the main source of new cells and major responsible for tissue repair after injury. SC have been classified as embryonic and adult. Embryonic stem cells (ES) are able to generate cell types of the three embryonic germ layers (ectoderm, mesoderm, and endoderm). This means they are totipotent; they can generate all cell types. In contrast, adult SC are more limited in their ability to generate cells. A common doctrine postulated that adult SC only give rise to a subset of cell lineages within the same embryologic origin. They are restricted in their differentiation and regenerative capability to the tissues in which they reside. For instance, a neural adult SC is only able to generate cells of the nervous system (neurons, astrocytes, and oligodendrocytes). This dogma was challenged after the publication of several papers from independent groups. The scientific community was concerned about the capability of tissue-specific adult SC to sustain, by themselves, the regeneration of a damaged organ throughout its life span. Bone marrow adult stem cells (BMSC) could be an additional source of SC capable of reaching many tissues through the blood stream. There are two types of BMSC: haematopoietic stem cells (HSC) and multipotent marrow stromal cells, also called mesenchymal stem cells (MSC) (8-10). HSC are able to generate all of the lineages of mature blood cell types. MSC are present in the stromal fraction of the bone marrow and are able to self-renew and differentiate into bone, cartilage, fat, tendon, and marrow stroma (11). In the late 90's, evidence emerged showing that HSC could have a greater plasticity than expected. To show their wider plasticity *in vivo*, several groups performed transplants of bone marrow derived cells, or cellular populations enriched in BMSC, or even a single HSC. Transplanted cells carried reporter genes such as LacZ or GFP to facilitate their tracking. After transplant, analysis of recipient animals showed the expression of these markers by non-haematopoietic cells in several organs. These cells presented the morphology of fully developed mature cells. The first of these types of studies was reported in 1997 by Eglitis and Mezey (12). After transplanting genetically engineered HSC, they observed micro and macroglia expressing the donor-derived reporter gene (NeoR) in the brain. Other groups also identified neurons in the cortex and cerebellum (13-17). Soon, new tissues were reported such as liver, pancreas, skeletal muscle, endothelium, and myocardium (18-29). A single HSC transplant showed reconstitution of the haematopoietic system, and, in addition, its contribution to epithelia, skin, and lung epithelium (18). In the meantime, MSC were also isolated and tested for their ability to generate tissues of different embryonic origin (30, 31). These cells were able to differentiate into cells of the three embryonic layers *in vitro*, and when introduced into an early blastocyst they contributed to most of the somatic tissues (32).

These results meant that adult BMSC were more plastic than expected, and they could be used to generate all kind of tissues. The clinical implications of these observations were enormous. Application of bone marrow cell subpopulations for regenerative medicine could now be seen as a reality, since bone marrow extraction is standardized in most hospitals. Additional works contributed to general enthusiasm. Several groups showed myocardial regeneration after direct grafting of bone marrow cells into an infarcted heart (29, 33). These observations led many groups to start several clinical trials to treat heart infarct by transplanting subpopulations of bone marrow derived cells (34). These trials were, to a certain degree, premature because the plasticity mechanism used by BMSC for regeneration remained, at that point, elusive.

4. MECHANISMS OF SC PLASTICITY

Plasticity broke with the idea of a rigid genetic program. A more flexible concept of differentiation of adult SC in response to microenvironmental or regenerative cues emerged. Adult SC seemed to be very plastic and, in theory, they could be totipotents. However, to take advantage of this capability and to control the fate of the adult SC it is necessary a deep knowledge of the plasticity mechanism. Two main hypotheses were postulated: transdifferentiation and cell fusion.

4.1. Transdifferentiation

The plasticity concept is linked to the idea of transdifferentiation, described as the conversion of a cell that belongs to a certain lineage into a cell of entirely distinct lineage. This is a simple and linear interpretation of *in vitro* and *in vivo* results. In general, SC respond to several factors *in vitro* that modify their differentiation program and make them even more plastic than *in vivo* (35). MSC are not an exception; they are able to differentiate into cells of the three germlines under the effect of specific culture conditions *in vitro* (32). Initial interpretations of the previously described bone marrow transplant (BMT) experiments followed this line of thought. BMSC could have penetrated the parenchyma of several organs where they would respond to tissue-specific factors, local niches, or injury signals. Thus, the new microenvironment would allow the transdifferentiation of BMSC into the specific cell types of that tissue.

