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THE IMPACT OF STEM CELLS IN REGENERATIVE MEDICINE

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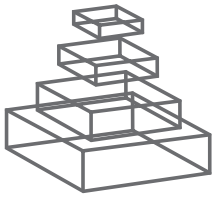

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THE IMPACT OF STEM CELLS IN REGENERATIVE MEDICINE

Topic Editor:
Anis Feki, Hopitaux Fribourgeois, Switzerland

Human embryonic stem cells (hESCs) are pluripotent cells which can differentiate into all cell types needed for cell therapy of severe diseases. However, the immunogenicity of hESCs is not yet well understood; therefore immuno-rejection after grafting is not excluded. In addition, the number of human embryos available for derivation of large numbers of hESC lines is limited and may raise ethical charges.

The recently successful reprogramming of mouse and human fibroblasts resulting in iPS cells has opened the door to the possibility of generating patient-specific pluripotent cells. Four RGs (either Oct-4, Sox2, c-Myc and Klf4 or Oct-4, Sox2, Nanog and Lin28) were shown to be sufficient to reprogram human fibroblast to undifferentiated iPS cells. The derived iPS cells had normal karyotypes, expressed telomerase activity, expressed cell surface markers and genes that characterize hESCs, and maintained developmental potential to differentiate into advanced derivatives of all three primary germ layers.

The impact of this field in basic research and in regenerative medicine led as to dedicate a Research Topic about this theme. This Research Topic will display the different methods of derivation of hESC and iPS cells, the understanding of the mechanisms ruling the reprogramming process, the use of such cells to understand organ physiology and to dissect diseases pathways, and of course we have to develop a chapter on the ethics point of view.

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EDITORIAL
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"XXIst century odyssey of Medicine" stem cells and their future

J. Bouquet de la Jolinière and A. Feki*

Department of Reproductive Biology and Obstetrics and Gynecology, Fribourg Hospital, Fribourg, Switzerland
**Correspondence: anis.feki@h-fr.ch*

Edited by:
Thimios Mitsiadis, University of Zurich, Switzerland

Keywords: pluripotent stem cells, iPS cells, IVF, stem cells, embryonic stem cells

The last century is undoubtedly the one of discoveries which have changed the destiny of humanity in a very short time. Quantum physics have opened the door of nanotechnology, information technologies, and their multiple applications in medicine. The discovery of penicillin and antibiotics has helped with the development of a safe and aseptic surgery. Moreover, microbiology, molecular biology, genetics, and the human genome sequencing have given us knowledge and wonderful tools to help to understand the mechanisms that rule life-sciences. They have also given us the technology to study gene transmission and their expression. Thus, it is nowadays possible to treat many diseases, for example in the field of oncology. Last, organ transplantation is part of these medical innovations.

During these discoveries, especially those related to IVF, ethical problems have arisen during the cloning of embryos, attempt of embryos manipulation, and embryonic stem cells derivation for therapeutic use. Pluripotent stem cells and deriving induced pluripotent stem cells represent the greatest medical advance of this century and were awarded the Nobel Prize for Medicine in 2012 (Yamanaka, 2012). Therefore, hope has arisen in several areas like neurology, cardiology, tooth regeneration, and reproductive medicine.

If it is now possible to consider repairing organs by autologous grafting like after myocardial infarct, or spinal cord injury (Hibaoui and Feki, 2012), the approach using pluripotent stem cells in reproductive medicine raised ethical and scientific questions. Obtaining functional sperm or oocytes from skin derived-iPS cells may lead to the disappearance of the term of infertility, and IVF programs would benefit from this development. Stem cell field also offers other possibilities like for pharmacology of drug testing, disease modeling, and the discovery of new gene functionalities.

The reviews of this special issue treated several of the aforementioned fields. This century will be the one of stem cell biology and genetics: the humankind will be attracted by findings of this science. It will not create but will try to repair, to live longer, and to rejuvenate. If Einstein believed that genius is intuition, we have enough models at our disposal to take over the following meaningful sentence of Master Eckhard who said: "it is not our actions that sanctify us, but, we who sanctify our actions."

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A new, most likely unusual approach is crucial and upcoming for the use of stem cells in regenerative medicine

Virginia Tirino and Gianpaolo Papaccio*

Tissue Engineering and Regenerative Medicine Laboratory, Department of Experimental Medicine, Second University of Naples, Naples, Italy
**Correspondence: gianpaolo.papaccio@unina2.it*

To date many articles and reviews have been devoted to the use of stem cells in regenerative medicine. Of the previous, some articles, about 20%, are interesting and constitute an advance in knowledge, the majority (50%) are of marginal interest, and some (30%) are completely not useful, although they can be found in literature.

The emerging major problems affecting the impact of this field in patient's cure can be mainly found in the high costs of procedures and in other problems that need to be fully addressed by the scientific community, that we evidence below.

The following major questions must be still addressed for a true enhancement of the practical use of stem cells in patient's cure:

(1) New methods for GMP stem cell cultures, dedicated to grafts, must be used, including, for instance, microgravity, omega3 fatty acids, new oxygen bioreactors; this is a major point, of interest and that can be addressed by both biologists, physicians, and engineers;
(2) Ascertain genetic stability of stem cells before and after grafting is another key step. A schedule should be delivered and published on this issue by researchers, often after having obtained data of graft trials;
(3) The problem of quality and quantity of cells must be unambiguous and a general rule established. Many researchers try to demonstrate the purity of stem cell populations and try to obtain a translation of their findings. This point presents several ambiguities, mainly residing in the purity judgment itself;
(4) Interactions with already existing cancer stem cells in patients to be grafted is another great problem to be discussed and rules needed. Niches of cancer cells may already reside in the patient where a stem cell graft has been prepared and performed. This can be a serious problem depending on the response of stem cells to cancer because opposite opinions and effects exist at present on this topic;
(5) Cell selection, as well as GMP procedures, must be deeply discussed. The search of a high purity and selection decreases the quantity of stem cells and their effectiveness; GMP procedures are difficult to be followed, their costs are too high and cannot be supported by a large patient number (d'Aquino et al., 2009). Bureaucracy procedures, not only in Europe, are long, difficult, often require to "*de novo*" design the graft design and are required for each protocol and often the trials cannot be done.

Therefore, is it possible to have a new, different, and provocative approach? Can we answer to the following questions?

(a) How is possible to perfectly measure and standardize quality and quantity of stem cells? Are we sure that we are in search of such a purity (Tirino et al., 2011)?
(b) A "full" characterization and selection of stem cell populations decreases their quantity and/or potentials: is this necessary or needed all the time?
(c) How is possible to overcome the high costs of GMP procedures and reach the goal of a large-scale use of stem cells?
(d) Do stem cells can be still studied and used as components of a whole tissue/organ (house to build) or as a vehicle of molecules and as a terrific recruitment for other cells?
(e) It would be possible and more "natural" to follow and recapitulate the embryonic development and physiological tissue repair processes (mainly niches for bone);
(f) It would be more easy and effective to activate inflammation or other mechanisms for endogenous stem cell recruitment and proliferation. In other terms, should we stimulate the physiological role of endogenous stem cells instead of their grafting?
(g) The research of "ideal" stem cells, with a full and standardized characterization/selection is difficult to be followed and requires high costs that cannot be supported, using the actual technology. Can we overcome this step?

In our opinion researchers must change their traditional approaches if the goal of a large-scale use of stem cells is compulsory.

This does not imply that basic studies on biology of stem cells must not be done: they should continue, independently from the practical use of stem cells, which, on the other hand, cannot wait for more.

Moreover, the studies on non-human stem cells (i.e. rat, mice, and other animals) must be reduced to the minimum, this because those cells show many differences with human stem cells and we need to study more human stem cells.

We are sure that our goal are patients, while several animal stem cells features or proteins or other features cannot be found in human stem cells.

In summary, a great effort must be tried in order to avoid that we will acquire a lot of knowledge but no translation of studies in patients will be performed.

The latter would really be a great failure or "default."

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Challenges for the therapeutic use of pluripotent stem derived cells

Magda Forsberg*[1] *and Outi Hovatta*[2]*

[1] *Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden*
[2] *Department of Clinical Science, Intervention and Technology, Karolinska Institutet, Stockholm, Sweden*

Edited by:
Anis Feki, Geneva University Hospitals, Switzerland

Reviewed by:
Yuji Mishina, University of Michigan, USA
Liang Ma, Washington University, USA

****Correspondence:***
Outi Hovatta, Department of Clinical Science, Intervention and Technology, H9 K 54, Karolinska Universitetssjukhuset, 141 86 Stockholm, Sweden.
e-mail: outi.hovatta@ki.se

Human embryonic stem cells (hESC) and induced pluripotent stem cells (hiPSC) are an attractive cell source for regenerative medicine. These cells can be expanded to vast numbers and can be differentiated to many desired pluripotent stem cells (PSC) derived therapeutic cells. Cell replacement bears promises, but also challenges. The introduction of exogenous cells in a recipient must address several different topics; its safety, the exclusion of tumor formation, the immunological response and possible rejection, the cells cleanliness and their biological quality, and quantity representing the functionality of the PSC derived therapeutic cells. Tumor formation requires the removal of any PSC remaining after differentiation. Immunological rejection can be addressed with immunomodulation of the cells and the recipient. Cleanliness can be optimized using good manufacturing practice quality systems. At last, the functionality of the cells must be tested in *in vitro* and in animal models. After addressing these challenges, precise strategies are developed to monitor the status of the cells at different times and in case of undesired results, corresponding counteracting strategies must exist before any clinical attempt.

Keywords: cell transplantation, human embryonic stem cells, human pluripotent stem cells, GMP, immunoreactivity, tumorigenic

CULTURE CONDITIONS OF PLURIPOTENT CELLS

After initial establishment and derivation of human embryonic stem cells (hESC; Thomson et al., 1998; Reubinoff et al., 2000) their potential therapeutic use in cell replacement strategies was clear. Before cell transplantation, several steps must be fulfilled. First the infection free status of the donors has to be addressed, In Europe couples are tested before any fertility treatment is offered, but the cells themselves have to be tested, too (Hovatta, 2011). Second, optimized good manufacturing practice (GMP) compliant systems must be implemented for derivation, scaling-up, banking of cells, and their corresponding quality assurance controls (Unger et al., 2008; Ausubel et al., 2011). The culture systems currently encounter the problem of suboptimal quality of xeno-free culture constituents. Thus strategies are needed to overcome this difficulty (Sidhu et al., 2008). Steps have been taken; initially, hESC were grown on irradiated mouse feeders, later human foreskin fibroblast were used (Hovatta et al., 2003), now we can use GMP compliant coating substrates specially designed for hESC growth (Rodin et al., 2010). Steps were also taken for the generation defined xeno-free GMP compliant medium for derivation and for expansion (Ludwig et al., 2006; Rajala et al., 2010; Ilic et al., 2012).

REPROGRAMMING AND hiPSC

The potential of somatic cell reprogramming via expression of specific transcription factors and thus the generation of hESC induced pluripotent stem cells (hiPSC; Takahashi and Yamanaka, 2006; Takahashi et al., 2007) has the advantage that they could be generated from the recipient patients own cells. This could overcome an allogenic immune rejection (Li and Zhong, 2009). But this potential although tempting, has not been proven. There is no deep understanding of the effects that the reprogramming events have; for instance on extracellular signaling (Okita et al., 2011), and the way that this could lead to immune reaction. It was shown that specific T cells were reactive toward Oct-4 antigens. Hence fast reactivity is already present in healthy individuals for controlling any rapidly amplifying cells (Dhodapkar et al., 2010). Un-silenced expression of the reprogramming factor Oct-4 might then cause undesired immunoreactivity on the transplanted cells. Immunoreactivity toward graft-derived hiPSC of the same genetic background was also shown in animal models (Zhao et al., 2011).

For successful reprogramming of somatic cells, many epigenetic changes must occur in an adequate manner. Failure will result in abnormal phenotypes (Mikkelsen et al., 2008). DNA methylation changes during reprogramming must occur in important developmental and oncogenic regions, which increases the oncogenic risk of the reprogrammed cells (Doi et al., 2009; Ohm et al., 2010). There is an additional risk for abnormalities and high tumorigenic potential, especially if c-myc is used as one of the transcription factors (Okita et al., 2007). Also, genetic and epigenetic stability and large-scale genomic rearrangements after reprogramming and subsequent culture (Kim et al., 2010; Gore et al., 2011; Hussein et al., 2011; Lister et al., 2011) might challenge the application of hiPSC under a clinical cell replacement therapy. Further studies to clarify these issues are needed (Pera, 2011). It is also important to address the safety of long-term culture, as shown recently; the occurrence of chromosomal rearrangements in long-term culture of 125 hESC and 11 hiPSC (Amps et al., 2011).

PLURIPOTENT STEM CELL DIFFERENTIATION

There is a consensus that undifferentiated pluripotent stem cells (PSC) will not be used directly in any clinical transplantations procedure, but instead their PSC derived differentiated cells Recently, results using hESC derived dopaminergic neurons have shown correct phenotype differentiation and grafting potential given by no tumor formation, maintenance of the grafted cells, and functional recovery in parkinsonian animal models in mice, rats, and monkeys (Kriks et al., 2011). The protocols designed for this cell replacement assay were optimal regarding the phenotype, quantity of the cells, functionality, and immunological properties. Integration of transplanted cells was achieved when single cells were transplanted, the use of proper biodegradable scaffolds must also be considered. In addition to this initial report regarding the neural lineage, differentiation protocols for other cell types are needed.

Even if transplantation in animal models is successful, it is important to generate safety strategies before clinical trials to appropriately remove undifferentiated PSC from their PSC derived therapeutic cells. Strategies such as inserting suicide gene (Drobyski et al., 2003; Uchibori et al., 2009) might have controversial outcomes under clinical trials given their safety (Yi et al., 2011). Alternatively, strategies such as removal of undifferentiated cells using antibodies might be safer (Tang et al., 2011).

THE IMMUNOGENIC PROFILES

As discussed earlier, an optimal engraftment and cell replacement strategy should account for a minimal immune reaction in the recipient. This immune reaction occurs because the immune system of the recipient recognizes the grafted cells as foreign material or mismatched cellular components and thus generates a cascade of events that ultimately results in destruction and rejection of the grafted cells. This destruction can also compromise the recipient's immune status (Petersen et al., 1975). Immunoreactivity toward the graft is mainly caused by T cell response toward unmatched major histocompatibility complex (MHC); in humans called human leukocyte antigen (HLA). If the profile is unmatched, it will result in rejection (Lechler et al., 2005). This rejection can occur via direct allorecognition of the donor antigen presenting cells (APC) or via indirect recognition of apoptotic cells ingested by the recipients APC, in both cases APCs presenting unmatched MHCs (Walsh et al., 2004). Several groups have studied MHC profiles of hESC and their differentiated cells (Swijnenburg et al., 2008; Pearl et al., 2011). Findings are that undifferentiated cells express MHC I antigens, though at low levels compared with somatic cells; but they do not express MHC II molecules (Drukker et al., 2002, 2006; Li et al., 2004). During *in vitro* differentiation toward germ lineages, embryoid body (EB) formation, or teratoma formation MHC I expression increases dramatically (Drukker and Benvenisty, 2004). Also culture methods of hESC can change antigen expression levels (Rajala et al., 2010). Careful selection of culture conditions, both for the undifferentiated hESC and for their differentiated derivatives is needed.

Human embryonic stem cells adopts the expression of non-human cell surface markers if exposed to such substances during culture (Martin et al., 2005; Hisamatsu-Sakamoto et al., 2008). Hence, optimal culture conditions must be xeno-free from the initial derivation and onward. These culture conditions must be carefully analyzed and scientific consensus must be achieved in order to raise current methodologies.

IMMUNOSUPPRESSION

Challenges with the immunoreactivity of the transplantable cells could be addressed by rigorous immunosuppressive treatments. Unfortunately, this is not desired, since there is a clear correlation between the length and intensity of exposure to immunosuppressive therapy and post-transplant risk of malignancy and tumor aggressiveness (Gutierrez-Dalmau and Campistol, 2007). An interesting solution is costimulatory blockage of T cell response (Grinnemo et al., 2006, 2008; Swijnenburg et al., 2008; Pearl et al., 2011). This immunosuppression strategy will generate tolerance to the grafted cells and thus increase graft survival; initial pharmaceutical agents have been developed and pending clinical applications to the FDA are to give in the near future more information.

DISCUSSION

In this mini-review we highlighted the most important areas to be considered under a cell replacement therapy. The possibility of using hiPSC derived therapeutic cells in cell replacement therapies requires still long-term studies in non-human animal models addressing the questions of immunogenicity, epigenetic and genetic stability of these cells, and the optimized differentiation of the cells.

The importance of profiling immunogenic markers as part of the stem-ness characterization and profiling of cells allocated in stem cell banks must be consider. The allocation and custody of the characterization data is equally important. Such information has to be well protected so that it will not be lost in any given situation. Adequate culture conditions, supporting correct immunogenicity of the cells under a transplantation assay is also required.

Next, the management of immunosuppression schemes must aim to a minimal time influencing the immunological status of the recipient.

From all the information obtained, profiles can be generated before and after differentiation. These profiles can then be used in combination with methodologies focused at monitoring the status of the transplanted cells. In a given scenario that undesired cells persist in the transplant, adequate counteracting actions have immediately to be taken. Such possibilities have to be tested and the removal of undesired effects confirmed before starting cell transplantations. Failure in any particular cell replacement clinical trial will imply disastrous effects. Responsible decisions are needed under complete scrutiny from medical agencies and the scientific community.

