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REVIEW ARTICLE

# ES, iPS, MSC, and AFS cells. Stem cells exploitation for Pediatric Surgery: current research and perspective

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**Abstract** Despite the advancements that have been made in treating infants with congenital malformations, these still represent a major cause of disease and death during the first years of life and childhood. Regeneration of natural tissue from living cells to restore damaged tissues and organs is the main purpose of regenerative medicine. This relatively new field has emerged by the combination of tissue engineering and stem cell transplantation as a possible strategy for the replacement of damaged organs or tissues. This review would like to offer an insight on the latest evolution of stem cells with a glance at their possible application for regenerative medicine, particularly in the Paediatric Surgery field.

**Keywords** Stem cells · Regenerative medicine · ES · iPS · MSC · AFS cells · Pediatric Surgery

## Introduction

Congenital malformations represent a major cause of disease and death during the first years of life and childhood and this is mostly due to complex conditions in which prosthetic materials are used because of the lack of biocompatible tissues able to replace or regenerate damaged organs. Besides the risk of infection, the major drawback of using a prosthetic patch closure is the risk of dislodgment and subsequent recurrence of the initial problem. Moreover, foreign body reactions and implant rejection occur when synthetic polymers are used. Regeneration of natural tissue from living cells to restore damaged tissues and organs is the main purpose of regenerative medicine. This relatively new field has emerged by the combination of tissue engineering and cell transplantation as a possible strategy for the replacement of damaged organs or tissues. So far, most of the attention has been focused on degenerative diseases such as Parkinson or Alzheimer, while very little has been done for the treatment of congenital conditions. However, the knowledge acquired in the last years from stem cell biology and regenerative medicine strategies could lead to new ways of repairing or replacing injured organs and systems, even during fetus development and therefore paediatric patients could largely benefit from the evolution of this new exciting field. In order to give rise to a new functional organ-like structure, several variables, such as local environment, nutrients, and metabolites are pivotal. These variables, in the contest of tissue engineering, are mainly dependent on the provision of a three-dimensional growth structure termed "scaffold" [1]. Scaffolds are usually made by natural materials, which are essentially bioactive but lack mechanical strength, or synthetic materials, which lack inherent bioactivity but are mechanically strong and can be engineered with the desirable macro-, microstructure, and might possess desired bioactive properties to make possible cellular growth and organogenesis [2]. Despite scaffolds could ultimately represent the exclusive tool for tissue engineering and several attempts to generate whole organs, such as liver, have been done by developing structures with vascular channels to ensure an adequate network of vascular supply [3], major developments in regenerative

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medicine have been achieved after the discovery of stem cells (SCs). These cells are unspecialized or undifferentiated cells with the capacity of self-renewal and the power to give rise to multiple different specialized cell types [4]. Three are the main sources SCs in human and animals: from embryonic, fetal, and adult tissues.

Adult SCs have a limited cellular regeneration or turnover that could represent a limitation for tissue engineering application where a large number of cells is necessary [5]. They can be identified in many adult mammalian tissues, such as bone marrow, skeletal muscle, skin, and adipose tissue, where they contribute to the replenishment of cells lost through normal cellular senescence or injury [6–10]. In contrast, SCs derived from embryonic sources have the ability to give rise to cells that not only proliferate and replace themselves indefinitely, but also have the potential to form any cell type [11, 12]. ES cells are derived from the inner cell mass of pre-implantation embryos, are pluripotent and demonstrate germ-line transmission in experimentally produced chimeras [13, 14]. More recently, cells with intermediate potential could be derived from the amniotic fluid (fetal SCs) [15] or reprogramed from adult SCs using various factors implicated with the maintenance of pluripotent potential of ES cells [16]. This review would like to offer an insight on the latest evolution of SCs with a glance at their possible application for regenerative medicine, particularly in the Paediatric Surgery field.