However, some groups were skeptical about this interpretation of the results. For instance, it is well known that brain development is achieved during the neonatal period. Later in adulthood, levels of growth factors, neurotrophins, and synaptic connectivity necessary for the correct differentiation of a neuron are no longer present, except for two neurogenerative regions: the subventricular zone and the hippocampus (36, 37). Putative transdifferentiated cells in the brain after BMT were observed outside of these two niches. Therefore, transdifferentiation hypothesis did not accurately fit in the

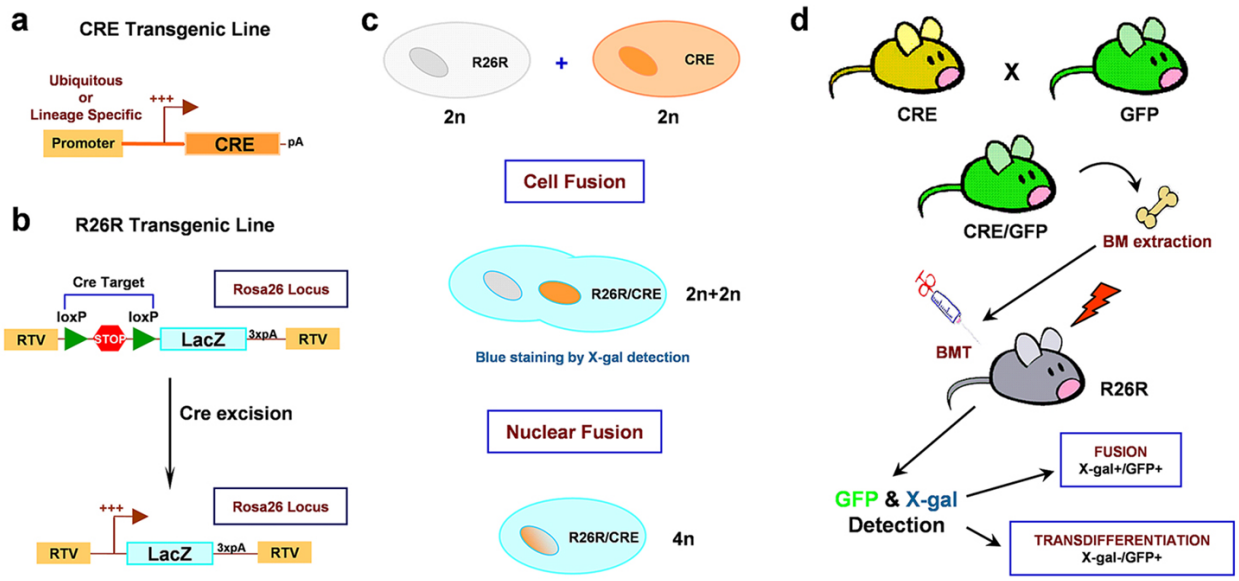

Figure 1. Cell Fusion Detection System. **A.** Schematic representation of the Cre recombinase transgene expressed by one of the mouse lines used in the system. **B.** Representation of the reporter transgene expressed by the R26R mouse line and its modification after Cre mediated recombination. **C.** When a cell expressing Cre recombinase (a) fuses with a cell bearing the LacZ reporter transgene (b), the floxed stop cassette is excised and the LacZ reporter is expressed in the fused cell. LacZ expression can be detected by the generation of a blue precipitate after X-gal staining. **D.** BMT strategy to detect cell fusion *in vivo*. A R26R mouse line was used as recipient of BM cells expressing GFP and Cre recombinase. Double detection of GFP and X-gal blue precipitate allows to discern between cell fusion and transdifferentiation events.

case of the adult brain. An alternative explanation was given: cell fusion.

4.2. Cell Fusion

This hypothesis postulates that a bone marrow derived cell fuses with a local precursor or mature cell, transferring its genetic material and mixing their cytoplasm. The newly formed hybrid cell would acquire a new phenotype. It would modify its genetic program and, in consequence, lineage restriction may be broken.

Cell fusion was not mentioned as an alternative to transdifferentiation until the publication by two independent groups of ES fusion *in vitro* with bone marrow cells and adult neural SC (38, 39). However, previous reports had already shown that cell fusion contributed to tissue repair (25, 27, 40, 41). Once transdifferentiation hypothesis was questioned, groups working on the field focused their efforts on finding evidence in favor of cell fusion in their experimental models. Liver regeneration was the first *in vivo* model where cell fusion was fairly shown. Cell fusion turned out to be the major source of bone marrow derived hepatocytes under hepatic degenerative conditions. Two independent groups, using a hepatic lethal mouse model with mutations in the fumarylacetoacetate hydrolase gene, showed the rescue of normal liver function after BMT (21, 22). Restoration of normal metabolism was due to repopulation of the liver with hepatocytes expressing the wildtype gene. *In vitro* cytogenetic analysis of these hepatocytes and southern blots showed karyotypes indicative of fusion between donor and host cells (21, 22). Previously, Wagers *et al*. already reported little evidence of transdifferentiation of HSC after transplantation and suggested that Purkinje neurons and hepatocytes could derive from cell fusion (42). Helen Blau's group, which previously showed the presence of neurons carrying donor derived markers after BMT, also turned its interpretation of the results towards cell fusion (13). This group studied brain biopsies from women who had received BMT from male donors. Some of the Purkinje neurons in these biopsies were tetraploid (XXXY), as detected by fluorescent in situ hybridization (FISH). The presence of both sets of chromosomes strongly suggested that Purkinje neurons had fused with haematopoietic cells from the bone marrow donor (17).