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Generation and applications of human pluripotent stem cells induced into neural lineages and neural tissues

Y. Martinez*, M. Dubois-Dauphin and K.-H. Krause

Department of Pathology and Immunology, Faculty of Medicine, University of Geneva, Geneva, Switzerland

Edited by:
Anis Feki, Geneva University Hospitals, Switzerland

Reviewed by:
Mohammad K. Hajihosseini, University of East Anglia, UK
Petros Papagerakis, University of Michigan, USA

****Correspondence:***
Y. Martinez, Department of Pathology and Immunology, University of Geneva, Faculty of Medicine, 1 Rue Michel-Servet, 1206 Geneva, Switzerland.
e-mail: yannick.martinez@unige.ch

Human pluripotent stem cells (hPSCs) represent a new and exciting field in modern medicine, now the focus of many researchers and media outlets. The hype is well-earned because of the potential of stem cells to contribute to disease modeling, drug screening, and even therapeutic approaches. In this review, we focus first on neural differentiation of these cells. In a second part we compare the various cell types available and their advantages for *in vitro* modeling. Then we provide a "state-of-the-art" report about two major biomedical applications: (1) the drug and toxicity screening and (2) the neural tissue replacement. Finally, we made an overview about current biomedical research using differentiated hPSCs.

Keywords: embryonic stem cell, induced pluripotent stem cells, engineered neural tissue, neurons, neural differentiation, cell transplantation, drug screening, cell therapy

INTRODUCTION

Human pluripotent stem cells (hPSCs) encompass human embryonic stem cells (hESC) and human induced pluripotent stem cells (hIPS); they are recently added tools in world of biological research. The first *in vitro* culture of hESC was established in 1998 and, even then, there was obvious interest in developmental biology, drug discovery, and transplantation medicine (Thomson et al., 1998). hIPS are cells in an embryonic stem cell-like state generated from non-pluripotent cells by induction of specific genes (Yu et al., 2007). hPSCs are functionally defined by their self-renewal and differentiation potential. They can be induced to differentiate *in vitro* into virtually all human cell types (Bhattacharya et al., 2009). A diseased or injured central nervous system (CNS) has little capacity to compensate for the loss of cellular elements (neurons, oligodendrocytes; Barrett et al., 2007), thus, cell replacement is an interesting prospective [i.e., missing dopaminergic neurons in Parkinson's diseased brain; missing motoneurons in amyotrophic lateral sclerosis (ALS) or spinal cord injury]. Significant progress has been made in culture and differentiation protocols to obtain cells suitable for transplantation. Further development of these technologies could lead to the scalable production of different neural cell types for toxicity screening and clinical therapies (Dantuma et al., 2010). Currently, 10 years after the first *in vitro* culture of hESC, the first therapy using hESC is being evaluated in clinical trials, beginning to make part of these promises a reality (Geron Corporation, 2009). However, in spite of numerous statements in the social media declaring that these cells can be used in medicine for therapeutic purposes, the clinical applications remain few (Aznar and Sanchez, 2011). hPSCs-derived neurons (-dN) are still too rarely used for drug screening and predictive toxicity. In these domains, requirements exist for efficient, predictive, and cost-effective *in vitro* models (Bal-Price et al., 2010). Such models have been established with hPSCs-dN but most *in vitro* models use mouse ESCs-dN. For each of these research domains, we will describe recent advances in hPSCs culture and we will focus on the clinical relevance of using hPSCs for *in vitro* nervous system disease modeling and therapies.

hESC AND iPSCs DIFFERENTIATION TOWARD NEURAL LINEAGE

CELL LINES

One major challenge in biomedical research is to recapitulate *in vitro* the biological events occurring *in vivo* in normal or diseased organs. There remain serious concerns with the relevance of the most commonly used model systems. For instance, human brain tissue obtained from postmortem samples is subject to numerous artifacts: abnormal brain pH resulting from near death hypoxia, a lengthy postmortem period, residual amounts of medications used. Although they are a major source for primary human neuron cultures, biopsies from the CNS are restricted, owing to the invasiveness of the procedure (Deep-Soboslay et al., 2011). Thus hESC-dN are an attractive alternative to primary neuron culture.

Human embryonic stem cells are derived from the inner cell mass of the 4- to 5-day-old blastocyst. These cells possess two hallmark characteristics: (1) they are able to proliferate *in vitro* and (2) under controlled culture conditions they are able to differentiate into all three germ layers (ectoderm, mesoderm, endoderm), and thereby represent a potentially inexhaustible source of somatic cells (Thomson et al., 1998). Growing knowledge about differentiation protocols allows the generation of cells found in neural tissue such as neurons and glia. However, the isolation of hESC raises ethical issues due to the destruction of human embryo. The development of hIPS avoids this ethical problem and is a good alternative to hESC.

There are several approaches to generate hIPS from adult somatic cells from various tissues, including nuclear transfer, cell fusion, and direct reprogramming (Hochedlinger and Jaenisch, 2006). The direct reprogramming of differentiated cells (i.e.,

fibroblasts) into hIPS provides a tractable source of pluripotent cells for regenerative therapy (**Figure 1**). Direct reprogramming was first realized by the transduction of four transcription factors in fibroblasts (Oct-3/4, Sox2, KLF4, and c-Myc – OSKM factors, Takahashi et al., 2007; Yamanaka, 2008). Cell reprogramming is usually achieved by methods involving viral-derived vectors, but there has been progress toward optimizing security. Several alternatives exist to replace some or all of the OSKM factors: pharmacological molecules, recombinant proteins, signaling factors or use of other transcription factors (Huangfu et al., 2008; Yoshida et al., 2009; Zhou et al., 2009; Gonzalez et al., 2011). More recently, the reprogramming of human somatic cells was driven by the expression of specific miRNA (Anokye-Danso et al., 2011). For therapeutic purposes, hIPS transgene-free were designed and some "safe" non-teratoma-forming cell lines have been identified (Okita et al., 2011). Although still subject to much controversy, hIPS proliferative and differentiation properties resemble hESC (Ohi et al., 2011). Both hESC and hIPS exhibit high intrinsic variability between different cell lines (Bock et al., 2011). Thus, the suitability of each cell line for clinical applications needs to be examined.

For disease modeling purposes, hIPS lines have been generated, for example, from patients affected by spinal muscular atrophy (SMA), familial dysautonomia (FD), Rett syndrome, and down syndrome (Baek et al., 2009; Hotta et al., 2009). Motor neurons derived from SMA or FD patients hIPS exhibited, *in vitro*, morphological features of the disease (Ebert et al., 2009; Lee et al., 2009). Since hIPS retain a "memory" and potential characteristics of the cells or related tissue they originate from (Tian et al., 2011), it was speculated that this memory could be helpful for modeling of late-onset neurological diseases such as ALS or Parkinson's disease (PD). Unfortunately, neurons derived from hIPS generated from ALS or PD patients do not readily recapitulate the diseases features (Dimos et al., 2008; Park et al., 2008; Soldner et al., 2009). The reprogramming of an adult cell to a pluripotent state may reset certain epigenetic hallmarks that developed during disease evolution. To avoid this problem, direct transdifferentiation of somatic cells to neural lineages could be considered. It is now possible to use direct reprogramming with human fibroblasts (with specific factors such as Ascl1, Brn2, Myt1l) to generate functional neurons (Vierbuchen et al., 2010; Kim et al., 2011; Pang et al., 2011) and more specifically, dopaminergic neurons (Pfisterer et al., 2011; **Figure 1**). However, these methods are inconvenient because they generate few cells; in the most recent protocols, about 20% of cells can be directly reprogrammed to functional neurons.

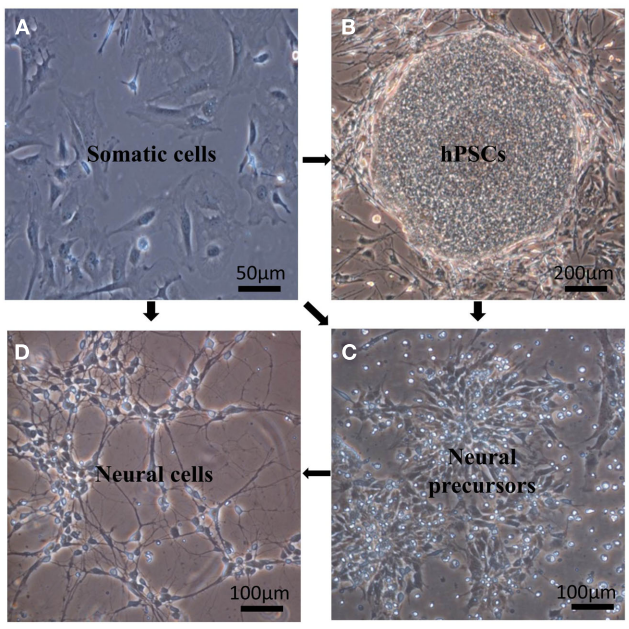

FIGURE 1 | Generation of neural precursors or neural cells from pluripotent stem cells using differentiation or somatic cells using transdifferentiation. (**A**) Fibroblasts used for direct reprogramming using the four transcription factors: Oct4, Sox2, KLF4, c-Myc. (**B**) hESC, H1 cell line cultured on mouse embryonic fibroblasts as feeder cells (MEFs). (**C**) Neural precursor cells obtained from differentiated H1. (**D**) Monolayer of neurons differentiated from H1.

DIFFERENTIATION

Withdrawing a key factor from the medium or forcing the hPSCs to grow in suspension is enough to induce cell differentiation (Thomson et al., 1998). However, the stochastic nature of differentiating hPSCs generates many different somatic cell types (Martinez et al., 2011). hPSCs-based applications, mainly in the biomedical domain, require specific *in vitro* differentiation toward the desirable cell population harboring a unique phenotype. Cell preparations containing undifferentiated or insufficiently differentiated hPSCs can lead to cell overgrowth or teratoma formation once transplanted in an organism (Lees et al., 2007; Aubry et al., 2008). For a given neurodegenerative disorder, hPSCs must be differentiated toward the specific neural cell type that could potentially restore the lost functions (**Table 2**). For example cell replacement therapy to treat PD aims dopaminergic neurons (Marchetto et al., 2010).

The crucial point is how to induce specific hPSCs differentiation toward the desired neural phenotypes. The first step is to obtain neural progenitor cells (NPCs; **Figure 1**). Essentially, specific differentiation depends on the addition of instructive factors and the removal, or inhibition, of preventive ones (Nat and Hovatta, 2004). To obtain NPCs, many different factors have been tested (Reubinoff et al., 2001; Dhara and Stice, 2008; Suter et al., 2009). The most commonly used are fibroblast growth factor (FGF), EGF, SHH, retinoic acid (RA), and bone morphogenetic protein-antagonists (BMPa); there is also the less well-defined stromal-cells derived inducing activity (SDIA). These factors are known to activate complex pathways such as Hedgehog, mesodermal, BMP, kinase, and WNT signaling but their roles are not entirely elucidated. To inhibit the differentiation toward lineages other than neural and promote neural differentiation, in most protocols, media supplements, such as N2 and B27, are added. N2 contain insulin, transferrin, putrescine, progesterone, and selenium. Insulin promotes proliferation, transferrin promotes proliferation and survival of mature neurons, putrescine is involved in axonal regeneration, and selenium protects against excitotoxicity. B27 contains more than 20 components including vitamins, hormone growth factors, antioxidants, and fatty acids (Suter and Krause,

Table 1 | Main factors used for differentiation toward specific neural lineages.

Cell type	Factors needed for differentiation
Neural precursor	βFGF, EGF
Dopamine neurons	FGF-8, Shh
GABA neurons	BDNF, Dkk1, Shh, cAMP
Motor neurons	RA, Shh
Astrocytes	CNTF, LIF, BMPs
Oligodendrocytes	PMN, VN, NGN, PDGF, cAMP, FGF-2
Retinal neural cells	Dkk1, Lefty-1
Auditory neural cells	βFGF, EGF, insulin-like growth factor, BMP4

BDNF, brain-derived neurotrophic factor; BMP4, bone morphogenetic factor 4; cAMP, cyclic adenosine monophosphate; CNTF, ciliary neurotrophic factor; Dkk1, Dickkopf-1; EGF, epidermal growth factor; FGF-8, fibroblast growth factor 8; GABA, g-aminobutyric acid; LIF, leukemia inhibitory factor; NGN, neurogenin; PDGF, platelet-derived growth factor; PMN, purmorphamine; RA, retinoic acid; Shh, Sonic hedgehog; VN, vitronectin (Suter and Krause, 2008).

2008). Ectodermal factors are also used to restrict mesoderm differentiation using P53 pathway (Sasai et al., 2008). Despite the numerous components tested and added, the effective maintenance and stable expansion of NPCs remains complicated, even with the most recently developed protocols (Li et al., 2011). Moreover, no protocol allows obtaining only NPCs; and a selection of cells of interest must be done with techniques like FACS sorting or with inducible suicide gene (Li, 2002; Kawaguchi et al., 2008).

The second step is to drive NPCs toward a specific neural phenotype (**Figure 1**). Many molecular pathways are involved in this step of differentiation. For example, Wnt/beta-catenin signaling is known to stimulate the formation of dopaminergic neurons (Ding et al., 2011). To get mature neural cell types, the presence of specific factors is necessary (**Table 1**). Yet, as for NPCs, the purity of neural cell population remains problematic (Pankratz et al., 2007). An additional consideration is that techniques for neural induction depend on the cell line used and the experimental practice (Schwartz et al., 2008; Suter and Krause, 2008; Daadi and Steinberg, 2009).

Two cell culture protocols are commonly used: suspension cultures and adherent cultures. In suspension, hPSCs form a cell mass. The most promising for 3D culture is in suspension. Adherent culture seems to provide better condition to obtain a homogenous cell population. An homogenous individual cell exposition to morphogens is not warranted due to the numerous cell layers. Thus, the concentration gradient can lead to the generation of cells at different developmental stages and subsequently the formation of multilayered structures that contain a heterogenous population of cells, including neural progenitors. The disadvantages of this protocol are: (1) the size of the cell mass varies, even with the same initial cell number and (2) there is variability in the percentage of each cell types generated and in the layer organization. In contrast, the adherent monolayer culture system allows a uniform cell exposition to morphogens and provides a more homogenous cell population. Static monolayer culture model does not mimic the *in vivo* microenvironment (Wilby et al., 1999) and none of the monolayer protocols used for cell differentiation yield structures and organization similar to those generated in suspension cultures or those with engineered neural tissues (ENTs).

ENGINEERED NEURAL TISSUES

The aim of hPSCs-derived neural tissue culture is to provide models for very early stage of nervous system development (neural tube and post neural tube early stages) and diseases, to provide models for toxicity and drug screening, and to explore the mechanism of action of different molecules. Three-dimensional cultures would allow for the study of interactions between various neural cell types and some intrinsic properties could be more readily compared with CNS physiological properties. To provide a relevant model for CNS modeling, the 3D culture system must adhere to three criteria: (1) to contain most CNS-related cell types (oligodendrocytes, neurons, astrocytes, microglia, endothelial cells, and meningeal fibroblasts); (2) to be biologically relevant (the *in vitro* system cell components must show similar behavior to those *in vivo*); (3) to recapitulate some of the developing or mature CNS features, including early neural tube organization. hPSC-derived ENTs have been produced by several laboratories with varying protocols and results (Wang et al., 2011). Amongst them, the use of air–liquid interface cell cultures device allows a 3D organization guided by endogenous developmental cues (Preynat-Seauve et al., 2009). Scaffolds with different materials like cellulose nanofibers, SiO_2, PLGA nanofibers or silicon can also been used with or without coating. Some coatings increase neural differentiation. Some frequently used coating are the laminin to support neural adhesion, the poly-L-lysine, or the alginate gel to induce slow drug release (Leach et al., 2010). All of these *in vitro* models recapitulate, at least partly, *in vivo* nervous system development.

Tissue engineering may provide advanced *in vitro* models for drug testing in combination with non-destructive techniques for long-term studies. Cell proliferation, migration, differentiation, and synaptogenesis could be followed in ENTs and give precious information. ENTs could reduce time, cost, and number of animals necessary for pre-clinical studies. However, the tissue thickness and variety of cell types found in the hPSC-derived culture may be challenging. It is difficult to monitor cell morphology and phenotype during cell differentiation process in ENTs. Compared to cell derived in a monolayer, hPSCs derived in 3D cultures could provide a more elaborate system for developmental neurotoxicity testing. The research aim will determine the choice between the two culture methods (**Figure 2**).

HUMAN CELL LINES AND *IN VITRO* MODELS

Until recently, the human *in vitro* models available were limited to the use of transformed cell lines (like SH-SY5Y cells) or of primary cells obtained from aborted fetuses' tissues or from resection during brain surgery. Transformed or primary cell lines used have obvious limits (**Table 2**). (**A**). Transformed cell lines derived from tumors and do not represent normal neural cells (Breier et al., 2010). Human primary cell lines raise ethical problems, are difficult to obtain, and, in the case of adult brain biopsies, contain very few neural progenitors, neurons whose developmental processes is achieved, and many reactive astrocytes. On the other hand, fetal biopsies contain more neural progenitors, which is advantageous for culture systems. Biopsies from patients with a

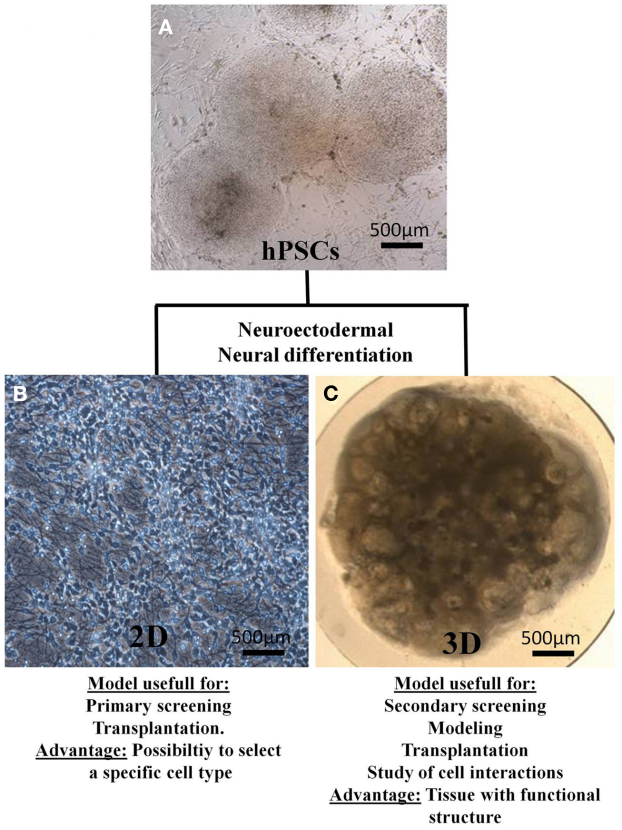

FIGURE 2 | Main biomedical applications and advantage of 2D cultures and 3D cultures. (**A**) hPSCs, H9 cell line on MEFs. (**B**) Monolayer of neurons obtained from differentiated hESC (H9 cell line). (**C**) Engineered neural tissue obtained from the differentiation of H9 on a semi-permeable membrane.

neurodegenerative disease or with epilepsy offer the opportunity to study real diseased human neurons (Radio and Mundy, 2008). Since recent developments of hIPS, especially hIPS from diseased humans, ethical problems are solved and hPSCs can now be considered a valuable tools for drug screening (Danovi et al., 2010).