## Embryonic Stem cells

Embryonic stem (ES) cells derive from the inner cell mass of a blastocyst stage embryo [17]. They are pluripotent and give rise during development to all derivatives of the three primary germ layers: ectoderm, endoderm, and mesoderm; hence, they possess the potential to develop into most of the cell types within the body [13, 18, 19]. The field of ES cell research began with the study of teratocarcinoma cells in 1950s, continued with first mouse ES cell lines derived from the inner cell mass of blastocysts using culture conditions (fibroblast feeder layers and serum) in 1981 and expanded in 1998 when Thomson et al. [13, 20, 21] first derived human ES (hES) cells. Optimal culture conditions have been developed employing both mouse embryonic stem (mES) or hES cells to evaluate and maintain both their proliferative and differentiative capacities. mES cells are grown on a layer of gelatin and require the presence of Leukemia Inhibitory Factor (LIF) while hES cells are grown on a feeder layer of mouse embryonic fibroblasts (MEFs) and require the presence of basic Fibroblast Growth Factor (bFGF) [18]. The maintenance of pluripotency in the hES is assured by the presence of different transcription factors like Oct-4, Nanog, and SOX2 that are essential to ensure the suppression of genes that lead to differentiation [22]. The cell surface antigens most commonly used to identify hES cells are the glycolipids Stem Cells Embryonic Antigen-3 and -4 (SSEA3 and SSEA4) and the keratan sulfate antigens Tra-1-60 and Tra-1-81 [9]. ES cells could be used not only to generate tissues, but also could be employed as "cellular models" to study a range of human diseases, and to test new drug candidates for efficacy and toxicity [23]. ES cells, being pluripotent, require specific signals for correct differentiation and if injected in vivo prior commitment, they will give rise to many different types of cells, causing teratomas. So far their potentials, together with the difficulties related to their allogenic origin, have limited their possible clinical applications [24]. In particular, the political debate surrounding SCs began suddenly after hES creation because of the destruction of the derivative embryo. Recently, researchers opened the possibility of generating ES cell lines without destroying embryos by deriving cells from the early development of the embryo without impairing their further development [25–27].

Somatic Cell Nuclear Transfer (SCNT) has also been adopted to create patient-specific SCs and avoid problems related to the creation of allogenic tissue. This procedure entails specifically the removal of an oocyte nucleus in culture, followed by its replacement with a nucleus derived from a somatic cell obtained from a patient. SCNT technique was first reported by Briggs and King [28] and some years after was used to obtain the first vertebrate (a frog) [29]. Cells yielded by this induction would be genetically identical to the donor and would not be rejected by the patient. SCNT can potentially be used for three purposes: (a) reproduction, leading to generation of an embryo for continuation of life (a notable example in 1996 was the generation of the first mammal, a sheep named Dolly, derived from an adult somatic cell by the use of this technique [30]); (b) therapy, generating blastocysts for SC derivation; and (c) research and regenerative medicine. The first is scientifically and ethical condemned. The second has important implications for the future of ES therapies, allowing the production of non-immunogenic ES lines. Besides, these cells could be stored and used subsequently for the treatment of future medical conditions. As a consequence this could be relevant for the creation of autologous tissues also in children who are born with complex malformations in which tissue viability represent a problem. Patient-specific cells could be created in vitro. ES cells derived using SCNT would have the same genetic background of the patient who has donated the initial genetic material and the tissue created would not be rejected after transplantation. ES cells have in fact the advantage of being extremely plastic facilitating the in vitro engineering of complex organ such as heart, liver, and

kidney [31–33]. Nevertheless, in spite of the ethical considerations, the limitation of this technique is related both to the low efficiency, leading to a high loss in cell yield and the inadequate supply of human oocytes [34].

## Induced Pluripotent Stem cells

Since the major objection to hES research is the destruction of embryos, it would be advantageous to develop a method of creating SCs that overcome this hurdle. A considerable step ahead was represented by the generation of the induced pluripotent stem (iPS) cells. The production of iPS cells with quasi-identical genetic and functional properties offers the possibility to bypass both moral conflicts and different genetic background inherent to the technologies mentioned above. iPS are pluripotent stem cell developed from a non-pluripotent cell, usually an adult somatic cell, by causing a forced expression of several genetic sequences and were first produced in 2006 by Takahashi and Yamanaka from mouse somatic cells. The key genes *Oct3-4* (POU5F1), the transcription factor *Sox2*, *c-Myc* proto-oncogene protein and *Klf4* (Krueppel-like factor 4) were sufficient to reprogram mouse fibroblasts to cells closely resembling mouse ES cells [16]. The insertion of these sequences is usually achieved through transfecting viral vectors, like retroviruses. After 3–4 weeks, small numbers of transfected cells begin to become morphologically and biochemically similar to pluripotent SCs, and are typically isolated through morphological selection, doubling time, or through a reporter gene and antibiotic selection. Although the initial mouse iPS cells did not contribute to full-term pregnancies chimeras, subsequent modification of the procedure to select iPS cells based on the reactivation of *Oct4* or *Nanog* promoter resulted in iPS cells that more closely resembled mouse ES cells, including the ability to contribute to germlines [35]. Despite the high similarity between mouse iPS and ES cells, tumor formation in iPS cell chimeric mice was high, presumably due to the expression of *c-Myc* in iPS cell-derived somatic cells [36]. Subsequently, Yamanaka successfully transformed human fibroblasts into pluripotent SCs using the same four key genes: *Oct3-4*, *Sox2*, *Klf4*, and *c-Myc* with a retroviral transfection. Subsequently, Thomson and colleagues [37, 38] used *Oct3-4*, *Sox2*, *Nanog*, and a different gene Lin28 using a lentiviral system, improving transduction output. The viral transfection systems used to insert the genes at random locations in the host's genome created concern for potential therapeutic applications of these iPS, because of the retroviral integration might increase the risk to form tumors [39]. To overcome these dangers, adenoviruses to transport the four sequences into the DNA of mice somatic cells have been used, resulting in cells identical to ES cells. Since the adenovirus does not combine any of its own genes with the targeted host, the increase danger of creating tumors is also eliminated [40]. Yamanaka and co-workers [41] demonstrated that reprograming can be accomplished via plasmid without any virus transfection system at all, although at very low efficiencies. Human iPS cells show morphological resemblance to hES cells, express typical human ES cell-specific cell surface antigens and genes, give rise to multiple lineages in vitro, and form teratomas when injected into immunocompromised mice. The efficiency of reprograming adult fibroblasts has been low (<0.1%) so far but, since reprogramed clones could consistently recovered and expanded with the existing gene combinations, for practical applications, the low reprograming efficiency itself is not really considered an issue, unless reprograming selects for abnormal genetic or epigenetic events that are stably propagated in the resulting iPS cell lines [42].