Despite these results, the scientific community remained skeptical about cell fusion. Liver observations were obtained from a damaged tissue model, and FISH was not an ideal technique to provide the most reliable results. So, there was no direct evidence of cell fusion or transdifferentiation under normal conditions. For this reason, our group developed a genetic system, reliable and easy to apply, to unequivocally discern fusion events from transdifferentiation in any tissue and under any pathological condition (43). Our detection method was based on the cre-lox technology (44). We used two different transgenic mouse lines (Figure 1a-b). The first line expressed the Cre recombinase under a specific promoter that can be ubiquitous or lineage restricted (Figure 1a). The second mouse line (R26R) carried the LacZ reporter gene, which is exclusively expressed after the excision of a loxP-flanked (floxed) stop cassette by Cre mediated recombination (Figure 1b). When Cre-expressing

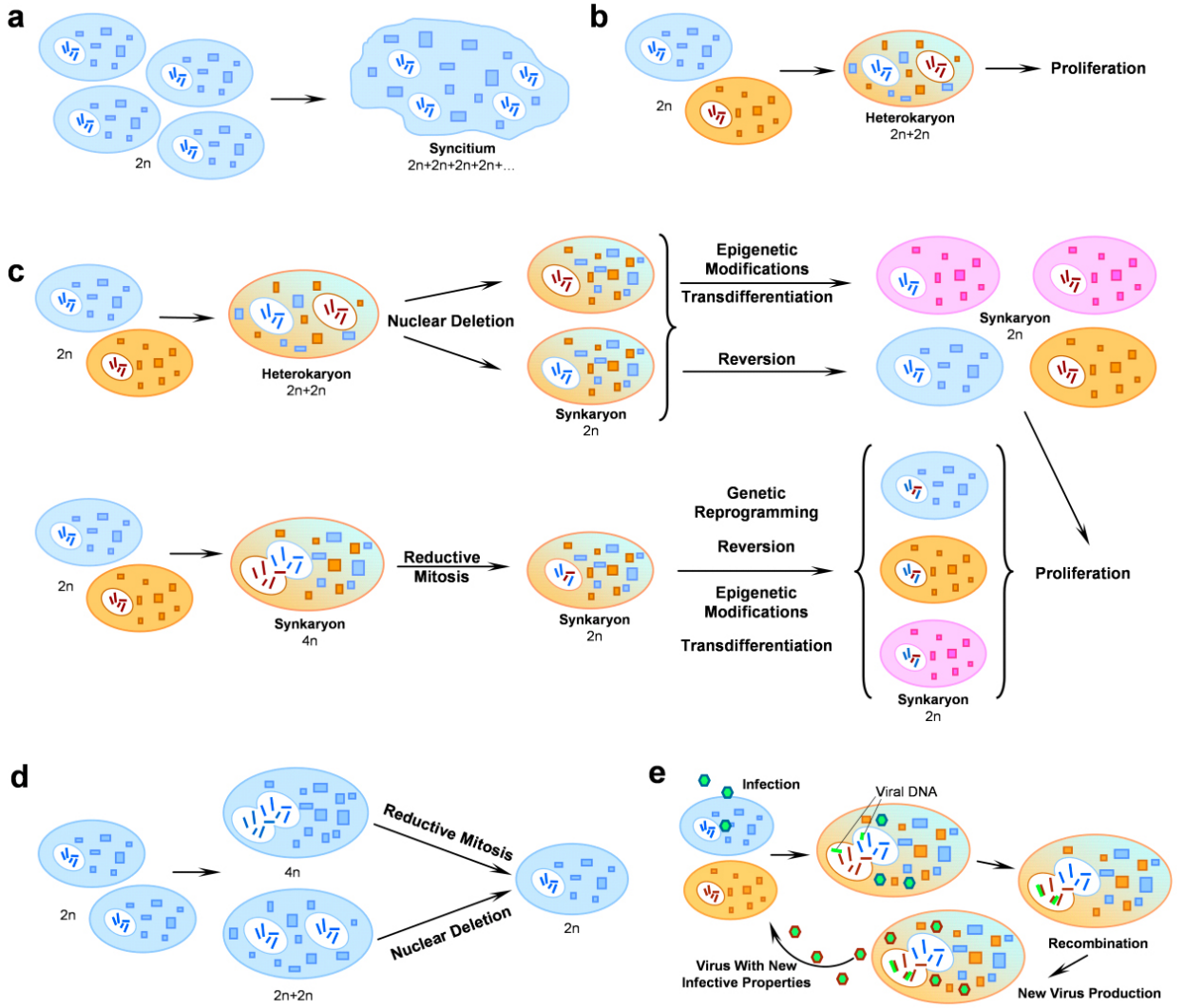

Figure 2. Cell Fusion Products. **A.** Cells of the same lineage fuse to form a giant cell with multiple nuclei, known as syncytium. Skeletal muscle and macrophages are examples of syncytia. **B.** Cells of different lineage fuse to form a cell with multiple nuclei, called heterokaryon. The stable heterokaryon might acquire new properties, being able to proliferate and differentiate. **C.** If a heterokaryon rearranges its multiple nuclei in a single nucleus we obtain a synkaryon. This process can take place by two different ways: deletion of supernumerary nuclei (upper panel), or by nuclear fusion and posterior reductive mitosis (lower panel). In the first case, fused cells mix their cytoplasm. This facilitates epigenetic modifications, what may lead to phenotype reversion obtaining a cell similar to the original one, or may lead to transdifferentiation originating a completely different cell type (pinkish colour). In the second case, nuclear fusion makes cells to mix DNA. This facilitates genetic reprogramming and acquisition of new phenotypes. It is important to note how, after all these processes (fusion, reprogramming, mitosis…), some of the final synkaryons are undistinguishable from the normal original cell types. **D.** Cells of the same lineage might suffer fusion and posterior nuclear rearrangement to obtain a single 2n nucleus. The new synkaryon would be very difficult to distinguish and detect due its similitude to original cells. Consequently, fusion events might be undercover and underestimated. **E.** Risks of cell fusion. Viral transfer is facilitated by cell fusion and posterior DNA recombination. After fusion, cellular and viral genomes are mixed suffering recombination in such a form that new virus might be able to infect new cell types.