NPCS derived from the H9 hESC line are commercially available ("Embryonic neural Stem-A" cells, Millipore, Inc.). Other neural stem cell lines are widely used by the research community such as ReNcell VM derived from the ventral mesencephalon or ReNcell CX derived from the cerebral cortex (ReNeuron group). To facilitate screening developments, these cells are defined by marketing features such as ideal culture and differentiation conditions, genomic stability, and phenotype expressed before and after differentiation. The recent production of hIPS from diseased patient represents a major advance for *in vitro* neurodegenerative disease models. The generation of *in vitro* assays with hPSCs facilitates early assessment of tested chemicals at a high throughput. Such assays become an area of interest for supporting "the 3 R's rule" (reduction, refinement, replacement) to alleviate animal use in biological research (Moors et al., 2009). The existing range of fundamental research protocols available to explore neural functioning allows investigation of all disease aspects. These protocols allow researchers to explore cellular phenotype (histological analysis), neuronal activity (electrophysiology, patch clamp, calcium imaging currents), connectivity (synapse maturation), circuitry (Rabies virus tracing, co-culture between neurons and glia), and cell migration (bioimaging).

DRUG AND TOXICITY SCREENING WITH hPSCs

The aim of drug screening is to find the most efficient molecule for a particular application, while avoiding deleterious effects. For efficient drug screening toxicity assessment, *in vitro* 3D culture models should yield a significant throughput. Because of their size and cell heterogeneity, these models are available only for the low-throughput approach. Considering the currently available protocols, it would be difficult to obtain the number of cultures required for regular use on 1536-well plates (Bal-Price et al., 2010).

The toxic properties of a large number of chemicals remain unknown, in particular in the CNS. hPSC-derived 3D systems could help to study the 1200 compounds known to be neurotoxic to humans or animals (non-confidential Toxic Substances Control Act, TSCA; Coecke et al., 2007). High-content/high-throughput screening (HCS/HTS) approaches to identify chemicals that may be toxic for nervous system cells are increasingly used (Lein et al., 2007; Breier et al., 2008). Present HCS/HTS approaches use imaging of biochemical or morphological endpoints in cells, such as neurite outgrowth, neurite number, average length, cell size, and shape, and nucleus/cytoplasm ratio (Pal et al., 2011). The use of hPSCs models in neurotoxicology and drug screening is an emerging field but that needs further expansion.

Although hPSCs are a reproducible and renewable source of cells, they do not offer all the main features required for screening, which are as follows: (1) It should be easy to produce the cell number needed to conduct HCS/HTS assays in multi-well plates; (2) Cell genotype and phenotype should be stable; (3) The proliferation, migration, and differentiation features of cells should be well-characterized and reproducible; (4) The relative percentage of neurons, astrocytes, and oligodendrocytes obtained during the differentiation should be standardized such that toxicity-induced changes in the proportions of each can be reliably detected (Breier et al., 2010). hPSCs do not satisfy to all of these characteristics. The major problems for their use in screening are: (1) The maintenance of stem cell colonies is an intensive and expensive labor; (2) Exact medium composition is rarely known because of commercial protections; (3) The time needed to accomplish neuronal differentiation is very long; (4) The conditions required for specific neuronal differentiation are not fully elucidated; (5) The neural progeny is asynchronous: mature and immature neural cell types are present in the final cell population (Breier et al., 2010; Azari et al., 2011). The above disadvantages explain why until now neurons derived from hPSCs have been rarely used to test the efficacy of drugs and their neurotoxicity (Barbaric et al., 2010). Recent hIPS-derived neuronal modeling establish alternatives tools for current drug screening platforms, at least as proof-of-principle (Ebert et al., 2009). Foremost, among hIPS derived from diseased patients' neural cells make it possible to target the screening against a specific disease. In some cases, such as with schizophrenia, screening could be complicated (Brennand et al., 2011). The complexity of this disease would require a subgrouping of hIPS based on pathways that are impacted for each specific patient. On

Table 2 | Cell types used in biomedical research with their advantages and inconvenients.

Cell types	Advantage	Disadvantage
Immortalized cell lines	Easy to obtain large quantities	Different from *in vivo* cells
	Inexpensive	Modified cell lines
		Relevance limited
Primary cell culture	Relevance	Hard to obtain
	Behavior similar to *in vivo*	Limited quantities
hESC	Unlimited quantities	Ethical issues
	Unmodified cells	Expensive
		Long differentiation time
		Cell lines hard to obtain
hIPS	Close to *in vivo* reality	Expensive
	Cell lines from patient with specific diseases easy to obtain	Not yet proven to have complete equivalence with hESC
Transdifferentiated cells	Relevance	Limited quantities
	Ability to obtain one specific cell type	Impact of transdifferentiation not well known

Table 3 | A specific cell type for a specific disease.

Target cell population	Markers	Potential treatment
NPC	Musashi, Nestin, Sox 2, Vimentin, Pax6, Sox1	
Astrocytes	GFAP, S100, Ran2	
Oligodendrocytes	O1, O2, MBP, RIP, CNPase, GalC	Vascular neuroencephalopaties, multiple sclerosis
GABA neurons	GABA, DARPP-32, GAD, VGAT	Huntington's disease
Dopamin neurons	DBH, DAT, l-DOPA, TH	Parkinson's disease
Cholinergic neurons	Acetylcholinesterase, ACh, ChAT, choline transporter	Alzheimer's disease
Motor neurons	ChAT, Chox10, En1, Evx1/2, Islet1/2, Lim3, REG2, Sim1	Amyotrophic lateral sclerosis, spinal cord injury
Auditory neural lineage	GATA3, phosphorylated NFH within Somata	Hearing loss (cochlear implant; Gunewardene et al., 2011)
Retinal cell lineage	Rhodopsin, RBP3	Blindness (Bharti et al., 2011)

GFAP, glial fibrillary acidic protein; MBP, myelin basic protein; GalC, galactocerebroside; DBH, dopamine beta hydroxylase; DAT, dopamine transporter; TH, tyrosine hydroxylase; SERT, serotonin transporter; Ach, acetylcholine; ChAT, choline acetyltransferase; RBP3, retinol binding protein 3.

the other hand, hIPS could bring the opportunity to identify the specific molecular factors in each subgroup. In this way, hIPS could hold the promise of individualized medicine in complex disease (Brennand et al., 2011; Buxbaum and Sklar, 2011).

hPSCs THERAPIES FOR NEURAL TISSUE REPLACEMENT

Effective treatments do not exist for neurodegenerative diseases. hPSCs hold enormous promise for cell-replacement based therapies. They are a potentially unlimited source of allogenic or autologous cells. The main goal of treatment-oriented research is to obtain appropriate cells able to repopulate diseased tissue *in vivo* without deleterious consequences. Cells must be free from xeno-contamination to avoid risks of zoonosis or activation of animal retroviruses (Swistowski et al., 2009). Then, appropriate cell differentiation and selection are critical to obtain enough specific cells to treat a targeted disease such as Parkinson's disease or Alzheimer's disease (**Table 3**). (**B**). The strict phenotype specificity and purity of transplanted cells is an absolute requirement.

One of the major problems is the teratoma, or overgrowth risk. It remains to be solved for most of the potential treatments. Following neural differentiation, neural precursors are able to proliferate in an uncontrolled manner, even if all of the undifferentiated cells are removed. For example, in a recent experiment, grafted IPS derived to striatal spiny neurons overgrew and lead to deleterious side effects after 13 weeks. The overgrowth problem was due to some nestin-positive NPCs and not to the presence of undifferentiated ESCs (Aubry et al., 2008). Three different ways have been investigated to increase transplantation efficiency as well as to avoid overgrowth or teratoma formation: (1) Cell sorting to isolate a specific population; (2) hPSC lines modified with an inducible suicide gene under the control of a promoter element used to maintain "stemness" (Schuldiner et al., 2003); (3) Targeted anti-human hPSC antibodies that induce apoptosis of undifferentiated hPSC (Choo et al., 2008; Tan et al., 2009; Lim et al., 2011). Finally, as with all transplantations, the risk of rejection must be considered (Preynat-Seauve and Krause, 2011).

With the aim to develop knowledge and potential therapies, many IPS lines have been produced from patient suffering from a variety of neurological diseases like HD, PD (Park et al., 2008), SMA (Ebert et al., 2009), ALS (Dimos et al., 2008), and schizophrenia (Chiang et al., 2011). "Proof-of-concept" for cell replacement therapy has been provided in the following two examples: PD and spinal cord injury (SCI; Roy et al., 2006; Erceg et al., 2010).

PARKINSON'S DISEASE

First described in 1817 by James Parkinson, this degenerative disorder results from the death of dopaminergic neurons in the ventral midbrain *substantia nigra* (Goto et al., 1990). The prevalence of Parkinson's disease (PD) is about 1–2% of the population over 65 years (Alves et al., 2008). Symptoms are severe motor deficits like muscle rigidity, tremors, and unstable gait and posture. Current treatments consists of the administration of drug levodopa (L-dopa), a dopamine precursor able to cross the blood–brain barrier and be metabolized into dopamine (Sethi, 2010). Deep brain stimulations are also used (Tuszynski, 2007). However, these treatments only alleviate symptoms; they do not correct deficits and are progressively ineffective with PD progression. Furthermore, long-term use of L-dopa induces dyskinesia (Calabresi et al., 2010). Research for more efficient alternative treatments are currently being investigated. Transplantation of neurons from fetal ventral midbrain to replace lost dopamine neurons shows varied and sometimes no benefit for the patients in clinical trials (Freed et al., 2001; Olanow et al., 2003). Moreover, due to ethical concerns and the difficulties in obtaining adequate tissue, this alternative will likely remain marginal. There has been progress in other areas though; hPSCs derived to dopaminergic neurons and then transplanted into a rat model of Parkinson's disease produced improvements in motor function (Ben-Hur et al., 2004; Roy et al., 2006; Chiba et al., 2008). As techniques have progressed to the point that researchers can obtain pure dopaminergic neurons from hPSCs (Cho et al., 2008; Swistowski et al., 2010; Kim, 2011). Moreover, derivation of specific dopaminergic neurons from patient IPS has been achieved and transplantation of these cells into a rodent PD model showed an alleviation of motor deficits (Cooper et al., 2010; Hargus et al., 2010). All together, these studies show that hPSCs are promising candidates for cell replacement therapy.

SPINAL CORD INJURY

The most advanced hPSC-derived therapy aims to treat SCI. It is the first treatment to be evaluated in clinical trials (Geron Corporation, 2009). This trial has been halted for economic reasons by Geron enterprise but continue to be monitored. In United States, incidence of SCI is estimated to be about 12,000 cases each year (Qin et al., 2010). After a spinal cord trauma, symptoms can vary depending on the localization of the damages as well as various internal and external factors (Jagatsinh, 2009). To treat motor deficit related to SCI the connection between motor cortex and muscles must be restored. For this purpose, the transplantation of motor neurons and oligodendrocytes can be considered. These two cells types can be derived from hPSCs (Kerr et al., 2010) and hPSCs induced to motor neurons promote functional recovery after SCI in a rat model (Rossi et al., 2010). Tissue engineering approaches has been tested to treat SCI. They combine hPSCs with collagen or fibrin-based scaffold. These scaffolds are able to deliver growth factors promoting hPSCs differentiation into oligodendrocytes and neurons. (Hatami et al., 2009; Johnson et al., 2010). These studies showed that implanted cells increase locomotor functions and enhance functional recovery in a rat model of SCI (Kerr et al., 2010; Niapour et al., 2011; Lee et al., 2012).

OTHER INJURIES AND DISEASES

Another promising trial is for Huntington's disease (HD), a neurodegenerative genetic disorder that causes dementia and affects muscle coordination. Prevalence of this disease is about 0.01% of the population (Warren and Yellowlees, 1990). As for PD, some studies have investigated the potential of fetal tissue transplantation as treatment and show more encouraging results for HD treatment than for PD treatment (Frank and Biglan, 2007; Gallina et al., 2010). Another experiment involved differentiation hIPS into neural progenitors and transplanting them into a rat model of HD; grafted animals had better performance than controls (Song et al., 2007). Unfortunately, in these tests, the mechanism of recovery was not clear: was it due to factors released by the graft or by the host tissue?

Human pluripotent stem cells were also occasionally used for traumatic brain injury and Alzheimer's disease (Molcanyi et al., 2007; Moghadam et al., 2009). Cell replacement therapy could be also investigated in some case of severe epilepsies by implantation of specific GABAergic neurons directly into affected areas. Most of these studies use mouse models and embryonic stem cells (mESCs), so much work would need to be repeated with hESC in pre-clinical testing to determine the viability of such therapies (Wang et al., 2006; Riess et al., 2007).

CONCLUSION

Despite the recurring front page media stories about hPSCs and therapeutic promises, we are still many years from clinical applications. hPSCs provide a renewable source of all somatic cell types, but important difficulties remain. The main ones stay to isolate and have a long-term expansion of specific cells. To achieve specific hPSCs differentiation requires protocols that are often complicated and expensive. Current cell selection protocols have intrinsic limits and cell cultures may still yield mixed populations containing neural cells at different developmental stages, which necessarily limits biomedical applications needing well-defined cells (Ebert and Svendsen, 2010). The recent development of hIPS allows generation of patient-specific neural cells and tissue, but we still do not know if these cells are equivalent to hESC since their respective potential can differ (Martinez-Fernandez et al., 2011).

The number of genetic mutations that are induced by the return to pluripotency can hamper future applications. Moreover, in the case of age-dependent diseases like HD, hIPS-derived cells do not always exhibit phenotypic differences compared with normal control cells. Some newer protocols involve culturing cells under oxidative stress conditions to reveal or accelerate aberrant neuronal phenotypes in late-onset diseases, but their relevance in drug screening is not yet established (Nguyen et al., 2011; Seibler et al., 2011). The use of hIPS in modeling diseases like Timothy syndrome or Down syndrome is only just beginning, and much work remains to obtain relevant models (Yazawa et al., 2011). Despites these limits, hPSCs have the potential to improve our knowledge in many biomedical domains. For example, hPSCs have obvious applications in neuroprostethics, leading to a better understanding of the inflammation process following implantation. Also, if performed early in the drug development process by pharmaceutical companies, relevant toxicological screenings would allow a substantial decrease in the cost of clinical studies. Moreover, the

introduction of hIPS adds a "personalized medicine" dimension to eventual biomedical applications. Considering these potential advantages, hPSCs are full of promise in the near future.

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Stem cell fate determination during development and regeneration of ectodermal organs

Lucía Jiménez-Rojo*, Zoraide Granchi, Daniel Graf and Thimios A. Mitsiadis

Institute of Oral Biology, Zentrum für Zahnmedizin, Faculty of Medicine, University of Zurich, Zurich, Switzerland

Edited by:
Anis Feki, Geneva University Hospitals, Switzerland

Reviewed by:
Francisco H. Andrade, University of Kentucky College of Medicine, USA
Petros Papagerakis, University of Michigan, USA

****Correspondence:***
Lucía Jiménez-Rojo, Institute of Oral Biology, Faculty of Medicine, University of Zurich, Plattenstrasse 11, 8032 Zurich, Switzerland.
e-mail: lucia@jimenez-rojo.com

The development of ectoderm-derived appendages results in a large variety of highly specialized organs such as hair follicles, mammary glands, salivary glands, and teeth. Despite varying in number, shape, and function, all these ectodermal organs develop through continuous and reciprocal epithelial–mesenchymal interactions, sharing common morphological and molecular features especially during their embryonic development. Diseases such as ectodermal dysplasias can affect simultaneously these organs, suggesting that they may arise from common multipotent precursors residing in the embryonic ectoderm. During embryogenesis, these putative ectodermal stem cells may adopt different fates and consequently be able to generate a variety of tissue-specific stem cells, which are the sources for the various cell lineages that form the diverse organs. The specification of those common epithelial precursors, as well as their further lineage commitment to tissue-specific stem cells, might be controlled by specific signals. It has been well documented that Notch, Wnt, bone morphogenetic protein, and fibroblast growth factor signaling pathways regulate cell fate decisions during the various stages of ectodermal organ development. However, the *in vivo* spatial and temporal dynamics of these signaling pathways are not yet well understood. Improving the current knowledge on the mechanisms involved in stem cell fate determination during organogenesis and homeostasis of ectodermal organs is crucial to develop effective stem cell-based therapies in order to regenerate or replace pathological and damaged tissues.

Keywords: stem cells, tooth, mammary gland, hair follicle, Notch, BMP, Wnt, ectodermal organs

DEVELOPMENT OF ECTODERMAL TISSUES AND THEIR APPENDAGES

During early embryogenesis, the ectoderm represents the external germ layer, which is composed of the surface ectoderm, neural crest, and neural tube. From the surface ectoderm the epidermis (i.e., skin) and other stratified epithelia (e.g., oral epithelium; Carlson, 2004) develop. Subsequently, these tissues give rise to diverse specialized structures (called ectodermal appendages) such as hair follicles, mammary glands, salivary glands and teeth.