Recently, Jaenisch group found a very elegant way to derive human iPS from somatic cells of patients free of reprograming factors using Cre-recombinase excisable lentiviruses. The efficiency of reprogramed iPS is very high with a low number of proviral vector integration, the cells maintain a gene expression profile more similar to hES than to human iPS and can be subsequently differentiated into specific tissue [43]. This methodology could be considered in the future as alternative to ES cells created by SCNT. Somatic cells could easily be derived from the skin of a child with a malformation, reprogramed and differentiated after obtaining patient-specific ES cells. The tissue obtained will match completely with the patient and it will not be rejected; however, the tumorigenic potential remains unclear and the clinical use is subjected to further animal experiments.

## Mesenchymal Stem Cells

Adult individuals equally contain stem cells but their characteristics are quite different from ES cells. Adult stem cells are considered as less proliferative, more mature with a narrower differentiation potential but a safer resource in respect of ES cells. They are virtually present in all adult tissues but, because of their implication for tissue regeneration in paediatric surgery, only mesenchymal stem cells (MSCs) will be discussed here. MSCs are multipotent SCs that can differentiate into a variety of cell types, first harvested from bone marrow via plastic adhesion, with a fibroblast-like morphology and differentiation potential into osteogenic (bone), chondrogenic (cartilage), and adipogenic (bone marrow stroma) lineages "in vitro" [44]. Some studies demonstrated that MSC can also differentiate to other cell types of mesodermal origin (skeletal muscle,