cells fuse with R26R cells, Cre recombinase excises the floxed stop codon of the reporter gene in the R26R nuclei, resulting in expression of LacZ in the fused cells (Figure 1b-c). Consequently, fused cells can be detected easily by X-gal staining. We performed BMT into R26R mice using as donor a Cre-expressing mouse that, in addition, expressed the GFP (Figure 1d). In this way we can distinguish fused cells ($X\text{-}gal^{+}/GFP^{+}$) from transdifferentiated cells ($X\text{-}gal^{-}/GFP^{+}$). The detection method contributed substantially to confirm cell fusion in several tissues under normal (healthy, but irradiated) conditions. We confirmed cell fusion of hepatocytes and Purkinje cells with cells of the haematopoietic lineage after BMT. In addition, we showed cell fusion of

cardiomyocytes (43). No evidence of transdifferentiation was observed in these tissues. The only X-gal$^-$/GFP$^+$ cells detected were macrophages and microglia. These cell types are reported to be of haematopoietic origin, and they were proposed as the fusion partners (45). These results have been corroborated by others in the following years. Thus, HSC were shown to generate cardiomyocytes at a low frequency through cell fusion, but not transdifferentiation in infarcted hearts (46-48). Weimann *et al*. showed that fused Purkinje neurons form stable heterokaryons and increase in number with age (16). The bone marrow derived nucleus within the heterokaryon was reprogrammed and activated the expression of Purkinje neuron-specific genes. Finally, heat-shocked small airway epithelial cells were shown to fuse with MSC and form single nucleated synkaryons with a new gene expression profile (49).

Cell fusion had been verified, however, identification of cell partner/s with fusogenic properties in the pool of haematopoietic cells remained elusive. Recently, it has been reported that myelo-monocytic cells can fuse with cardiac muscle cells, and macrophages with hepatocytes, yielding heterokaryons in the case of cardiac muscle, and both heterokaryons and synkaryons in the case of hepatocytes (50-52). In contrast, in the skeletal muscle, a macrophage precursor in the myelo-monocytic population, but not the macrophage itself fuse (53).

4.3. Cell Fusion vs. Transdifferentiation: The Controversy

It's important to note that cell fusion and transdifferentiation processes do not exclude each other. They might take place independently under different conditions or could work in a succession of events. For instance, a bone marrow derived cell may fuse with a resident cell, reprogramming its genome and conferring the ability to transdifferentiate (Figure 2). This happens after fusion of cardiomyocytes with bone marrow cells *in vitro*. The new heterokaryon keeps the cardiomyocyte phenotype and, in addition, acquires a new proliferative ability (54).

If transdifferentiation and cell fusion processes are compatible, why is this issue so polemical? Mainly, the omission of alternative explanations of results and the wide variety of experimental conditions have led to confusion in the fusion/transdifferentiation fields. Let's analyze in detail the controversy and the interpretations of the results.