All ectodermal appendages develop through similar cellular mechanisms that involve an intimate and controlled crosstalk between the epithelium and mesenchyme, and share common morphological features during early organogenesis (Pispa and Thesleff, 2003). The first morphological sign of an appendage development is a thickening of the epithelium that forms the appendage placode. In most of the cases the placode invaginates into the mesenchyme and due to an important proliferation activity gives rise to a bud, while the surrounding mesenchyme starts to condense. The bud epithelium grows further into the adjacent mesenchyme and finally acquires an organ-specific configuration (**Figure 1**).

SPECIFICATION OF ECTODERMAL APPENDAGES: THE ROLE OF EPITHELIAL–MESENCHYMAL INTERACTIONS

The precise mechanisms that define the specificity of the ectodermal appendages along the body are not yet well understood. Tissue recombination assays are valuable for investigating the role of the epithelium and mesenchyme in regulating the initiation and morphogenesis of ectoderm-derived organs.

Classical heterotopic tissue recombination experiments, in which epithelium and mesenchyme from different origin are re-associated, have demonstrated the importance of the mesenchyme for initiating the development, and establishing the pattern, of ectodermal organs (Kollar and Baird, 1969; Kratochwil, 1969; Sakakura et al., 1976; Sengel, 1990). The capacity of the mesenchyme to ectopically induce the formation of ectodermal organs has been shown in a large variety of experimental models. For instance, it has been demonstrated that the embryonic mammary mesenchyme has the ability to induce the formation of mammary buds in areas where mammary glands normally do not develop, such as in mid-ventral and dorsal epidermal regions (Cunha et al., 1995). Similarly, it has been established that embryonic dental mesenchyme is able to induce the formation of dental structures when combined with non-dental epithelia *in vitro* (Mina and Kollar, 1987). Likewise, *ex vivo* experiments have shown that the oral epithelium of birds is capable of forming tooth-like structures when in contact with mouse neural crest-derived mesenchyme (Mitsiadis et al., 2003, 2006). It is now broadly accepted that the mesenchyme regulates the morphogenetic events of the various ectodermal organs. However, it remains controversial whether the cytodifferentiation events occurring later in the epithelium are tissue-autonomous or strictly mesenchyme-dependent. For

example, the recombination of mammary epithelium with salivary gland mesenchyme gives rise to salivary glands (**Figure 2**) that secrete milk proteins instead of salivary proteins (Sakakura et al., 1976). In contrast to the previous findings, embryonic chick mesenchyme (dermis) has been shown to control the epithelial (epidermal) cell differentiation (Dhouailly et al., 1978): tarso-metatarsal scale-forming dermis instructs the dorsal epidermis to develop scales, while dorsal feather-forming dermis induces the formation of feathers in tarso-metatarsal epidermis. One possible explanation is that mammary epithelial cells may be committed to express tissue-specific proteins earlier than feather and scale epidermal cells. Along these lines, tissue recombination experiments can provide valuable information on the developmental stage in which tissue-specific stem cells are irreversibly committed. This has been elegantly demonstrated by heterochronal recombination assays using pituitary epithelium and salivary gland mesenchyme

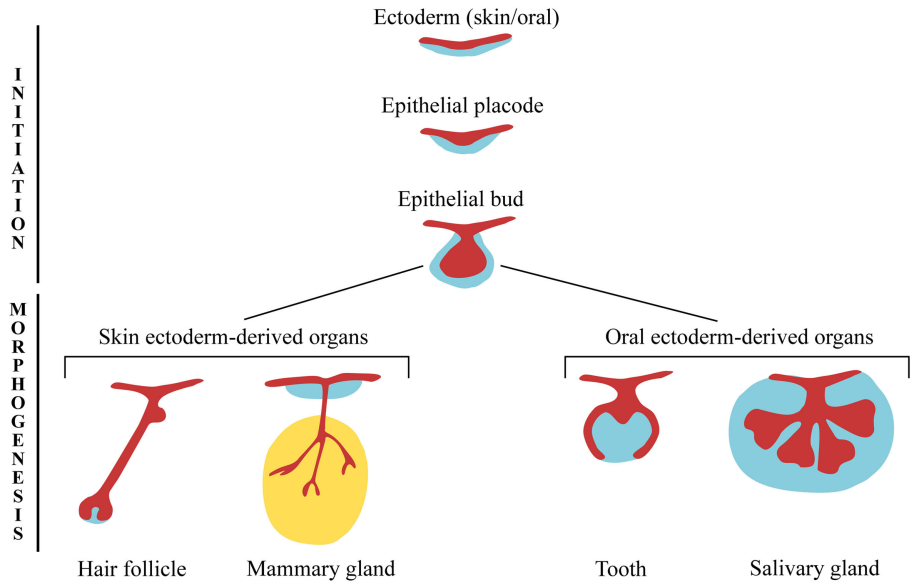

FIGURE 1 | Ectodermal appendages during early stages of development. Ectoderm-derived structures start to develop from embryonic ectoderm upon mesenchymal inductive signals. The formation of the epithelial placodes, and their subsequent growth into the mesenchyme, is common to the early development of all ectodermal organs. At later developmental stages, epithelial buds undergo different morphogenetic programs resulting in the formation of highly specialized structures.

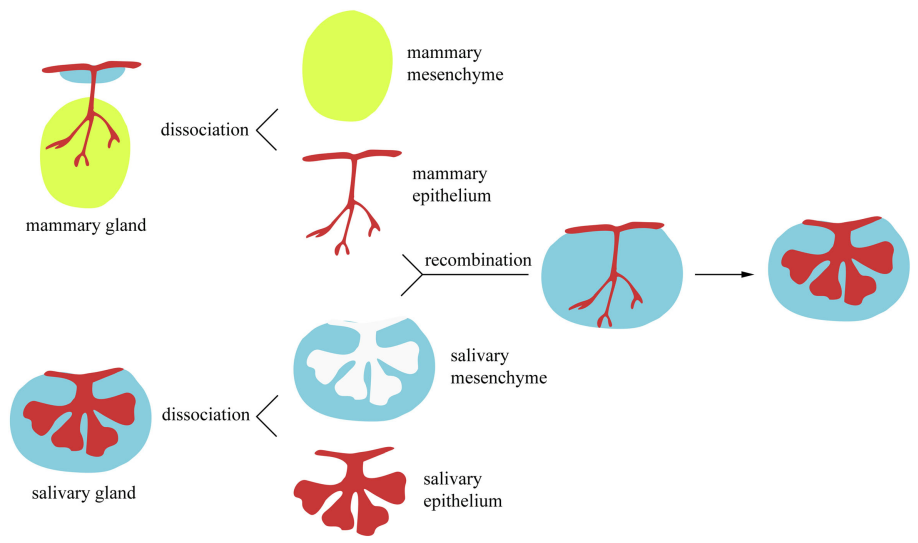

FIGURE 2 | *In vitro* heterotopic tissue recombination assay. Epithelial and mesenchymal components from ectodermal appendages can be enzymatically and mechanically dissociated. Subsequently, they can be recombined with epithelium and mesenchyme from a different organ.

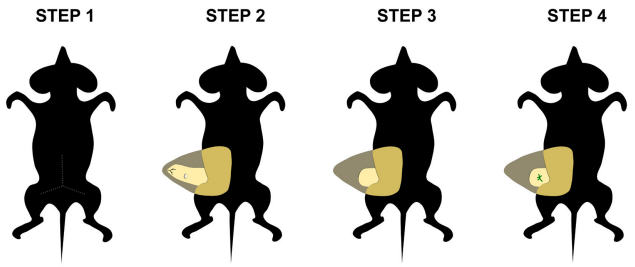

FIGURE 3 | Transplantation into postnatal mammary fat pad. This *in vivo* tissue recombination assay can be used to assess the effect of the adult mammary mesenchyme in the fate determination of stem cells. First, an incision is made in the abdominal skin of the host mouse (step 1) and the skin is pulled away from peritoneal wall to expose the mammary fat pad (step 2). Subsequently, the fat pad tissue from the lymph node to the nipple area is removed (step 3). Finally, donor tissue is transplanted into the cleared fat pad (step 4).

(Kusakabe et al., 1985). Recombination of embryonic day 8 (E8) to E11 pituitary epithelia with salivary gland mesenchyme results in the formation of salivary epithelial structures. However, this was not the case when pituitary epithelium from more advanced developmental stages was used.

Although it has been well established that the mesenchyme or environment plays an essential role in the epithelial cell fate determination in several ectodermal organs, this may vary by tissue and developmental time point. Does the mesenchyme retain its inductive capacity after birth? Is the microenvironment able to redirect the fate of stem cells? The mouse mammary gland constitutes an ideal model to address and clarify these questions *in vivo*, because epithelium can be easily removed from the fourth inguinal mammary gland of pre-pubertal mice (**Figure 3**). Thus, adult and embryonic mammary epithelium can reconstitute a functional mammary gland when transplanted into a cleared or epithelium-free mammary fat pad (Smith and Medina, 1988). By contrast, epithelia from another origin such as salivary gland, lung, and pancreas, fail to undergo morphogenesis when transplanted into the mammary fat pad of postnatal mice (Sakakura et al., 1987). However, it has been demonstrated that postnatal mammary mesenchyme has the potential to induce the differentiation of non-mammary cells into milk-secreting luminal cells after mixing with mammary epithelial cells and subsequently injecting them into empty fat pads (Boulanger et al., 2007, 2012; Booth et al., 2008). Based on their common origin, it would be interesting to study the morphogenetic and differentiation potential of stem cells from various ectodermal-derived organs after their transplantation into mammary mesenchyme, either alone or in combination with mammary epithelial cells.

LINEAGE COMMITMENT OF STEM CELLS DURING DEVELOPMENT OF ECTODERMAL ORGANS

As mentioned above, skin and oral epithelia are derivatives from the embryonic surface ectoderm. Thus, during embryogenesis, a putative common stem cell population residing in the surface ectoderm would be determined to give rise to multipotent stem cells in skin and oral epithelia that would be further specialized to form ectodermal appendages (**Figure 4**). For instance, during mammalian embryogenesis, stem cells from the oral epithelium are committed to form a stratified oral mucosa, the highly specialized dental epithelium and salivary and pituitary gland epithelia, whereas stem cells from the skin ectoderm can give rise to epidermis, hair follicles as well as sebaceous, and mammary glands.

Most of the ectodermal organs undergo their terminal differentiation and become functional at postnatal stages. This holds true for salivary glands that complete their functional differentiation during postnatal stages. Similarly, mammary glands reach their major specialization during adulthood and notably during pregnancy. Finally, hair follicles fully develop and cycle only postnatally and teeth erupt after birth. However, the main morphogenetic processes during the development of ectodermal-derived organs occur during embryonic stages. During the morphogenetic stage, tissue-specific stem cells divide and give rise to more committed progenitors that will eventually differentiate into all the tissue-specific lineages (**Figure 4**).

In the hair follicle, eight different cell types originate from the epithelium. The cells directly responsible for the hair shaft (medulla, cortex, cuticle) formation; the IRS (Inner Root Sheath) lineages (cuticle, Huxley's layer, Henle's layer, and companion layer), which constitute the external support to the shaft; the outer root sheath (ORS) linage, which separates the hair follicle-organ from the epidermis (Niemann and Watt, 2002).

In the adult mammary epithelium, two main cell lineages can be found: the contractile myoepithelial cells and the luminal cells that can be further subdivide into the ductal and the milk-producing alveolar lineages (Woodward et al., 2005).

The salivary gland is composed of acinar, ductal, and myoepithelial cells. Acinar cells can be subdivided into serous or mucous, depending on the composition of their secretion. Similarly, ductal cells can be grouped into three different lineages based on the duct type in which they reside, i.e., intercalated, striated, or granular ductal cells (Tucker, 2007).

Regarding the tooth, epithelium can give rise to outer enamel epithelium, stellate reticulum, stratum intermedium, and inner enamel epithelium (**Figure 5**) that eventually will differentiate into enamel-secreting ameloblasts (Mitsiadis and Graf, 2009). Lineage tracing experiments using lipophilic carbocyanine dyes, such as the fluorescent dye DiI, have contributed to the understanding of lineage commitment during odontogenesis (**Figure 5**). In order to identify putative multipotent stem cells in the epithelium of embryonic mouse molars, cells from different regions of the dental epithelium were labeled before the morphogenesis stage. At further stages, DiI dye is retained in the same area where it was injected, suggesting that the labeled cells were committed progenitors rather than multipotent stem cells (Mitsiadis et al., 2008). However, DiI labeling faces several limitations such as the dilution of the dye concentration after each cell division, and it cannot be ruled out the possibility of the existence of multipotent epithelial stem cells in the embryonic molars that were not targeted with DiI.

ADULT EPITHELIAL STEM CELLS IN ECTODERMAL ORGANS

There is increasing evidence for the existence of a pool of adult stem cells in several tissues that serves as a source for tissue

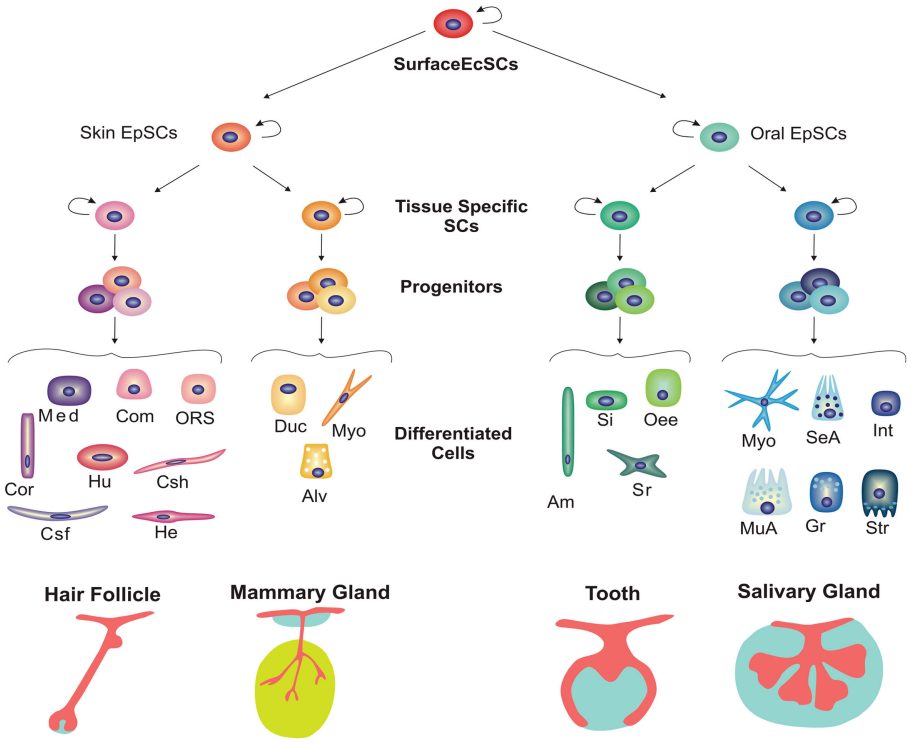

FIGURE 4 | Hierarchical model for stem cell commitment during development of various ectodermal organs. Skin and oral epithelia arise from the surface ectoderm. Putative multipotent stem cells residing in both epithelia can take different fate decisions, giving rise to diverse appendages. During further development of ectodermal organs, more committed tissue-specific stem cells arise from these putative multipotent stem cells in the skin and oral epithelium. Subsequently, tissue-specific stem cells give rise to progenitors or transient amplifying cells that differentiate into functional cell populations. Abbreviations: Alv, alveolar cell; Am, ameloblast; Com, companion layer; Cor, cortex; Csf, cuticle of the hair shalf; Csh, cuticle of the hair sheath; Duc, ductal cell; EcSCs, ectodermal stem cells; EpSCs, epithelial stem cells; Gr, granular duct cell; He, Henle's inner root sheath layer; Hu, Huxley's inner root sheath layer; Int, intercalated duct cell; Med, medulla; Myo, myoepithelial cell; MuA, mucous acinus; Oee, Outer enamel epithelium; ORS, outer root sheath; SeA, serous acinus; Si, stratum intermedium cell; Sr, stellate reticulum cell; Str, striated duct cell.

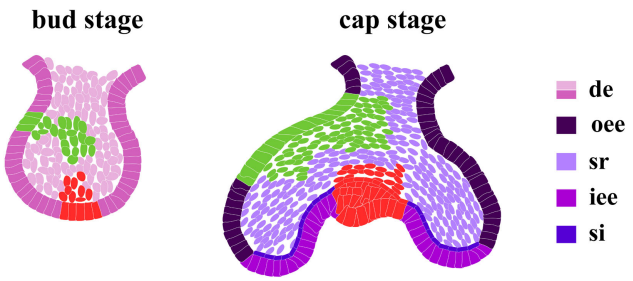

FIGURE 5 | Dil labeling in dental epithelium. At the cap stage, the fate of cells from the dental epithelial bud is already determined. Four different, phenotypically distinct, cell compartments compose the enamel epithelium: outer enamel epithelium (oee); inner enamel epithelium (iee); stellate reticulum (sr); stratum intermedium (si). Two independent Dil labeling experiments (in green and red) traced the fate of cells from different regions in the dental bud epithelium. At a more advanced developmental stage (cap stage) the Dil dye is detected in the same compartments where it was previously injected.

homeostasis and injury-repair (Li and Xie, 2005; Mitsiadis et al., 2007; Barker et al., 2010). Adult stem cells are considered poorly differentiated cells that have self-renewal capacity and can give rise to every cell type in a given tissue (Potten and Loeffler, 1990). Adult stem cells reside in niches that provide them with the proper signals to regulate their function and maintenance according to the tissue requirements (Li and Xie, 2005). Deregulation of adult stem cells can result in pathologies such as tumorigenesis or early senescence (Iglesias-Bartolome and Gutkind, 2011).