smooth muscle, cardiac muscle, endothelial cells) but not solid experiments with in vivo transplantation of the progeny of a single cell could finally demonstrate terminal differentiation. To date, MSCs have been isolated in the fetus from blood, liver and bone marrow, amniotic fluid, lung, pancreas, dental pulp, and periosteum [45–50]. They have also been isolated from umbilical cord blood, Wharton's jelly, placenta, and amniotic fluid [51–54]. The definition "mesenchymal stem cells" has been considered unclear through the years; "mesenchymal" was based on the hypothesis that multiple tissues beyond skeletal lineages, such as skeletal muscle, myocardium, smooth muscle could be generated by MSCs and secondly during embryonic organogenesis. The postnatal MSCs related tissues, are generated by a system of distinct progenitors, rather than from a common precursor. Dealing with this problem, three major criteria have been introduced to define MSCs by International Society for Cell Therapy [55]. First, cells must be plastic-adherent when maintained under standard culture conditions. When measured by flow cytometry, >95% of the cell population must express CD73 (5′-nucleotidase ecto, NT5E), CD90 (Thy-1) and CD105 (SH2 or MCAM or endoglin), LNGFR (Low affinity Nerve Growth Factor Receptor), CD166 (ALCAM adhesion protein), CD146 (P1H12), CD29, CD106 (vascular adhesion molecule-1, VCAM-1) and >98% of the cells should be negative for hematopoietic cell surface antigens: CD45, a pan-leukocyte marker; CD34, a marker of primitive hematopoietic progenitors and endothelial cells; either CD11b or CD14, markers for monocytes; either CD19 or CD79a, B-cells markers and Human Leukocyte Antigen II (HLA Class 2). Finally, to be defined as MSCs, cells should be capable to differentiate into osteoblasts, chondroblasts, and adipocytes when placed into an appropriate induction/differentiation medium. Among the MSCs collected from different tissues, there is no clear evidence of phenotypic differences in surface antigen expression. However, the success rate of MSCs isolation varies among tissues. MSCs can be isolated from only 63% of cord blood samples, while they can be easily derived from 100% of both bone marrow and adipose tissue processed [56]. Many scientific reports indicate that MSCs possess immunomodulatory properties and may play specific roles as immunomodulators in transplantation tolerance, autoimmunity, as well as fetal-maternal tolerance [57]. MSCs suppress T cell proliferation, but express different ligands that are recognized by activating NK receptors that trigger NK alloreactivity. Treatment of MSCs with IFN-gamma up-regulate expression of HLA class I molecules and decrease NK activity [58]. Recently, it has been supposed that MSCs may exert a more significant role through the release of different factors via paracrine action, rather than adopt a particular differentiated state after engraftment in target tissue [59]. In contrast with the aforementioned cells, MSCs also have a limited life span and become senescent when cultured in vitro. Several mechanisms over the progressive loss of telomeres were invoked to explain the acquisition of this phenotype and various experimental strategies have been adopted to extend MSCs life span [60–62]. Proliferation capacity of MSCs can be significantly increased by the presence of oncogenes (E6–E7) from HPV. Unexpectedly, transfected MSCs showed no signs of neoplastic transformation [63]. Nevertheless the acquisition of neoplastic features in these engineered cells could not be totally excluded and might occur. Regardless the isolation procedure, MSCs quantity obtained from primary tissues is not sufficient for any downstream application in clinical settings. In vitro expansion can affect biological properties of the cells; in fact MSCs go through very significant changes in phenotype and gene expression as a result of cell culture adaptation. Although considered a safer source, if compared to ES, the prospective clinical applications of MSCs require a meticulous examination. Some approaches aiming at improving safety have been established to evaluate the possibility of eliminating xenoproteins or xenoproducts like fetal calf serum in the feeding medium, to reduce the risk of potential viral-transmission-like unidentified zoonoses or prions and reduce immunogenicity related to serum component absorption [64]. The ability of MSCs to give rise to different lineages has been a matter of intense studies and plasticity and mechanisms of action have been studied in models of small and big animals. MSCs can differentiate beyond their traditional mesodermal lineage, at least in vitro, into both ectodermal (neurons) and endodermal (hepatocytes) nature [65–67]. However, broad abilities of MSCs are questionable and in several publications it has been demonstrated that MSCs do not undergo a proper trans-differentiation (irreversible switch of one differentiated cell into another), but rather fuse with specialized differentiated cells, thus more studies are required to a better understanding of this issue [68]. To date, MSCs have been tested on pediatric patients for several clinical indications, like inborn error of metabolism (Metachromatic leukodystrophy, Hurler syndrome, Infantile hypophosphatasemia), osteogenesis imperfecta, and GVHD [69–73]. Preliminary studies have been assessed in patients with amyotrophic lateral sclerosis and autologous MSC transplantation has also been evaluated in patients after acute myocardial infarction [74, 75]. For the engineering of mesodermal-derived tissues, MSCs certainly represent at the moment the optimal source: in the close future children with bone, smooth muscle, or cartilage defects could have their tissue loss replace using MSCs derived from their bone marrow. MSCs derived from other sources such as the placenta or the amniotic fluid have also shown to be beneficial in animal models of congenital malformations

[76, 77]. While clinical applications of MSCs are progressing, basic research is carrying on big efforts to understand better cell properties and abilities through the analysis of the molecular mechanisms causing the evident clinical benefits after MSCs therapy.