Most of the early transplantation experiments based their interpretations on the tracking of single markers such as LacZ, GFP or sex chromosomes. The first groups interpreted that bone marrow derived cells transdifferentiated only based on the presence of these markers in mature resident cells (12-15, 18, 27). However, the single presence of GFP or LacZ is not direct evidence of transdifferentiation. Moreover, ploidy analysis by FISH was not performed properly in early reports. FISH probes were exclusively used for Y sex chromosome detection; so, these groups never checked for the possibility of additional X chromosomes. When this was done, for instance in the liver and in brain biopsies, evidence of fusion arose (17, 21, 22). Posterior experiments have examined the presence of Y and X chromosomes. Detection of non-haematopoietic mature cells carrying a normal XY ploidy after a sex mismatched BMT has led some groups to argue against cell fusion and in favor of transdifferentiation. However, ploidy alone does not exclude the possibility that any given cell is derived from a fusion event. Chromosomal DNA might be eliminated by reductive division, as in somatic meiosis (55-57), or multipolar mitosis (the formation of multipolar spindles in mitosis) (58, 59). Thus, a fused polyploid cell may suffer a reductive mitosis, generating fused diploid cells that would be indistinguishable from a transdifferentiated or a resident cell (Figure 2c).

Experimental conditions have also contributed to confusion. Cell types used in transplantation experiments have been different in each research group. Whole bone marrow cells, MSC, and HSC isolated by sorting using different cell markers have been transplanted with diverse results. This variability makes difficult to compare results and reach a definitive generalized conclusion. Cell fusion seems to be present under normal conditions or low level injury (i.e. irradiation). In contrast, when tissue damage is extensive, a high level of contribution of transdifferentiated cells is observed. This has been used to refute cell fusion because the low frequency of fusion events cannot explain the observed high number of cells after BMT that contribute to the regeneration of some tissues. Here, an alternative explanation has often been omitted: fusion events may take place with few local precursors or resident SC. The new hybrid cells can later proliferate to generate thousands of cells to repair the damaged tissue. I already mentioned that fused cells may reprogram their genetic profile and acquire proliferative properties (54). I shall come back to the important topic of genetic reprogramming in section 6.

Neither transdifferenciation nor fusion can be discarded as cell plasticity mechanisms. Transdifferentiation has been shown *in vitro*, and examples *in vivo* have been reported, including BMDC-derived kidney epithelium, pulmonary epithelium, and pancreatic islets (18, 23, 32, 60). Cell fusion emerges as a better explanation for some results, rather than to contradict transdifferentiation. It is possible that both processes coexist in some organs. Alternatively, each process may be only able to generate certain cell types under tissue restrictive conditions that facilitate/impede any of them. Further efforts should be devoted to discern between these processes. A gold standard criterion for transdifferentiation or fusion demonstration should be applied (7). This would contribute to a better understanding of SC plasticity and posterior development of appropriate therapeutic strategies.

5. MECHANISMS AND BASIS OF CELL FUSION

Despite the importance of cell fusion in the development and physiology of multicellular organisms, little is known about the mechanisms underlying this process. What induces cell fusion? What controls the specificity of different cell fusion events? How do cells fuse? Is there a specific set of molecules specialized in this

process? These questions are open to speculation. The diversity of cell types that undergo fusion during development may indicate a wide range of molecules involved. However, mechanisms of membrane fusion, such as intracellular vesicle fusion and virus-cell fusion are the same for all cell types. The underlying processes during a fusion event, including cell-cell adhesion, alignment, and membrane mixing, are similar irrespective of the cell type. These observations suggest that different cell-cell fusion events may share common mechanisms. Fusion mechanisms and molecules involved are out of the scope of this review. For further information on membrane fusion and viral induced fusion refer to (1, 3, 61-65). I shall focus on what we can discern from our findings. We observed cell fusion of hepatocytes, Purkinje neurons, and cardiomyocytes with bone marrow derived cells (43). Why do these cells fuse and not others? What is in the base of these fusion events? To start answering these questions we should ask whether these cell types share any common characteristics.

Macrophages, the other fusion partner, migrate to injury locations and activate their fusion machinery for syncitia formation (4, 66). This cell type forms giant multinucleated cells in response to infections and to eliminate necrosed tissue or foreign bodies (4) (Figure 2a). Molecular machinery implicated in macrophage syncytia formation includes adhesion molecules, ligand interactions, and the induction of fusion by cytokines such as interleukin-3 (IL-3), interleukin-4 (IL-4), interleukin-13 (IL-13), gamma interferon (gamma-IFN), and granulocyte-macrophage colony stimulating factor (GM-CSF) (4, 67, 68). These molecules play an important role in organogenesis and inflammation, and, interestingly, their expression is shared by neurons, cardiomyocytes**,** and hepatocytes during development (69, 70).