Ectodermal organs are exposed to a high risk of being damaged and it becomes extremely important that they retain the ability of wound repair and regenerate throughout the organism's lifespan. Similarly to other organs, the injuries suffered after birth by epidermis and oral epithelium, as well as their appendages, are overcome with the help of adult stem cells.

EPITHELIAL ADULT STEM CELLS DURING DEVELOPMENT AND HOMEOSTASIS OF ECTODERMAL APPENDAGES

In a number of ectodermal organs, epithelial adult stem cells have already been identified. However, in the vast majority, the existence of an adult stem cell pool in the epithelium is suspected although it has not been yet well characterized. Fluorescence-activated cell sorting (FACS) has been widely used to separate the different cell populations residing in a tissue based on their differential expression of several cell surface proteins. Subsequently, their stemness can be assessed either *in vitro* or *in vivo*. *In vitro*, the ability of cells

to form colonies or spheres containing various lineages has been used to define adult epithelial cells with stem/progenitor properties (Barrandon and Green, 1987; Dontu et al., 2003; Blanpain et al., 2004; Lombaert et al., 2008). Moreover, when disaggregated and replated as single cells, putative stem cells can form new colonies or spheres, showing self-renewal capacity (Barrandon and Green, 1987; Dontu et al., 2003). *In vivo*, transplantation assays have largely been used to prove that a given cell behaves as a stem cell in hair follicle (Blanpain et al., 2004), tooth (Ohazama et al., 2004a), mammary (Sleeman et al., 2006), and salivary glands (Lombaert et al., 2008). The previously mentioned transplantation into cleared mammary fat pads and the transplantation of dental cells under kidney capsules to prove their ability to develop tooth-like structures, are some examples. In salivary gland stem cell biology, ligation–deligation techniques forcing the putative adult stem cell expansion are used. Briefly, closing or ligating the main duct in a salivary gland causes wide organ damage and atrophy. The subsequent duct deligation, triggers a very intense process of gland regeneration which has been extensively studied to identify the putative adult stem cells (Scott et al., 1999; Carpenter et al., 2009).

Although very informative, most of these techniques do not reflect the physiological situation. Indeed, lineage tracing experiments using inducible Cre–lox technology have recently demonstrated that the potential of cells can be different during normal homeostasis and experimental conditions (van Keymeulen et al., 2011). Thus, there is a need to overcome such technical limitations in order to be able to unequivocally define epithelial adult stem cells in ectodermal appendages. Hereby, we provide an overview of the current knowledge on epithelial adult stem cells in some ectodermal appendages such as hair follicles, mammary glands, teeth, and salivary glands during normal development.

Similar to other rapidly renewing organs, quiescent, and active adult stem cells coexist in hair follicles (Li and Clevers, 2010). Cotsarelis and colleagues first proposed the presence of slow cycling and label retaining stem cells. Those cells are residing in the bulge, a small area close to the attachment-site of the *erector pili* (Cotsarelis et al., 1990). Bulge stem cells express the surface marker CD34 as well as a truncated isoform of Keratin 15, and they have the ability to form colonies *in vitro* and produce fully functional hair follicle and interfollicular epidermis *in vivo* (Trempus et al., 2003; Blanpain et al., 2004; Morris et al., 2004). Apart from the slow cycling bulge stem cells, actively proliferating stem cells have been also described. These stem cells express Leucine-rich G protein-coupled receptor 5 (Lgr5) and reside not only in the bulge but also in the hair germ. Lgr5-positive stem cells contribute to the renewal of the lower hair follicle components under physiological conditions, although they can be forced to regenerate the entire hair follicle, the sebaceous gland, and the interfollicular epithelium in transplantation assays (Jaks et al., 2008).

The existence of adult mammary stem cells (MaSCs) is strongly supported by the fact that any portion of the adult mouse mammary epithelium, at any developmental stage, is able to grow and give rise to an entire new ductal system when transplanted into epithelium-free fat pads (DeOme et al., 1959; Smith and Medina, 1988). However, the newly formed mammary epithelium can only be re-transplanted for approximately seven generations, suggesting that MaSCs have a finite life span (Daniel et al., 1968). Further attempts to characterize MaSCs using FACS revealed that $Cd24^{low}Cd49f^{high}$ and $Cd24^{low}Cd29^{high}$ populations are enriched for MaSCs, based on their high regeneration ability when injected into cleared fat pads (Shackleton et al., 2006; Sleeman et al., 2006; Stingl et al., 2006). However, recent data based on lineage tracing experiments suggest that during postnatal mammary gland development, newly generated luminal or myoepithelial cells arise from unipotent progenitors rather than from multipotent adult stem cells (van Keymeulen et al., 2011).

Multiple lines of evidence support the existence of adult stem cells in salivary glands. Radiolabeling experiments have demonstrated the presence of multipotent cells in the intercalated ducts that can contribute to both acinar and ductal cell lineages (Zajicek et al., 1985; Man et al., 2001). More recently, Kishi et al. (2006) used a clonal assay to demonstrate the existence of common progenitors among neonatal rat submandibular gland cells. Damage-induced regeneration assays have served to isolate adult stem cell enriched populations from adult salivary glands based on their c-Kit expression (Lombaert et al., 2008). Moreover, Lombaert and colleagues isolated mouse submandibular gland cells from the duct area and cultured them *in vitro* in a sphere-forming assay. Thus, when c-Kit-positive cells were purified by FACS and transplanted into an irreversibly damaged mouse submandibular gland they were able to regenerate a functional gland, exhibiting stem cell properties.

Mouse incisors present a source of dental epithelial stem cells that allows them growing continuously throughout the animal's life (Harada et al., 2002). These stem cells reside in the so-called cervical loop present in the labial side of the incisor. The differentiation process occurs along the epithelium from the labial side of the incisor starting from the cervical loop that constitutes the niche for such adult epithelial stem cells. Nevertheless, in mouse molars and human teeth, most of dental epithelium disappears shortly after tooth eruption. The only remaining epithelial cells are the epithelial cell rests of Mallassez (ERM), located in the periodontal ligament tissue. Although their function still remains unclear, sub-cultured ERM have the potential to differentiate into ameloblast-like cells, suggesting that they may be quiescent stem cells (Shinmura et al., 2008).

FLEXIBILITY OF ADULT STEM CELLS DURING REGENERATION

Adult stem cells have the capacity to give rise to all the tissue-specific progeny required during normal development and tissue homeostasis. However, epithelial adult stem cells retain some flexibility (or plasticity) that provides them the ability to contribute to the formation of different organs during regeneration processes triggered by wound repair or in the previously mentioned recombination assays. For instance, this plasticity has been observed in hair follicle adult stem cells during wound healing of epidermis. Stem cells from the bulge region of hair follicles contribute only to hair follicle progenies during both embryonic development and adult tissue homeostasis but they can also contribute to interfollicular epidermis during wound healing (Levy et al., 2005, 2007; Blanpain, 2010). Similarly, lineage tracing experiments in the mammary gland have recently demonstrated that although during normal postnatal development and homeostasis, cells arise only from committed progenitors, myoepithelial progenitors can be forced to be multipotent in mammary reconstitution assays

(van Keymeulen et al., 2011). In addition, putative salivary adult stem cells have the ability to give rise to pancreas and liver lineages *in vitro* (Hisatomi et al., 2004).

Thus, the plasticity of adult stem cells represents an advantage for the potential use of stem cell therapies in regenerative medicine. For instance, due to the lack of dental epithelium in erupted human teeth, obtaining functional enamel-producing ameloblasts from a non-dental source of stem cells constitutes a major challenge in the dental regeneration field. Hence, it has been shown that stem cells derived from other tissues such as bone marrow can contribute to the ameloblasts layer in the *in vivo* formed tooth after recombining them with dental and mesenchymal cells (Hu et al., 2006). Nevertheless, in this case, dental epithelial cells were still needed for an organized tooth reconstitution. Given their common origin, it is conceivable that adult stem cells from other ectodermal-derived tissues may be able to differentiate into dental epithelium. In fact, recombination of postnatal non-dental oral epithelium and embryonic dental mesenchyme gives rise to tooth-like structures demonstrating that, similar to hair follicle stem cells, adult oral epithelial stem cells retain a certain plasticity (Nakagawa et al., 2009). Further investigations to gain a better knowledge on the molecular and cellular mechanisms involved in ectodermal organs' development and injury response are still required in order to successfully apply the regenerative therapies to human diseases and tissue damage.

SIGNALING PATHWAYS INVOLVED IN THE REGULATION OF ECTODERMAL STEM CELLS

Several signaling pathways coordinately regulate the initiation and further development of ectodermal organs. The study of transgenic animals has shed some light on which molecules regulate the processes of specification of the skin or oral ectodermal stem cells into diverse tissue-specific progenitors, as well as the consequent fate choices to give rise to the distinct cell populations that will build up the organ.

BONE MORPHOGENETIC PROTEINS

Bone morphogenetic proteins (BMPs) are secreted growth factors that belong to the transforming growth factor beta (TGFβ) superfamily (Zeng et al., 2010). They were first described by their ability to induce bone formation, but since then, they have been implicated in several processes such as proliferation, apoptosis, tumorigenesis, tissue repair, stem cell maintenance, and epithelial cell fate determination (Wagner et al., 2010). BMP signaling is activated when BMP ligand dimers bind to the receptor complex. Two different types of serine/threonine kinase receptors form the BMP receptor complex: BMP receptor type I (BMPRI) and type II (BMPRII). When the signaling is activated, BMPRII receptors phosphorylate BMPRI receptors, which in turn phosphorylate the cytoplasmic common mediator Smad (co-Smad or Smad4) inducing its binding to the receptor-activated Smads (R-Smads). Subsequently, the complex formed by the co-Smad and R-Smads is translocated to the nucleus where it binds to the BMP response elements (BRE) in the promoter of BMP target genes, inducing or inhibiting their expression. BMP signaling is regulated at the extracellular level by various molecules that function as BMP antagonists, including Noggin, Chordin, Follistatin and follistatin-related gene product (FLRG), Ventroptin, twisted gastrulation (Twsg1), and the Dan/cerberus family of proteins (Zeng et al., 2010).

During early embryogenesis in vertebrates, BMP4 is considered to be the signal that induces the epidermal vs neural fate of the ectoderm at gastrulation stage (Wilson and Hemmati-Brivanlou, 1995). The levels of BMPs have shown to be important in the fate determination of later epidermal progenitors during embryogenesis. Thus, lowering the levels of BMP signaling by conditional overexpression of Noggin, a BMP antagonist, results in defects in ectodermal organs due to inappropriate fate inductions. For instance, K14-Noggin overexpressing mice show replacement of sweat glands by pilosebaceous units (Plikus et al., 2004) and conversion of the nipple to hair-bearing epithelia (Mayer et al., 2008). Similarly, the blockage of BmpRIb-mediated signaling in chicken embryos leads to the transformation of dorsal scales to feather filaments in the dorsal surface of the foot (Zou and Niswander, 1996). Expression of BMP4 in the early oral epithelium has been shown to be necessary for the induction of tooth formation (Ohazama et al., 2004b) and Bmp7 deficient mice occasionally lack teeth (Zouvelou et al., 2009). Moreover, Cre-mediated loss of epithelial *Bmpr1a* results in striking defects in postnatal hair follicle differentiation, as well as in an early arrest of tooth morphogenesis at bud stage (Andl et al., 2004). Thus, BMP signaling plays an essential role in the organ-type specification and development initiation of different organs from ectodermal origin.

NOTCH

Notch signaling is a highly conserved pathway that involves cell-cell contact. In mammals, 4 receptors have been described (Notch1–Notch4) with 5 ligands (Jagged1, Jagged2, Delta-like1, Delta-like3, and Delta-like4). Upon ligand-receptor binding, the extracellular domain of Notch receptors is proteolytically cleaved. Subsequently, the intracellular domain (NICD) is translocated to the nucleus where it binds to the transcription factor CSL (CBF-1 in humans or RBP-J in mice), forming a complex that regulates the transcription of Notch target genes (**Figure 6**). Notch signaling has diverse and multiple functions on cell differentiation, proliferation, and survival.

The Notch pathway is a master regulator of cell fate determination. In invertebrates, Notch activation directs accurate cell fate choices by restricting differentiation toward alternative fates and permits the self-renewal and survival of multipotent cells (Artavanis-Tsakonas et al., 1999). In vertebrates, diverse cell types express the different Notch receptors and ligands, indicating that Notch signaling may have more complex functions (**Figure 7**). Notch signaling regulates the switch from basal to suprabasal fate in epidermis (Blanpain et al., 2006) and controls differentiation and homeostasis in hair follicles (Pan et al., 2004). In the mammary gland, Notch activation specifies the luminal cell fate (Yalcin-Ozuysal et al., 2010). In the developing tooth, Notch signaling has been proposed as an important regulator of dental cell type specification in the enamel organ (Mitsiadis and Graf, 2009). Notch1 is expressed in stratum intermedium, whereas Jagged2 and Delta-like1 are expressed in the adjacent cell layer of inner enamel epithelium, suggesting that Jagged2 and Delta-like1 proteins may function as ligands for Notch1 receptor during tooth development

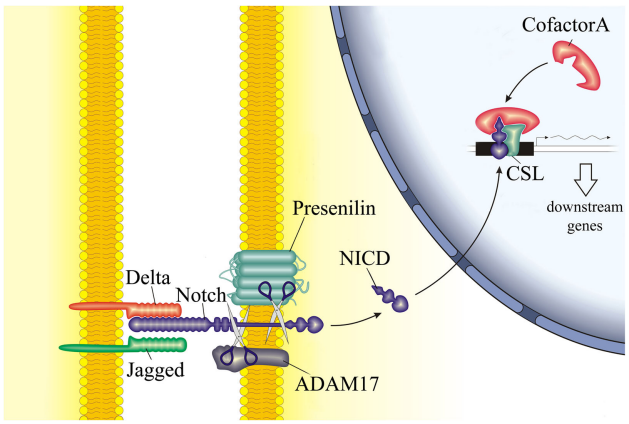

FIGURE 6 | Schematic representation of the Notch signaling pathway. The interaction between Notch receptor on one cell and its ligands on the neighboring cell results in the proteolytic cleavage of Notch receptor and release of its inter-cellular domain (NICD) into the cytoplasm. NICD therefore translocates to the nucleus and interacts with cofactors to regulate the expression of downstream genes.

(Mitsiadis et al., 1995, 1997, 1998, 2010). It is possible that cell fate determination in developing dental epithelium occurs through inhibitory interactions between adjacent epithelial cells. Notch signaling seems to have an essential role in those interactions, influenced by extrinsic signals belonging to other pathways such as fibroblast growth factor (FGF) and BMP signaling pathways (Mitsiadis and Graf, 2009; Mitsiadis et al., 2010).

WINGLESS-TYPE MMTV INTEGRATION SITE (Wnt)

Wnt ligands are secreted, cysteine-rich proteins that signal through transmembrane receptors belonging to the Frizzled family (Wodarz and Nusse, 1998). Wnt signaling can activate both canonical and non-canonical pathways. The canonical transduction pathway involves the activation of the intracellular protein Disheveled (Dsh). β-Catenin is consequently stabilized and then translocated to the nucleus where it forms complexes with TCF/LEF transcription factors regulating the expression of target genes.

Wnt signaling has been implicated in the induction of placode formation during early developmental stages of several ectodermal organs (Andl et al., 2002; Chu et al., 2004). Hyper-activation of canonical Wnt signaling in the mouse oral epithelium results in the formation of supernumerary teeth due to a continuous induction of dental epithelium thickening and subsequent tooth development throughout life (Järvinen et al., 2006). Lef-1 is a Wnt target transcription factor that it is also involved in the transduction of canonical Wnt signaling. Deletion of *Lef-1* in mice results in the developmental arrest of ectodermal organs such as mammary gland, hair, and tooth at the bud stage. Lef-1 overexpression under the Keratin14 (K14) promoter in mice leads to an inappropriate positioning of hair follicles in the skin (Zhou et al., 1995). Moreover, an ectopic formation of tooth-like structures and hair follicles in lip furrow epithelium is apparent in these mice. Interestingly, the expression of K14, and thus Lef-1, is higher in that region than in the rest of the oral epithelium. Taken together, these data

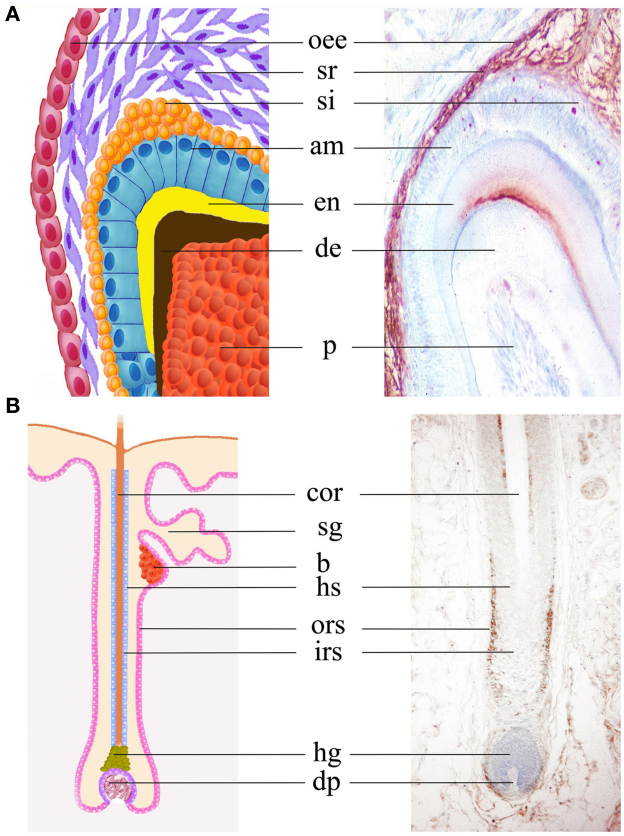

FIGURE 7 | Expression of the Notch2 protein in molar teeth and hair follicles. Immunohistochemical staining (brown color) on sections shows Notch2 expression in stellate reticulum, stratum intermedium, outer enamel epithelium, and dental papilla in postnatal day 6 mouse molars **(A)**. In human hair follicles, Notch2 protein is mainly detected in outer root sheath **(B)**. Abbreviations: am, ameloblast; b, bulge; cor, cortex; de, dentin; dp, derma papilla; en, enamel; irs, inner root sheath; hg, hair germ; hs, hair sheath; oee, outer enamel epithelium; ors, outer root sheath; p, pulp; si, stratum intermedium; sg, salivary gland; sr, stellate reticulum.

suggest that Wnt signaling activation is necessary for the induction of ectodermal appendages during normal development. Furthermore, its activation levels are crucial in regulating ectodermal stem cell fate, forcing them to form appendages in ectopic sites when altered.