## Amniotic Fluid Stem cells

While ES or iPS cells have the limitation of being difficult to program and could let tumor formation in vivo and MSCs are difficult to expand in vitro, it would be ideal to have a source of cells capable to overcome all the different problems. We have recently described the possibility of deriving pluripotent stem cells from the amniotic fluid. Amniotic Fluid Stem (AFS) cells represents about 1% of the whole cells in cultures of human amniocentesis specimens obtained for prenatal genetic diagnosis and can be harvested by immunoselecting the antigen c-Kit (CD117) positive population [78]. AFS cells are described as broadly multipotent SCs that can differentiate into a variety of cell types. AFS cells have been shown to differentiate to adipogenic, osteogenic, myogenic, endothelial, neurogenic, and hepatogenic lineages, inclusive of all embryonic germ layers [15]. This group of cells can be steadily expanded in cultures, has a typical doubling time of 36 h and do not need any feeder layer. Sub-confluent cells showed no evidence of spontaneous differentiation, nevertheless, under specific inducing conditions these cells are able to differentiate and if injected in vivo, showed no evidence of tumor growth in severe combined immunodeficient mice. The AFS cells are positive for a number of surface markers characteristic of mesenchymal and/or neural SCs, but not ES cells, as CD29, CD44 (hyaluronan receptor), CD73, CD90, and CD105 (endoglin). Human AFS cells are positive for stage-specific embryonic antigen (SSEA)-4, also expressed by ES cells. Moreover, more than 90% of the cells express the transcription factor Oct4, which has been associated with the maintenance of the undifferentiated state and the pluripotency of ES and EG cells [79]. Retroviral marking using a vector encoding green fluorescent protein identified differentiated positive subclones descended from a single cell. AFS cells appeared to be less plastic than ES cells, nevertheless reproducibility of the generation and differentiation of these SCs has not yet been widely reported and future studies are required to assess the potential broad use of these promising resource. In the paediatric field, however, they could play an important role for prenatally diagnosed structural defects, there is the possibility of obtaining homologous cells at the time of invasive sampling; fetal cells could be harvested, cultured, and manipulated in vitro, during the remainder of pregnancy and later used for tissue engineering of graft material that will be used for postnatal reconstruction. Moreover, they could also be stored for future use (Table 1).

## Conclusion

In this scenario, the use of AFS and iPS [80, 81] cells could bring together researchers working for the common aim to develop new protocols to treat diseases and congenital malformations without ethical problems, although, the most up-to-date work of elegant iPS derivation is burdened with problems related to teratoma formation and possible altered epigenesis of iPS derived tissue. Regarding ES cells

**Table 1** Main characteristics of the described stem cell populations: ES, iPS, AFS, MSCs

| | ES cells | iPS cells | AFS cells | MSCs |
|---|---|---|---|---|
| Source | Early stage embryo | Somatic cells | Amniotic fluid | Bone marrow and other adult tissues |
| Feeders | Required | Required | Not required | Not required |
| Markers | SSEA3/4 Tra1-60/1-81 | SSEA3/4 Tra1-60/1-81 | SSEA4-c-kit | CD73 CD90 CD105 |
| Plasticity | Pluripotent | Pluripotent | Broadly multipotent | Multipotent |
| Tumorigenesis | Yes | Yes | No | No |
| Doubling time (h) | 31–57[a] | 48[b] | 36[c] | Variable[d] |
| Lifespan in vitro | Long | Long | Long | Short |
| Ethical issues | Yes | No | No | No |
| Animal model | Therapeutic | Therapeutic | Therapeutic | Therapeutic |
| Clinical application | No | No | No | Yes |

[a] Ref. [87]

[b] Ref. [38]

[c] Ref. [15]

[d] Different sources (from 12 h up to several days)

generation, this year a new impulse to research has been given in the USA by an executive order lifting restrictions on federal funding for stem cell research, which erased limits imposed 8 years ago by the former government. Despite the hurdles represented by cell expansion (doubling time 36–48 h), immunorejection and safety concerns, hESC-derived tissues after modification may have a promising future for transplantation thanks to the typical versatility of these cells. Adult SCs like MSCs have already been used for infusion in various clinical therapies in a relatively large number of individual, including patients of pediatric age, without any serious adverse effects [64]. Apart from the direct infusion of SCs, other applications such as in the surgical field have also been proposed. Encouraging results from the clinical application were achieved lately by the first successful transplantation of a tissue-engineered trachea built in a bioreactor, seeded with autologous cells in an adult with bronchial stenosis [82]. Adequate preclinical models together with the conclusion of ongoing clinical trials, will contribute to the establishment of SCs therapeutic potential in pediatric patients with congenital defective malformation such as oesophageal atresia that requires segmental replacement, diaphragmatic hernia, abdominal wall defects, in addition it might be possible to shift traditional surgical disease like Hirschsprung's toward a medical setting by injection of enteric nervous system SCs, harvested from postnatal gut and transplanted into aganglionic to refill the insufficient neuronal network of the intestine wall [83]. Ultimately, SCs may protect the injured intestine in diseases such as necrotising enterocolitis (NEC), reducing the severity of bowel damage, promoting proliferation, and enhancing vascularization [2, 84], and in case of massive intestinal resection occurred after neonatal volvulus or NEC, might be in the future a valid alternative to intestinal transplantation [85, 86].

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