Of special interest are the chemokine stromal-cell-derived factor 1 (SDF-1) and its receptor CXCR4. SDF-1 is secreted by bone marrow fibroblasts and is detectable in heart and skeletal muscles, liver, neural tissue**,** and kidney (69, 71, 72). It plays an important role in the homing/retention of HSC in these tissues. It works as a potent chemoattractant of CXCR4-positive cells, including macrophages and HSC (69, 71). CXCR4 mediates migration of resting leukocytes and HSC in response to SDF-1(73). It has been reported as a mediator in the fusion of HIV-1 virus to macrophages (74). CXCR4 plays an important role in heart and cerebellar development, and is involved in inflammatory or regenerative response to ischemia (73, 75). Accordingly, we can hypothesize that hepatocytes, cardiomyocytes, and Purkinje cells may express the same set of molecules involved in macrophage syncytia formation in situations such as, stress or tissue injury (71). In fact, SDF-1/CXCR4 expression is increased under pathological conditions such as, brain and muscle ischemia, toxic liver damage or total body irradiation (24, 72, 75, 76). In this way a cell of haematopoietic lineage (macrophage) expressing CXCR4 may be attracted and perform a fusion out of confusion. Alternatively, the macrophages could recognize these factors as real S.O.S. signals from the cells. Thus, fusion would take place to aid a cell in danger. In fact, response to injury has been proposed as a fusion inductor. Assays with parabiotic mice show that fusion occurs with no lesion (42). However, there are indications suggesting that tissue injury may stimulate the cell fusion process. Fusion events under normal conditions are scarce. In contrast, fusion levels are high in hepatic degeneration, likely due to positive selection of fused cells under degenerative conditions (21, 22).

There is an alternative motive for cell fusion. A direct relationship exists between cell size and ploidy status of the cell (77-81). If a cell needs to enlarge, its growth will be limited by the fact that a nucleus can control only a finite amount of cytoplasm. Purkinje neurons, hepatocytes, and cardiomyocytes are among the largest cells in the organism. They are extremely rich in mitochondria and consume energy avidly. We can speculate that a simple way for these cells to cope with their high metabolic demand would be the duplication of their energy factory (78). To achieve this, you would need a new foundation plan; an extra nucleus; a new set of genes. A well known mechanism used by cells to increase its genome is the endoreplication (78, 82). Alternatively, cell fusion with another cell emerges now as a possible mechanism.

6. IMPACT OF FUSION ON CELL BIOLOGY

Robert Hooke's observations of cork slices under the microscope led to the very first description of a cell in the 17th century. Later, in 1838, Schwann and Schleiden formulated the cell theory officially, adding a codicil in 1847 that described how walls and cavities of cells coalesce together. These observations suggested, for the first time, a cell fusion process. Since then, our knowledge of cell fusion has been gradually expanding. Currently, the discovery that haematopoietic cells fuse with several cell types arouses new biological implications (83). What is the contribution of the haematopoietic system to general development by cell fusion? Do haematopoietic cells participate by cell fusion more actively than expected in the formation of some organs, such as liver or brain? Do fused cells play a specific function in these organs different than non-fused cells?

An important consequence of cell fusion is the modification of cell theory. Ogle *et al*. have proposed to revisit the cell theory for a more plastic and dynamic notion of the cell (83). If cell fusion is occurring between cell types to generate a new cell with a single diploid nucleus, we will be unable to differentiate this fused cell from a normal cell (Figure 2c-d). Cell fusion might be occurring invisibly in many more organs than we previously thought. Certain cells would be able to fuse and interchange genetic and cytoplasmic material dynamically.

This dynamic concept of the cell leads to a second very important implication: cell fusion as a modifier of cell fate and gene program. Cell biologists have been fusing cells *in vitro* to study gene regulation since the 70's (84-86). They observed that both of the original sets of chromosomes are expressed and can interact with one another. Fused cells sometimes acquire the identity of their

partners, but on other occasions certain properties that formerly typified the cells disappear in the hybrids (84-86). Cell fusion, at least *in vitro*, can reprogram cell fate (85, 87). Fusion might reverse the developmental program of a mature cell towards a more immature cell with progenitor properties (88). Alternatively, a SC might change its fate, and therefore its function, by fusing with a mature somatic cell. Reversion or modification of cell fate/gene program by cell fusion can be achieved not only by the influence of one nucleus on the other, but also cytoplasmic factors might induce important epigenetic modifications (Figure 2c) (87). For instance, embryonic germ cells induce reprogramming of somatic nucleus in hybrid cells (89). Striking changes in methylation of the somatic nucleus, resulting in demethylation of several imprinted and non-imprinted genes has been observed. Changes affected gene expression with re-activation of the silent maternal alleles in the somatic nucleus (89). These observations *in vitro*, in conjunction with the regeneration of a damaged liver *in vivo*, are evidence that cell fusion might be a tool to modify gene expression patterns and owns a powerful therapeutic potential for post-mitotic tissue with lethal mutations. I shall develop this topic in the next section.