FIBROBLAST GROWTH FACTORS

The FGF family is composed of 22 secreted polypeptides that bind to specific transmembrane tyrosine kinase receptors. To date, four different *Fgfr* genes have been described (*Fgfr1–Fgfr4*) encoding 7 different major proteins (FGFRs 1b, 1c, 2b, 2c, 3b, 3c, and 4) with differing ligand-binding specificity (Itoh and Ornitz, 2011). FGFs are among the main regulators of embryonic development controlling cell proliferation, survival, and differentiation (Szebenyi and Fallon, 1999).

The study of different mouse models in which FGF signaling is altered has demonstrated their involvement in epithelial–mesenchymal interactions that characterize the development of ectodermal organs. Abrogation of FGF signaling has been related

to skin alterations and a defective wound healing capacity (Werner et al., 1994; Ortega et al., 1998). Regarding skin appendages, a number of FGF mutants with hair defects exist (Hébert et al., 1994; Petiot et al., 2003). In addition, FGF8, FGFR1, and FGF10/FGFR2b signaling have been shown to be important during the early stages of mammary gland development (Mailleux et al., 2002; Eblaghie et al., 2004) pointing to FGFs as essential regulators of the specification of the ectodermal stem cells from the ventral skin to form mammary placodes during embryogenesis. FGFs have also been implicated in the development of oral ectoderm-derived organs such as salivary gland and tooth. In the salivary gland, FGF10 is crucial for the initiation and correct patterning of submandibular salivary gland (Ohuchi et al., 2000). During early odontogenesis, FGF signaling determines the position and patterning of teeth (Neubüser et al., 1997). Moreover, FGFs have been described as important regulators of dental epithelial stem cells. In the absence of FGF10, the putative stem cell niche termed cervical loop cannot be maintained and mouse incisors cease their growth (Harada et al., 2002). In conclusion, FGFs function as inductors of ectodermal appendage development and regulators of tissue-specific stem cells residing in the epithelium of different ectodermal-derived organs such as epidermis and teeth.

ECTODYSPLASIN A (EDA)

EDA is a member of the tumor necrosis factor (TNF) superfamily. Mice carrying mutations in *Eda* gene present defects in the development of several ectodermal organs such as hair, teeth, and sweat glands (Mikkola et al., 1999). Interestingly, mice overexpressing EDA in developing epidermis present supernumerary teeth and mammary glands. Furthermore, treating embryonic skin with recombinant EDA induces the formation of epithelial placodes (Mustonen et al., 2004).

ECTODERMAL DYSPLASIAS

Several types of tissue-specific diseases often affect ectoderm-derived organs. The existence of disorders affecting the development of more than one of these organs suggests that the function of a putative stem cell residing in the embryonic ectoderm may be altered. Ectodermal dysplasias are defined as a highly heterogeneous group of rare heritable disorders characterized by defects in two or more organs of ectodermal origin (Pinheiro and Freire-Maia, 1994). Dysregulation of genes involved in the normal ectodermal organogenesis can lead to ectodermal dysplasia. This is the case of the transcription factor p63, in which mutations in humans can be the cause of various ectodermal dysplasias (Vanbokhoven et al., 2011). Studies on p63 knockout mice have demonstrated that the *p63* gene is crucial for the appropriate stratification of skin epidermis and the correct development of several ectodermal appendages (Mills et al., 1999; Yang et al., 1999). Furthermore, other mutations in molecules involved in the EDA signaling pathway cause severe pathologies in both humans and mice, such as hypohidrotic ectodermal dysplasia (Itin and Fistarol, 2004). Ectodermal dysplasias are often characterized by the absence of some ectodermal appendages from the embryonic stages. Thus, these disorders are likely the consequence of a failure during the process of stem cell specification that may be caused by either the loss of the potency or the self-renewal and proliferation capacity of early ectodermal stem cells.

CONCLUDING REMARKS

Ectoderm-derived skin and oral cavity, as well as the highly specialized appendages that arise from them, are in close contact with the environment. Consequently, these organs are exposed to a great risk of being damaged and it becomes extremely important that they retain regeneration capacity throughout the organisms' life. Adult stem/progenitor cells represent the source for the repair of ectoderm-derived organs during tissue homeostasis as well as upon injury conditions. However, this source may be limited due to aging, or due to certain pathological conditions, leading to the inability of stem cells to repair the damaged tissue. Regenerative medicine tries to overcome these limitations.

Major efforts are being made to isolate and characterize putative adult stem cells in different ectodermal organs. However, in most of the cases, only stem cell enriched populations have been isolated, probably including not only putative stem cells but also more differentiated cell types. Several assays have been developed to assess the regeneration potential of putative stem cells in order to define their stemness. It is essential to bear in mind that those assays may not reflect the normal developmental process but instead they reproduce an injury-repair situation where stem cells may be forced to be more plastic. Thus, *in situ* lineage tracing experiments provide valuable information concerning cell fate determination under more physiological conditions.

Data obtained from the study of diverse knockout and transgenic mice have improved the knowledge on the regulation of stem cell fate determination at different developmental stages of ectodermal appendages. Signaling molecules belonging to different pathways have been shown to have important roles in such processes. Nevertheless, the tightly controlled interaction in time and space between these signaling pathways still remains unclear. Thus, a better understanding of the molecular mechanisms underlying cell fate choices during normal development and homeostasis of ectodermal-derived organs is mandatory in order to successfully make use of stem cell biology-based therapies in regenerative medicine.

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Impact of stem cells in craniofacial regenerative medicine

Pedro A. Sanchez-Lara[1,2,3,4,5]*, Hu Zhao[4], Ruchi Bajpai[4], Alaa I. Abdelhamid[6] and David Warburton[1,2,3,4,5]*

[1] Children's Hospital Los Angeles, Keck School of Medicine, University of Southern California, Los Angeles, CA, USA
[2] Department of Pediatrics, Keck School of Medicine, University of Southern California, Los Angeles, CA, USA
[3] Department of Pathology, Keck School of Medicine, University of Southern California, Los Angeles, CA, USA
[4] Center for Craniofacial Molecular Biology, Ostrow School of Dentistry, University of Southern California, Los Angeles, CA, USA
[5] Developmental Biology and Regenerative Medicine Program, Saban Research Institute, Children's Hospital Los Angeles, Los Angeles, CA, USA
[6] Tissue Engineering and Biomaterials Research Unit (TEBRU), Qassim College of Dentistry, Qassim University, Kingdom of Saudi Arabia

Edited by:
Anis Feki, Geneva University Hospitals, Switzerland

Reviewed by:
Christian Morsczeck, University of Regensburg, Germany
Ivo Lambrichts, Hasselt University, Belgium
Catherine Chaussain, Université Paris Descartes Paris Cité, France

***Correspondence:**
Pedro A. Sanchez-Lara and David Warburton, Developmental Biology and Regenerative Medicine Program, Saban Research Institute, Children's Hospital Los Angeles, 4650 West Sunset Blvd, MS#100, Los Angeles, CA 90027, USA.
e-mail: Pedro.Sanchez@usc.edu; dwarburton@chla.usc.edu

Interest regarding stem cell based therapies for the treatment of congenital or acquired craniofacial deformities is rapidly growing. Craniofacial problems such as periodontal disease, cleft lip and palate, ear microtia, craniofacial microsomia, and head and neck cancers are not only common but also some of the most burdensome surgical problems worldwide. Treatments often require a multi-staged multidisciplinary team approach. Current surgical therapies attempt to reduce the morbidity and social/emotional impact, yet outcomes can still be unpredictable and unsatisfactory. The concept of harvesting stem cells followed by expansion, differentiation, seeding onto a scaffold and re-transplanting them is likely to become a clinical reality. In this review, we will summarize the translational applications of stem cell therapy in tissue regeneration for craniofacial defects.

Keywords: stem cell, craniofacial, regeneration

INTRODUCTION

Although conventional surgical treatments for congenital and acquired craniofacial problems continue to make progress, the final functional and cosmetic outcomes can be varied, unpredictable and sometimes unsatisfactory mostly because of complications, infections, and scar tissue. The promise of regenerative medicine brings new energy and hope for improved outcomes by replacing damaged or absent tissues with healthy regenerated tissue (**Figure 1**).

One potential stem cell based strategy for repairing craniofacial defects is the use of embryonic stem (ES) cells. ES cells are derived from the inner cell mass (ICM) of the blastocyst and possess the capacity to differentiate into all cell types (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998). However, the application of ES cells for clinical purposes has been limited by ethical issues, dysregulated ES cell differentiation, and immune rejection. In addition, the possibility of genomic instability and tumorigenesis still needs to be examined before any large-scale clinical experiments are planned.

The ability to generate induced pluripotency stem (iPS) cells is one of the major breakthroughs in stem cell study in recent years (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). Somatic cells from human fibroblast cells can be reprogrammed into a primordial, ES-like state and are able to differentiate into all three germ layers (ectoderm, endoderm, and mesoderm). This technology offers a revolutionary approach for the introduction of autologous multipotential stem cells into patient-specific, tissue-specific regeneration and repair.

Applications of iPS cell technology to the clinic are still at a preliminary stage and face some of the same concerns as their ESC counterpart. Foremost amongst them is the issue of dysregulated growth (Li et al., 2008) and lack of methods for is ensuring accurate and complete reprogramming of differentiated somatic cells from progeny. The second issue of major concern is the potential for tumor growth and development from even micro contamination of undifferentiated cells. This is further compounded by the persistence of ectopic gene expression, since iPS cells are often produced by transduction of somatic cells with lentivirus encoding ectopic transgenes. There are concerns that the continuous expression of transgenes may bring the risk of abnormal tumor growth (Nelson et al., 2010). The next generation technologies using small molecules or alternative approaches to gene induction may address these limitations. Coupled with recent advances in identifying biomarkers to select against tumor forming pluripotent cells and robust techniques to differentiate the iPS and ES cells into lineage restricted stem cells, the potential therapeutic use of stem cells ideally suited for craniofacial repair is gaining ground (Alvarez-Manilla et al., 2010; Bajpai et al., 2010; Curchoe et al., 2010).

Tissue-specific postnatal stem cells have been isolated from a variety of organs and tissues, including but not limited to, bone marrow (Castro-Malaspina et al., 1980; Civin et al., 1984), neural tissue (Flax et al., 1998; Johansson et al., 1999), muscle (Chen and Goldhamer, 2003; Huard et al., 2003), and skin (Janes et al., 2002; Lavker and Sun, 2003). Compared to ES or iPS cells that self-renew indefinitely, tissue-specific adult stem cells also have

significant self-renewal capability, but severely limited differentiation ability. In the craniofacial region, multiple types of stem cells have been recognized, including bone marrow mesenchymal stem cells (BMMSC), muscle satellite cells (MSCs), dental pulp stem cells (DPSCs), periodontal ligament stem cells (PDLSCs), and stem cells from human exfoliated deciduous (SHED teeth; Gronthos et al., 2000, 2002; Miura et al., 2003; Seo et al., 2004; Akintoye et al., 2006) (**Figure 2**).

In the current review, we aim to summarize the application of stem cell therapies in tissue regeneration of various craniofacial defects.

SCAFFOLDS AND BIOMATERIALS

Craniofacial reconstructive surgery manipulates available tissues in a three dimensional field, either by transferring tissue from a donor site or supporting and shaping the repair with artificial scaffolds and biomaterials. Biomaterials in stem cell tissue engineering and regeneration not only provide a supportive scaffold but also create an artificial niche that allows natural processes of stem cell renewal, proliferation, and differentiation while promoting vascularization, integration, adhesion, and survival of the newly generated tissue (Rossi et al., 2010a). Incorporating small molecules and growth or differentiation promoting factors within the biomaterials can further potentiate these natural repair processes resulting in efficient biological repair. The basic requirement for all biomaterial used for tissue engineering purposes is that it be inert and does not provoke a significant inflammatory response. However the tensile strength, biostability or biodegradability are features that will be favored in a context dependent manner. Inert stable scaffolds provide rigidity but lack the ability to remodel with age. While biodegradable scaffolds that provide transient three-dimensional contour for the regenerating tissue are especially appealing for soft tissue repair but raise the concern of inadequate regeneration, inadequate mechanical properties of the newly formed tissue and sustained function over long periods of time.

CRANIOFACIAL BONE TISSUE ENGINEERING AND STEM CELLS

Current clinical approaches for reconstructing craniofacial bone defects include autologous bone grafts, allogeneic bone grafts, and prosthetic grafts such as titanium frameworks (Marchac, 1982; Shenaq, 1988; Goodrich et al., 1992; Cowan et al., 2004). Stem cell based strategies are currently a promising approach in craniofacial bone tissue engineering (**Figure 1**).

Different cell sources have been used for repairing craniofacial bony defects. BMMSCs have been reported to be capable of multipotential differentiation. When cultured in the presence of dexamethasone, inorganic phosphate, and vitamin C, BMMSCs can be induced to become osteoblast-like cells *in vitro* and form calcified nodules (Gronthos et al., 1994). However, when transplanted into immunocompromised mice, only a subset of the BMMSCs was able to form ectopic bone in the host, suggesting that heterogeneity exists among BMMSCs (Kuznetsov et al., 1997; Gronthos et al., 2003). Successful repair of bony defects has been demonstrated in both calvarial and long bone in various animal models (Bruder et al., 1998; Krebsbach et al., 1998; Kon et al., 2000; Mankani et al., 2001). Autologous BMMSCs have also been used in clinical experiments to repair bony defects in the mandible. In a study conducted by Warnke and coworkers, autologous BMMSCs were seeded onto custom-made mandible scaffolds composed of titanium and bone mineral blocks containing BMP7. The construct was placed under the patient's skin for 7 weeks and then transplanted into the patient's mouth to repair the mandible defect. The patient showed significantly improved masticatory function and was satisfied with the esthetic outcome (Warnke et al., 2004).

Adipose-derived mesenchymal stem cells (AMCs) are also used for repairing craniofacial bone defects. AMCs are readily obtained via lipo-aspiration and expand easily *in vitro*. They are multipotential and capable of forming different types of tissue including muscle, bone, neural, and chondrocyte tissues (Zuk et al., 2001; Gimble and Guilak, 2003; Hicok et al., 2004). AMCs taken from human sources were shown to be able to form bone when seeded in an HA-TCP scaffold and transplanted into immunocompromised mice (Hicok et al., 2004). In a clinical experiment conducted by Cowan and coworkers, AMCs were expanded *in vitro* and seeded in apatite-coated, PLGA scaffolds. The construct was then transplanted into a human patient to repair a critical size calvarial bone defect. New bone formation was observed 2 weeks after transplantation and complete bony bridging was observed by 12 weeks. Over 90% of the new bone formation was contributed by the transplant (Cowan et al., 2004).

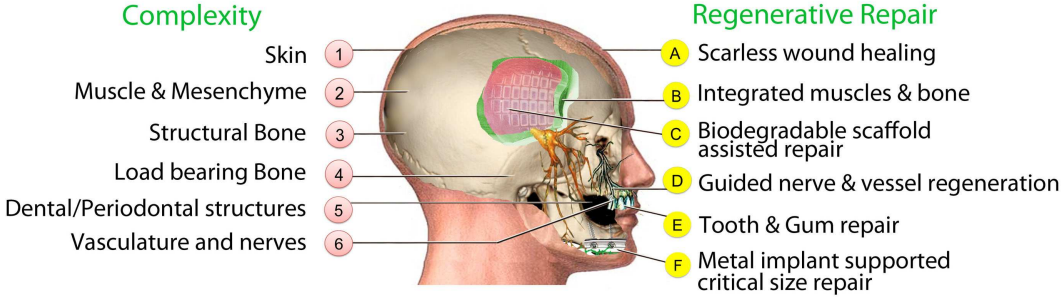

FIGURE 1 | Craniofacial structures and potential for regenerative repair.

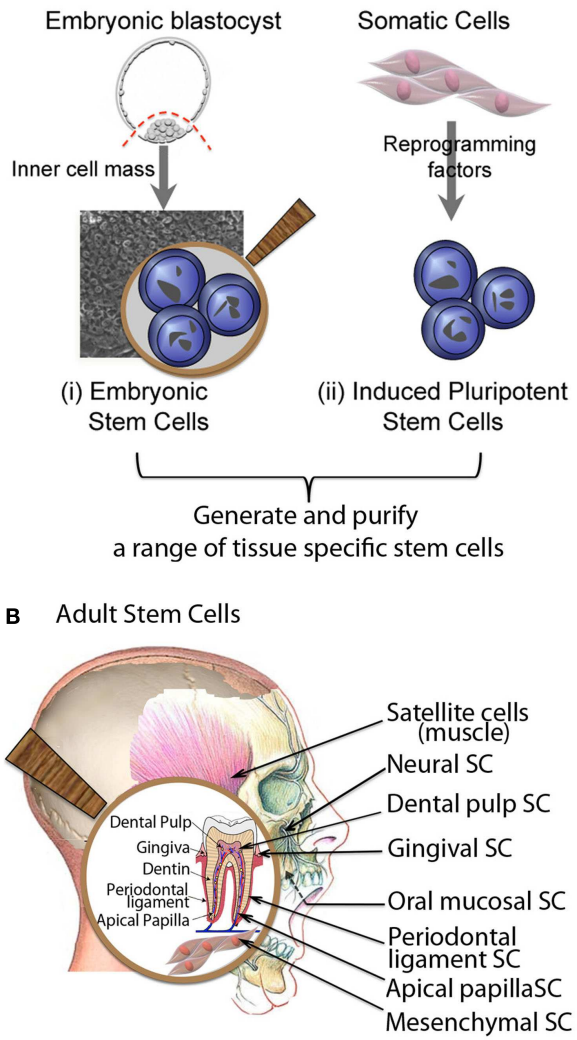

FIGURE 2 | Major sources of cells for craniofacial repair and regeneration. (A) Pluripotent stem cells, **(B)** Adult stem cells.