7. BIOLOGICAL POTENTIAL OF CELL FUSION

Apart from its normal contribution to development, cell fusion now has a broader biological potential than previously expected. It might contribute to tissue regeneration and be used therapeutically alone or in combination with other techniques such as gene therapy (90-92). However, the cell fusion process is not exempt of risks. Let's analyze its pros and cons.

7.1. Pros

I already mentioned throughout the text several examples of tissue regeneration by cell fusion. Muscle (skeletal and cardiac), brain, and liver are the organs where cell fusion has been demonstrated more accurately. Liver regeneration by cell fusion is so far the best documented and largely accepted. In this organ, it was convincingly shown that normal haematopoietic cells can restore the liver function in mice with a recessive lethal mutation by regeneration through the fusion of wild-type with mutant cells (21, 22). Furthermore, the exact subpopulation of bone marrow cells responsible for fusion in the liver has been identified: the macrophages (51, 52). The liver is the most promising organ where an effective therapy by cell fusion could be developed for humans in an immediate future (93, 94).

Rescue of muscular function by cell fusion has been shown in *mdx* mice, which have a condition that resembles Duchenne muscular dystrophy. Gibson *et al.* transplanted dermal fibroblasts into *mdx* mice and observed the formation of heterokaryons containing nuclei of mutant and wild-type fibroblasts (40). This fusion causes the phenotypic and functional reversion of muscular dysgenesis. Similarly, bone marrow derived cells were also able to migrate and fuse with skeletal muscle**,** restoring the expression of dystrophin in *mdx* mice, and recovering muscle function (27, 41, 95). Cell fusion not only works under pathological conditions derived from recessive genetic alterations; normal regeneration of a stress-induced or mechanical injured skeletal muscle is achieved by cell fusion with bone marrow derived cells, as well (24, 53). In humans, the ability of exogenous bone marrow cells to fuse with skeletal muscle of patients with Duchenne muscular dystrophy has also been reported (25).

In contrast to liver or skeletal muscle, the contribution of cell fusion to cardiac muscle regeneration after heart infarct has been very controversial (96). The work of Anversa's group generated great enthusiasm when they showed that HSC significantly repaired the infarcted myocardium (33). According to these investigators, HSC injection into the myocardium of rats undergoing ischemia could repair 60-70% of the damaged tissue by originating smooth muscle, endothelial and cardiomyocytic cells (33). This group also analyzed heart biopsies of female organ donors in male recipients a few weeks after heart transplant. Y chromosome (but not, in addition, the X chromosome to check for fusion) was used to follow recipient cells integrating into the parenchyma of transplanted heart (97). They found that the proportion of cells containing the Y chromosome and expressing markers of smooth muscle, endothelium or cardiomyocytes was very high (>20%). Several attempts to reproduce these results by other groups concluded with a significant reduction in these percentages (>1%), probably due to differences in tissue histology detection techniques and experimental conditions (98, 99). Transdifferentiation was postulated as the mechanism of generation of the myocardial cell subtypes after BMSC grafting. However, direct demonstration of transdifferentiation into cardiomyocytes after *in vivo* transplantation failed, and cell fusion emerged as the most likely mechanism to explain the low frequency generation of cardiomyocytes after heart infarct (46-48). Nonetheless, this low percentage of new cardiomyocytes regenerated by cell fusion was not able to explain the observed improvement of cardiac function after BMSC injection. Likely, these cells synthesize growth factors and have an anti-apoptotic effect on cardiomyocytes *in vivo* (96). In addition, BM cells contain endothelial precursors, which promote angiogenesis in the infarcted areas improving myocardial perfusion and viability (100).

In the brain, several groups have reported the presence of donor-derived neurons and glia after BMT. However, cell fusion has been exclusively demonstrated in Purkinje neurons of the cerebellum (16, 17, 43). These results suggest a cell fusion potential for the treatment of pathologies related with Purkinje neurons, such as ataxias, or neurodegenerative diseases. Interestingly, focal implantation or intravenous delivery of bone marrow derived cells improved brain function in models of cerebral ischemia, Parkinson's disease, Huntington's disease, and trauma (101-106). However, whether these pathologies are improved thanks to the generation of new neurons by cell fusion or, alternatively, due to delivery of growth factors and cytokines by transplanted cells remains to be resolved.