For tissue engineering purposes, stem cells are usually delivered with scaffolds. One critical requirement for the bone-engineering scaffold is osteoconductivity, which means the ability of the scaffold construct to integrate with the host bone (Zaky and Cancedda, 2009). It has been widely accepted that the HA-TCP combination provides the best bone integration ability and maintains a proper resorption rate (Cancedda et al., 2007). In addition, polymeric materials have also been widely tested as the scaffold. These scaffold materials are based on alpha-hydroxy acids and are usually composed of polyglycolic acid, poly-L-lactic acid, or both. They have limited osteoconductive ability but can be excellent scaffold materials when combined with HA for bone repair (Cancedda et al., 2007).

Besides supporting the cells, another important function of the scaffold is to deliver growth factors. Various growth factors promote angiogenesis and osteogenesis. For example, BMP2, TGF-beta, and VEGF all enhance bone formation from osteogenic cells (Rose et al., 2004; Yang et al., 2004; Montjovent et al., 2007; Oest et al., 2007). Transgenic BMMSCs that over express BMP-4 also enhance bone formation (Savarino et al., 2007). Despite the many informative and promising results, the effects of growth factors still need to be studied more thoroughly, considering some unexpected effects such as oncogenicity (Hunter and Avalos, 2000).

SKELETAL MUSCLE TISSUE REGENERATION AND STEM CELLS

Functional muscle regeneration is likely the most challenging task in craniofacial tissue regeneration, yet with the potential for the greatest impact. Muscle reconstruction plays a critical role in rebuilding functional craniofacial structures like cleft palate repair, whole face reconstruction from congenital or acquired deformities and tongue reconstruction after tumor resection. Current therapies require surgical tissue transfer from local or distant donor sites, which can generate secondary morbidity of volume loss and pain, with potential additional risks of infection and functional loss.

Multiple stem cell sources of skeletal muscle cells have been identified either within or outside the muscle compartments. Skeletal muscle precursors (myoblasts) can be derived from satellite cells (reserve cells located on the surface of mature myofibers underneath the basal lamina) or from cells lying beyond the myofiber, e.g., interstitial connective tissue or bone marrow. Both of these classes of cells may have stem cell properties (Grounds et al., 2002). *In vivo*, SCs can be characterized by their expression of Pax7 (Seale et al., 2000). SCs can be isolated through either mechanical or enzymatic disassociation from muscle fibers and can be expanded *in vitro* (Rosenblatt et al., 1995; Collins et al., 2005; Cerletti et al., 2008; Rossi et al., 2010b). After transplantation, they maintain a strong myogenic potential both *in vitro* and *in vivo*. SCs obtained from single fiber explants, then expanded and injected intramuscularly, showed poor proliferation and regeneration ability, yet freshly isolated SCs present better proliferative and regeneration potential (Beauchamp et al., 1999; Collins et al., 2005; Montarras et al., 2005; Rossi et al., 2010b). Therefore, the preferred method of delivering SCs might be to isolate SCs freshly from a muscle biopsy and deliver them directly, to avoid the disadvantages of *in vitro* culture.

Several other cell types have been identified as myogenic within the muscle compartment. The mesoangioblasts are associated with blood vessels and express early endothelial markers including Flk1, CD34, stem cell antigen-1, and VE-Cadherin (Barberi et al., 2007). They can proliferate for several passages with no obvious tumorigenic potential. Using co-culture with myoblasts, they can be easily induced into myoblasts (Cossu and Bianco, 2003). Some factors including integrin-alpha4, stromal cell derived factor-1 (SDF-1), and TNF-alpha promote the migration of wild type mesoangioblasts to dystrophic muscles by five fold, which enables the production of new muscle fibers expressing a normal amount of the mutated genes (Galvez et al., 2006). Another myogenic stem cell population within the muscle compartment is the pericyte. The pericyte can be characterized by expression of NG2 and PDGF receptor beta. Pericytes isolated from numerous

human organs have been shown to be myogenic both *in vitro* and *in vivo* (Quattrocelli et al., 2011). Skeletal myogenic progenitors (SMPs) have been isolated based on beta1-integrin and CXCR4 expression from skeletal muscle (Cerletti et al., 2008). SMPs were able to restore dystrophin expression and improve muscle function and histology when transplanted into dystrophic mice. SMP transplantation resulted in the rebuilding of a functional stem cell pool within the muscle compartment of the recipient mice.

Muscle stem cells (MuSCs) were also isolated by the specific expression of integrin-alpha7 and CD34. MuSCs injected into muscles of mice damaged with notexin were able to rebuild the host SC niche and generate new muscle fibers (Sacco et al., 2008).

Outside of the muscle compartment, BMMSCs are capable of undergoing myogenic differentiation (Bianco et al., 2008). After direct injection, BMMSCs can migrate to damaged muscle sites and undergo myogenic differentiation (Bianco et al., 2008). CD133+ cells into the blood stream have also been tested for their myogenic potential (Torrente et al., 2007). They can be induced *in vivo* and *in vitro* into myogenic progenitors and may be able to contribute to the treatment of muscular diseases together with other bone marrow derived progenitors.

Until now, there have been only a few human clinical trials of stem cell based strategies to treat muscular dystrophy, myocardial infarction and stress urinary incontinence by using SC or CD133+ cells (Tedesco et al., 2010). There have been no reports of regenerating the craniofacial skeletal muscles. Although they share many common properties with the limb skeletal muscles, craniofacial skeletal muscles also possess many unique features. Their embryonic origins are different from the limb muscles (McLoon et al., 2007). Craniofacial skeletal muscles express some immature myosin heavy chain isoforms not present in the limb muscles (McLoon et al., 2007). Moreover, craniofacial skeletal muscles, especially extraocular muscles (EOM) and laryngeal muscles (LM), contain a population of activated satellite cells that is nearly twofold more than that of the limb skeletal muscles (Renault et al., 2002). This raises the interesting possibility that craniofacial muscles might be a better source than limb muscles for obtaining progenitor cells to treat Duchenne and related muscular dystrophies or other musculoskeletal defects.

DENTAL STEM CELLS AND TOOTH REGENERATION

Human teeth are comprised of enamel, dentin, tooth, pulp, and cementum covering the root surface. The periodontal ligament surrounds and supports the tooth. Unlike bone, most hard tissue in the tooth does not undergo renewal after its formation; only dentin can regenerate itself internally upon injury, suggesting the existence of stem cell populations within the tooth pulp. One of the first dental related stem cell populations identified are the DPSCs (Gronthos et al., 2000). DPSCs are capable of differentiating into multiple types of tissue including odontoblast, bone, adipocyte, and neuron (Gronthos et al., 2000; Miura et al., 2003; Huang et al., 2009). In addition, SHED teeth pulp also possess multipotential differentiation ability (Miura et al., 2003). Both SHED and DPSCs are able to generate tissue resembling human tooth pulp under appropriate conditions (Cordeiro et al., 2008; Casagrande et al., 2010; Demarco et al., 2010; Sakai et al., 2010). Several studies have attempted to rebuild teeth *in vitro* by combining tooth pulp derived stem cells with proper scaffold materials (Young et al., 2002; Ohazama et al., 2004; Cordeiro et al., 2008). In the Ohazama study, different types of non-dental derived mesenchymal cells including ES cells, neural stem cells and adult BMMSCs were mixed with embryonic oral epithelium cells. The mesenchyme-epithelium cell mixtures were then delivered into kidney capsules of adult mice in an effort to recapitulate the classical dental epithelium-mesenchyme interactions which initiate and direct tooth development. All the mixtures resulted in the development of tooth-like structures and surrounding bone. This experiment indicated that it is possible to regenerate a tooth by mimicking the natural developmental process (Ohazama et al., 2004). Other studies have used DPSCs or SHED for treatment of disease of non-dental tissue such as muscle dystrophies, critical size bony defect, spinal cord damage, corneal injury, and even systemic lupus erythematosus (Nosrat et al., 2001; Kerkis et al., 2008; Seo et al., 2008; Monteiro et al., 2009; Ishkitiev et al., 2010; Yamaza et al., 2010).

Scaffolds provide a 3-D framework for cells and serve as an extracellular matrix for a finite period of time. Scaffolds provide an environment that allows both cell migration and proliferation, and may be fabricated in pre-determined shapes and composition (Nakashima and Akamine, 2005). The first scaffold material used successfully for tooth tissue engineering was a copolymer of PGA/PLLA and PLGA (Young et al., 2002), which are the most commonly used scaffold materials for tissue engineering studies. These scaffolds are biodegradable and biocompatible. Changing the component ratio can control the degradation rate of the PLLA/PGA scaffold. PLLA has also been used in many tooth tissue engineering studies, and tissue with morphology and structure resembling that of human tooth pulp has been generated after seeding dental pulp stem cells onto the PLLA scaffolds (Cordeiro et al., 2008; Casagrande et al., 2010; Demarco et al., 2010; Sakai et al., 2010). Odontoblast specific marker DMP-1 is detectable within scaffolds generated with gelatin or salt porogens (Demarco et al., 2010). In the future, the ability to control the shape of the tissue engineered tooth generated with appropriate scaffold materials will be a crucial step towards bringing the technique to the clinic (Modino and Sharpe, 2005).

PERIODONTIUM TISSUE REGENERATION AND STEM CELLS

Periodontal diseases affect 15% of the human adult population, with periodontal soft tissue loss and subsequent supporting bone resorption leading to loss of teeth (Mase et al., 2006). Current treatment approaches include the use of guided tissue regeneration, bioactive grafting materials, and application of bioactive molecules to induce regeneration, but the overall effects of these approaches are relatively modest and limited in practical applications. Regenerating the periodontium is a challenge in the treatment of periodontal diseases due to its complex structure, consisting of cementum, periodontal ligament, gingiva, and supporting bone. Thus, regeneration of the periodontium will require either multiple cell populations or a multipotential stem cell population.

The Periodontal ligament is unique among the ligament and tendon tissues of the body, because it is the only soft tissue connecting two distinct hard tissues (McCulloch et al., 2000). The periodontal ligament suspends the tooth like a cushion in order transduce the mechanical load from the teeth evenly onto the supporting bone. Early studies of different animal models demonstrated that the periodontal tissues possess some regeneration activity, suggesting the existence of stem cell population within the periodontium (Karring et al., 1980; Nielsen et al., 1980; Nyman et al., 1980, 1982; Parlar et al., 2005). After depletion of various periodontal tissues, not only the periodontal ligaments, but also cementum and alveolar bone, can be regenerated, suggesting the presence of multipotential stem cell populations (Nielsen et al., 1980; Nyman et al., 1982; Parlar et al., 2005). Studies conducted on the human periodontium indicate the presence of a putative PDLSCs population (Seo et al., 2004), positive for MSC markers including STRO-1 and CD146 and able to differentiate into osteoblasts, adipocytes, and cementoblasts (Seo et al., 2004). Human PDLSCs expanded *in vitro* can contribute to periodontal tissue regeneration when transplanted into immunocompromised mice (Seo et al., 2004). PDLSCs are multipotential and can differentiate *in vitro* into various mesodermal (adipocyte, osteoblast, and chondrocyte), ectodermal (neuron), and endodermal (hepatocyte) cell types (Seo et al., 2005).

Cells of non-dental origins have also been tested for periodontal tissue regeneration. In 2004, Kawaguchi et al. and coworkers transplanted *ex vivo* expanded bone marrow MSCs into recipient dogs with periodontal defects. After a month, the transplanted cells were able to repair the defective periodontal tissue, including cementoblasts, periodontal ligament, and bone. This study suggested that bone marrow MSCs could be used as a source for periodontal tissue regeneration (Kawaguchi et al., 2004). Their following study indicated that regeneration by MSCs could be enhanced by addition of brain-derived neurotrophic factor (BDNF). BDNF increased the expression level of multiple bone and periodontal tissue related markers including OPN, BMP2, collagen I, ALPase, and VEGF (Takeda et al., 2005).

Cell sheet engineering has emerged as a novel alternative approach for periodontal tissue engineering without the disruption of critical cell surface proteins such as ion channels and growth factor receptors or cell-to-cell junction proteins. In this approach, PDL cells are isolated from an extracted tooth and cultured on temperature-responsive culture dishes at 37°C. Transplantable cell sheets can be harvested by reducing the temperature to 20°C and transplanted into a bony defect (Huang and Zhang, 2011). This method results in an obvious cementum layer and Sharpey's fibers (Flores et al., 2008). Cell sheet engineering therefore allows for tissue regeneration by either direct transplantation of cell sheets to host tissues or the creation of three dimensional structures via the layering of individual cell sheets. By avoiding the use of any additional materials such as carrier substrates or scaffolds, the complications associated with traditional tissue engineering approaches, such as host inflammatory responses to implanted polymer materials, can be avoided. Thus, cell sheet engineering presents several significant advantages and can overcome many of the problems that have previously restricted tissue engineering with biodegradable scaffolds (Yang et al., 2005).

ORAL MUCOSA STEM CELLS AND TISSUE ENGINEERING

The human oral mucosa is highly active in terms of cell turnover and regeneration, which suggests the existence of one or more types of stem cell populations. Recently a stem cell population was identified from the lamina propria of adult human oral mucosa. This population was identified by positive expression of ES cell markers Oct4, Sox2, Nanog, and p75. These cells were localized *in vivo* to cord-like structures. They are highly proliferative *in vitro* and are able to differentiate into tissue of mesodermal (osteoblast, chondrocyte, and adipocyte), endodermal (endothelium), and ectodermal lineages (neuronal cells). Surprisingly, when transplanted into nude mice and treated with dexamethasone, these cells were able to form tumors containing mixed types of tissue (Marynka-Kalmani et al., 2010). This study indicates caution needs to be taken when applying stem cells for tissue engineering. In addition, Tran et al. (2003) reported the transdifferentiation of BMMSCs into buccal epithelial cells in human patients. By tracing the Y-chromosome of the bone marrow MSC male donor in the female recipient patients, they were able to localize the distribution of donor bone marrow MSCs cells on the buccal epithelial cells of the recipients. 1.8% of the recipients' cheek epithelial cells originated from the donor MSC and were detectable 56–1964 days after the procedure (Tran et al., 2003).

Regenerative therapy aims to reduce wound healing time and minimize scar formation. Wound healing of the skin is comprised of three phases: coagulation/early inflammation phase, late inflammation phase, and proliferative phase (Nauta et al., 2011). Although oral mucosa healing goes through the same three phases, it proceeds with an accelerated rate and reduced scar formation (Whitby and Ferguson, 1991). Fibroproliferative scars such as keloid and hypertrophic scars are rarely seen in the oral cavity (Wong et al., 2009). The only exception is the hard palate of the mouse which heals at a much slower rate than any other area of the oral mucosa (Graves et al., 2001). This unique property of the oral mucosa is critical to consider for any tissue engineering study. The first reason for the difference between oral mucosa healing and skin healing processes is the distinctive inflammatory response to the injury. The ratio of TGF-beta1 to TGF-beta3 is much lower in the oral mucosa than in the skin (Schrementi et al., 2008). In addition, fewer inflammatory cells infiltrate the mucosa wound at the initial stage and fewer inflammatory cytokines and chemokines are activated in the wound. Also, angiogenesis is less active in the oral mucosa wound than the skin, so that oral wound healing is quite similar to fetal skin wound healing (Mak et al., 2009).

To date, no satisfactory FDA-approved therapy is available for the treatment of scar tissue. Some reagents have been shown to possess anti-scarring effects. Topical hyaluronic acid and saponin may reduce scar formation by stimulating hyaluronic acid production (Mast et al., 1991). Some TGF-beta3 formulations and neutralizing antibody to TGF-beta1 or 2 have been shown to be effective at reducing scar formation (Rhett et al., 2008). Decorin can limit the duration of TGF-beta effects on inflammation and fibrosis (Jarvelainen et al., 2006). Other factors including TNF-alpha, PDGF, FGF, VEGF, ILGF, EGF, and others have also demonstrated various effects on preventing scar formation (Lawrence, 1998).

TMJ TISSUE ENGINEERING AND STEM CELLS

The temporomandibular joint (TMJ) is comprised of both osseous and cartilaginous structures. It can deteriorate due to injuries, osteoarthritis, or rheumatoid arthritis. The cartilage tissue has a limited capacity of intrinsic repair, so even minor lesions of injury may lead to progressive damage. Severe TMJ lesions need surgical replacement of the mandibular condyle (Sarnat and Laskin, 1992). Currently, a few studies on TMJ tissue engineering have been conducted in animal models. In one study, bone marrow MSCs were isolated from the long bone marrow and expanded *in vitro* under either osteogenic or chondrogenic culture conditions (Alhadlaq and Mao, 2003, 2004). The expanded osteogenic and chondrogenic cells were mixed with PEGDA hydrogel and seeded onto an adult human cadaver mandible condyle in two stratified yet integrated layers. These bi-layer constructs were then placed under nude mice skin for culture. After 4 weeks of implantation, *de novo* formation of human condyle-like structures was detectable replicating the relevant shape and dimensions. Chondrocytes and osteocytes of donor origin were identified in separated layers, and the two cell types infiltrated into the territory of each other, resembling the native condition. However, both chondrogenic and osteogenic layers showed suboptimal maturation, possibly due to an insufficient amount of cells. The same group also constructed a mandibular condyle scaffold by using CAD/CAM techniques and combined it with autologous bone marrow MSC cells. The construct was then transplanted into minipig TMJs. Evaluation and analysis after 1 and 3 months indicated bone regeneration of condyle shape and thus improvement of masticatory function (Mao et al., 2006).