This compilation of regenerative experiments evidences the potential of cell fusion to correct recessive

mutations and to restore tissue functionality. As stated above, a serious handicap of the therapeutic application of cell fusion is its low frequency of events. However, the detection of cell fusion *in vivo* has been limited basically to multi-nucleated cells. If the heterokaryons so formed were to eliminate a nucleus or undergo nuclear fusion with posterior reductive mitosis, the frequency of fusion might be grossly underestimated (Figure 2d). Furthermore, I already mentioned the proliferative properties of newly formed synkaryons, which might contribute to regeneration more substantially than expected. The identification of stimuli and the molecular machinery of cell fusion will make possible the increment of fusion events up to effective levels for therapy.

An additional advantage of cell fusion mediated regeneration, when considering ways to effectively repair in highly specialised and integrated environments, such as the adult brain, is the preservation of the structural complexity in the damaged tissue. In contrast to focal cell transplants that need to recreate the whole organ structure, cell fusion takes place within the original organ scaffold.

Finally, an advantage of fusion process is its utilization as a means of gene transfer. Thus, cell fusion may be used in conjunction with gene therapy. For instance: macrophages, the main fusion partner, enter the brain specifically attracted to the sites of neuronal damage (66). Genetically modified macrophages might be used as vectors for delivery of drugs, growth factors, or cytokines. Cell fusion could help to target this delivery into a specific cellular subpopulation, such as Purkinje neurons (91, 92).

7.2. Cons

Cell fusion is not excluded of some risks. It is recognized as a factor in cancer promotion and even tumorigenesis (107). The result of cell fusion is a polyploid heterokaryon or synkaryon. Polyploidy is not always associated with disease; some polyploid animals are viable (108). In fact, it is beneficial and contributes to the development of new species, including the primates (109-111). However, it is clear that acquisition of additional chromosomes and centrosomes in the fusion process may lead to aberrant chromosome segregation and aneuploidy on proliferation of the fusion product. I already mentioned that macrophages are the main fusogenic partners. Interestingly, fusion of tumour cells with lymphocytes or macrophages can render a tumour metastatic (112, 113). Cell fusion can also promote tumour progression by increasing malignancy in the resulted hybrid cell, amplifying its drug resistance, conferring the ability to metastasize, and contributing to tumoural diversity (114-117). On the other hand, cell fusion might be used as a therapeutic tool against cancer. Cells with recessive mutations of tumour suppressor genes or other genomic defects could be rescued by cell fusion, especially if they emit the appropriate signals, similarly to stressed hepatocytes, myocytes, and Purkinje neurons, which are recognized by macrophages. Cell fusion might also be used to eliminate existing cancer cells by targeted incorporation of antitumoural agents with the help of genetically modified macrophages (118, 119).

More recently, cell fusion has been shown to be harmful due to its potential as a mechanism of viral transfer (Figure 2e). Ogle. *et al*. engrafted human bone marrow cells in foetal pigs for xenotransplant studies (120). They analyzed whether porcine endogenous retrovirus (PERV), which is quiescent in pigs, might transfer to human cells. They found that human cells that remained in the pigs contained both human and porcine chromosomal DNA, confirming cell fusion. These hybrid cells divided, expressed human and porcine proteins, and contributed to porcine non-haematopoietic tissues. More disturbing, the hybrid cells were able to transmit this virus to uninfected human cells *in vitro* (120). Thus, spontaneous fusion can occur *in vivo* between the cells of disparate species and could explain the generation of novel pathogens by recombination of selected DNA sequences (83, 120).

8. CONCLUSIONS

Cell fusion has emerged as a powerful process with a prominent role in biology. Together with transdifferentiation provides a mechanism to explain SC plasticity. Both mechanisms are valid, depending on tissue and conditions. But, beyond this controversy, cell fusion opens new expectations in reparative medicine. Its newly revealed biological implications range from modification of cell theory to correction of genetic alterations and tissue regeneration, which have an enormous clinical potential. However, cell fusion may also promote or transfer diseases. We need to fully understand the cell fusion mechanisms before to consider it as clinically relevant. For this, we should face a rigorous identification of the tissue-specific and injury-related signals that recruit, stimulate or regulate the fusion process. A better characterization and expansion of the cell populations with fusogenic properties would be necessary as well. We should be hopeful in view of the experimental results with mice, but we should not forget that effectiveness and safety must be warranted before cell fusion can be used as a therapy for human diseases. Cell fusion is an exciting and promising research field. Further efforts should be devoted to investigate the mechanisms that govern it.

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Abbreviations: SC: Stem cells, ES: Embryonic stem cells, BMSC: Bone marrow stem cells, HSC: Hematopietic stem cells, MSC: Mesenchymal stem cells, BMT: Bone marrow transplant

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