SUMMARY

The impact of tissue engineering and potential applications of stem cells to reconstruct different dental, oral, and craniofacial tissues and structures extend well beyond craniofacial and dental practices. It is to be hoped that future stem cell based therapeutics will replace allograft and autologous tissue grafts, while improving long-term function and eliminating donor site morbidity.

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Human pluripotent stem cells: applications and challenges in neurological diseases

Youssef Hibaoui [1] **** and Anis Feki*** [1,2] *******

[1] *Stem Cell Research Laboratory, Department of Obstetrics and Gynecology, Geneva University Hospitals, Geneva, Switzerland*
[2] *Service De Gynécologie Obstétrique, HFR Fribourg – Hôpital Cantonal, Fribourg, Switzerland*

Edited by:
Gianpaolo Papaccio, Second University of Naples, Italy

Reviewed by:
Pier Paolo Claudio, Marshall University, USA
Vincenzo Desiderio, Seconda Università degli Studi di Napoli, Italy

****Correspondence:***
Youssef Hibaoui, Stem Cell Research Laboratory, Department of Obstetrics and Gynecology, Geneva University Hospitals, 30, Bld de la Cluse, CH-1211 Geneva, Switzerland.
e-mail: youssef.hibaoui@unige.ch;
Anis Feki, Service De Gynécologie Obstétrique, HFR Fribourg – Hôpital Cantonal, Chemin des Pensionnats 2-6, Case postale, 1708 Fribourg, Switzerland.
e-mail: fekia@h-fr.ch

The ability to generate human pluripotent stem cells (hPSCs) holds great promise for the understanding and the treatment of human neurological diseases in modern medicine. The hPSCs are considered for their *in vitro* use as research tools to provide relevant cellular model for human diseases, drug discovery, and toxicity assays and for their *in vivo* use in regenerative medicine applications. In this review, we highlight recent progress, promises, and challenges of hPSC applications in human neurological disease modeling and therapies.

Keywords: pluripotent stem cells, neurological diseases, neurodegenerative diseases, neurodevelopmental diseases, disease modeling, drug screening, regenerative medicine

INTRODUCTION

A major challenge in human neurological diseases is the understanding of the detailed mechanisms responsible for the clinical features. In fact, the lack of access to the affected tissue has limited the study of the molecular and cell biological aspects of the pathogenesis. For instance, several studies reported genotype-phenotype correlations using genetic analysis approaches; however, in most of the studies the molecular mechanisms responsible for the pathogenesis were not fully addressed. Human cell lines and tissues have been used for the study of the pathogenesis of such diseases; however these models are often not relevant as they usually do not recapitulate the human phenotype. Indeed, "healthy" fibroblasts from patients affected by neurological diseases are readily available but these cells are not the neural cells of interest. Neural stem cells (NSCs) have been isolated from human fetal and adult brains in post mortem conditions. While these cells might be an excellent model for the study of human neural development in physiological and pathological conditions (Svendsen et al., 1998; Carpenter et al., 1999; Vescovi et al., 1999; Bahn et al., 2002), they are scarce and do not support systematic analysis. Moreover, long term culture of NSCs has been shown to promote glial differentiation pattern at the expense of neuronal differentiation (Anderson et al., 2007) and to promote cell senescence (Bhattacharyya et al., 2009). Therefore, such effects reduce the potential of these cells for research and therapy. Many notable insights into the neurological disorders have been provided via studies using animal models (mouse principally; Gama Sosa et al., 2012). For some of them, animal models display the neurological phenotype (behavioral abnormalities, anatomical, and cellular perturbations) consistent with human disease (Baker, 2011; Winner et al., 2011). However, the others are not accurately recapitulated in animal models and thus cannot be investigated by this approach (Schnabel, 2008; Scott et al., 2008; Schulz et al., 2009; Chesselet and Richter, 2011). In fact, several neurological phenotypes such as mental retardation or cognitive behavior have human specific manifestations. The incomplete synteny between animal and human genetics together with behavioral and physiological discrepancies account for this.

An innovative way to study human neurological diseases is through the use of human pluripotent stem cells (hPSCs; Park et al., 2008; Mattis and Svendsen, 2011; Zhu et al., 2011). These cells are defined by two criteria: (i) their ability to continually self-renew and (ii) their ability to differentiate into cells of the three primitive germ layers (endoderm, mesoderm, ectoderm). These cells include embryonic stem cells (ESCs), induced pluripotent

Abbreviations: AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; AS, Angelman syndrome; CNS, central nervous system; DA, dopaminergic; DS, Down syndrome; EBs, embryoid bodies; ESCs, embryonic stem cells; FD, familial dysautonomia; FDA, U.S. Food and Drug Administration; FRDA, Friedreich's ataxia; FXD, fragile X syndrome; HD, Huntington's disease; HPRT, hypoxanthine-guanine phosphoribosyltransferase; iPSCs, induced pluripotent stem cells; MSCs, mesenchymal stem cells; NPCs, neural progenitor cells; NSCs, neural stem cells; PD, Parkinson's disease; PGD, pre-implantation genetic diagnosis; PSCs, pluripotent stem cells; PWS, Prader–Willi syndrome; RTT, Rett syndrome; SCZD, schizophrenia; SMA, spinal muscular atrophy; SOD, superoxide dismutase; TALENs, transcription activator-like effector nucleases; VLCFA, very long chain acids; X-ALD, X-linked adrenoleukodystrophy-iPSC; ZFNs, zinc finger nucleases.

stem cells (iPSCs), embryonic germ cells, and embryonic carcinoma cells. In fact, the generation of disease-specific hPSCs offers the opportunity to reproduce normal and pathological neural tissue development (Lee and Studer, 2010). The differentiation of hPSCs into multiple neuronal lineages is a powerful tool for studying early embryonic neurogenesis and the mechanisms involved in the pathogenesis of human neurological diseases. Also, it provides a unique opportunity to generate a number of cells of neural lineage for regenerative medicine (Lee and Studer, 2010; Lee et al., 2010) and should provide new therapies for such diseases.

In this review, we explore the growing interest in using hPSCs and in particular human ESCs (hESC) and iPSCs: *in vitro* as research tools for modeling human neurological diseases and drug screening and *in vivo* in regenerative medicine. We will also highlight the challenges and limitations in the field.

HUMAN EMBRYONIC STEM CELLS

Embryonic stem cells are derived from the inner-cell mass of blastocyst stage embryos (**Figure 1**). Historically, since the isolation of the first mouse embryonic stem cells (mESC) in 1981 (Evans and Kaufman, 1981), it took another 17 years before the generation of the first hESC lines (Thomson et al., 1998). ESCs held great promise in biology and medicine as these cells showed the potential to proliferate over prolonged period of time and to differentiate *in vivo* and *in vitro* into derivatives of the three germ layers endoderm, ectoderm, and mesoderm (Keller, 2005; Murry and Keller, 2008). Typically, ESCs are maintained in the undifferentiated state by co-culture on fibroblasts cells (also called feeder cells) where they retain their ability to self-renew indefinitely. When these ESCs are removed from the feeder cells and transferred in suspension condition, they aggregated to form embryoid bodies (EBs) that contain derivatives of the three germ layers. In this regard, huge efforts have been made to simplify the protocol for maintaining the ESCs in the undifferentiated state; such as culture of ESCs on Matrigel™in the absence of feeder cells (Xu et al., 2001) or the addition of a selective inhibitor of Rho-associated coiled-coil kinase (p160-ROCK) to the culture medium after dissociation and passaging of the ESCs (Watanabe et al., 2007). At least three general approaches have been used to promote neural differentiation of ESCs: as EBs, as adherent cells and in co-culture with appropriate support cells or in a combination of these three approaches (Reubinoff et al., 2001; Tabar et al., 2005; Lee et al., 2007a). More recently, a feeder-free monolayer culture method for neural differentiation has been established via dual inhibition of SMAD signaling. This approach uses a combination of bone morphogenetic protein 4 inhibitors (such as Noggin or Dorsomorphin) and inhibitors of Lefty/activin/TGFβ pathway (such as SB431542) to improve the efficiency of the differentiation (Chambers et al., 2009). At present, differentiation protocols do not exist for the generation of all cell types of the central nervous system (CNS), however over the past decade progress has been made for directed differentiation of hESCs into several neural cell types of the CNS (Suter and Krause, 2008; Liu and Zhang, 2011; see also in the same issue Martinez et al., 2012) including specific subtypes of neurons (Wichterle et al., 2002; Ying et al., 2003; Yan et al., 2005; Lee et al., 2010), oligodendrocytes (Hu and Zhang, 2009, 2010; Hu et al., 2009), astrocytes (Krencik et al., 2011; Liu and Zhang, 2011), and retinal cells (Meyer et al., 2009, 2011; Osakada et al., 2009; Lamba and Reh, 2011).

REPROGRAMMING OF SOMATIC CELLS INTO A PLURIPOTENT STATE

Epigenetic reprogramming of somatic cells into a pluripotent state has been achieved using several approaches including nuclear transplantation, cell fusion (for review see Jaenisch and Young, 2008; Yamanaka and Blau, 2010) and more recently, direct reprogramming by the expression of reprogramming factors. Takahashi and Yamanaka reported a significant advance in the stem cell field with the reprogramming of somatic cells into ESC-like cells (**Figure 1**). They demonstrated that the ectopic expression of four factors *Oct4*, *Sox2*, *klf4*, and *c-Myc* reprogrammed mouse embryonic fibroblasts into iPSCs (Takahashi and Yamanaka, 2006). As ESCs, these iPSCs could differentiated *in vivo* and *in vitro* into cells of the three germ layers and generate chimeras when injected into blastocyst embryos (Takahashi and Yamanaka, 2006). One year later, two independent groups had successfully reprogrammed human fibroblasts into human iPSCs (hiPSCs) using two different sets of reprogramming factors; the former using *Oct4*, *Sox2*, *klf4*, and *c-Myc* (Takahashi et al., 2007) and the latter using *Oct4*, *Sox2*, *Nanog*, and *Lin 28* as reprogramming factors (Yu et al., 2007). Direct reprogramming is a slow and inefficient process with efficiencies ranging from 0.002 to 0.02% (Takahashi et al., 2007; Yu et al., 2007). During and after this stochastic process (Hanna et al., 2009), the generated iPSCs have to be carefully tested for their pluripotency properties and their differentiation potentials. In particular, the ESC-specific transcription factors *Oct4* and *Nanog* have to be demethylated upon reprogramming of the somatic cells into iPSCs (Takahashi et al., 2007; Mikkelsen et al., 2008; Ebert et al., 2009). The differentiation into derivatives of the three germ layers *in vitro* and *in vivo* (in the teratoma formation assay) is also a necessary hallmark of a fully reprogrammed iPSCs. Moreover, the efficiencies of iPSC generation and differentiation depends on the stoichiometry of the reprogramming factors (Papapetrou et al., 2009; Tiemann et al., 2011) and the silencing of the vector-encoded reprogramming factors (Maherali and Hochedlinger, 2008; Ramos-Mejia et al., 2010).

As the introduction of the reprogramming factors using lentivirus or retrovirus for the generation of iPSCs may render these cells unuseful for research applications and regenerative medicine due to potential insertional mutagenesis, non-integrating reprogramming strategies have been developed including plasmids (Okita et al., 2007), episomal vectors (Yu et al., 2009), piggyBac transposition (Woltjen et al., 2009), Cre- or Flp-recombinase-based excisable viruses (Soldner et al., 2009; Voelkel et al., 2010), membrane soluble protein-induced methods (Kim et al., 2009; Zhou et al., 2009), modified RNA (Warren et al., 2010), and miRNA (Anokye-Danso et al., 2011). Reprogramming into iPSCs has been also achieved using small molecules that can either replace reprogramming factors or enhance reprogramming efficiency (Feng et al., 2009). Up to now, iPSCs have been reprogrammed from several types of somatic cells including fibroblasts (Takahashi et al., 2007; Park et al., 2008; Ebert et al., 2009), neural progenitor cells (Shi et al., 2008), keratinocytes (Aasen et al., 2008), peripheral blood (Loh et al., 2009), pancreatic B cells (Stadtfeld

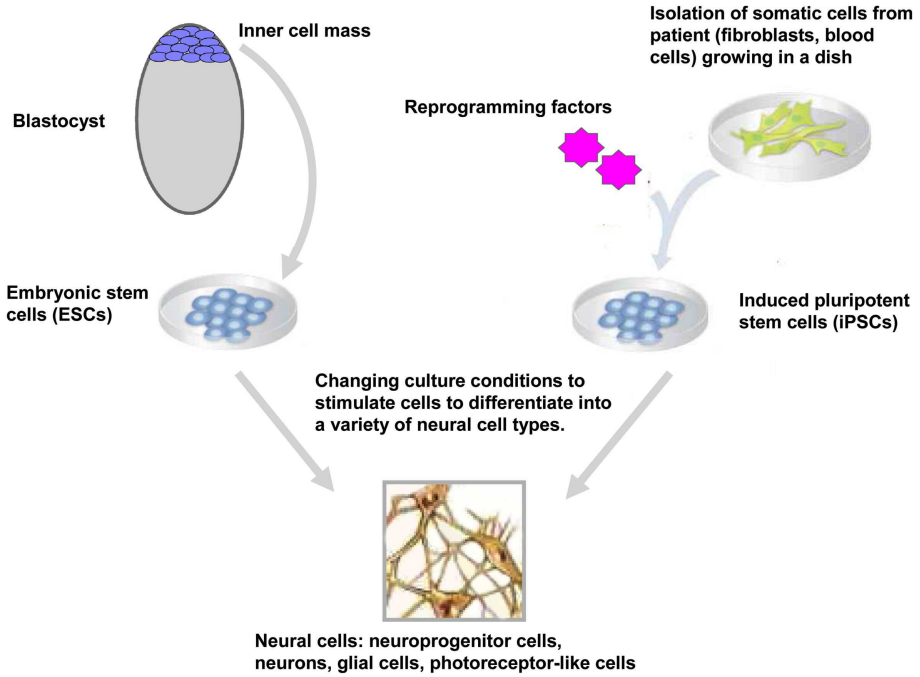

FIGURE 1 | Generation and neural differentiation potential of pluripotent stem cells. Human embryonic stem cells (hESCs) are derived from the inner-cell mass of blastocyst stage embryos. Human induced pluripotent stem cells (hiPSCs) are reprogrammed from somatic cells after the ectopic expression of reprogramming factors. After neural induction using specific stimuli, hESCs, and hiPSCs differentiate into neuroprogenitor cells and further mature into neurons, glial cells, retinal pigment epithelium, and other neural cells (only cells of the neural lineage are represented).

et al., 2008), and hepatocytes (Aoi et al., 2008). Like hESCs, hiPSCs have been successfully differentiated into NPCs (Chambers et al., 2009; Liu and Zhang, 2011), specific subtypes of neurons (Di Giorgio et al., 2008; Dimos et al., 2008; Ebert et al., 2009; Soldner et al., 2009), oligodendrocytes (Czepiel et al., 2011), astrocytes (Krencik et al., 2011), and retinal cells (Buchholz et al., 2009; Meyer et al., 2009, 2011; Osakada et al., 2009; Jin et al., 2011).

APPLICATIONS

In this section, we discuss four major applications of hPSCs that will advance our understanding of human neurological diseases through deciphering the targets and mechanisms involved in the pathogenesis. The first two applications are the study of neural development and differentiation processes in physiological and pathological contexts. Then, the identification of the detailed mechanisms that contribute to the pathogenesis of the disease will provide targets for drug screening and cell-based therapies for neurological diseases (**Figure 2**).

BASIC DEVELOPMENTAL BIOLOGY

Over the last decade, hPSCs have emerged as a valuable and powerful material for studying the pathways governing human embryogenesis and development (Keller, 2005). Such studies were previously unattainable due to technical and ethical concerns regarding the use of human fetuses. Thus, hPSCs enable the investigation of the basic mechanisms involved in pluripotency, neural fate specification, and differentiation (Munoz-Sanjuan and Brivanlou, 2002; Levine and Brivanlou, 2007; Hanna et al., 2010).

The knowledge accumulated by embryologists from frog, fish, chicken, and mouse embryos has allowed the development of strategies to direct neural fate specification from hPSCs *in vitro* (Munoz-Sanjuan and Brivanlou, 2002; Stern, 2005, 2006; Levine and Brivanlou, 2007). As in the developing embryo, neural differentiation of hPSCs appears to be a default lineage differentiation when self-renewal is not maintained. It explains why early protocols used spontaneous differentiation for generating neural cells even with low efficiency (Reubinoff et al., 2001; Tropepe et al., 2001; Stern, 2006). Subsequent studies have used factors and patterning signals that mimic embryogenic neurogenesis to improve fate specification and differentiation efficiencies. Bone morphogenetic proteins (BMPs), wingless-type MMTV integration site family (WnT), and Smad signaling are pathways that suppress the induction of ectoderm (Munoz-Sanjuan and Brivanlou, 2002). Based on this, addition of BMP, WnT, or Smad inhibitors promote specification of hESCs into neuroectoderm (Pera et al., 2004; Watanabe et al., 2005; Smith et al., 2008). This conversion was further improved by dual inhibition of SMAD signaling using noggin and SB431542 (Chambers et al., 2009). At the same time, elegant work has demonstrated the successful differentiation of hPSCs into specific subtypes of neurons (Ying et al., 2003; Di Giorgio et al., 2008; Dimos et al., 2008; Chambers et al., 2009; Ebert et al., 2009; Soldner et al., 2009; Lee et al., 2010; Liu and Zhang, 2011), oligodendrocytes (Hu and Zhang, 2009, 2010; Hu et al., 2009), and astrocytes (Krencik et al., 2011; Liu and Zhang, 2011). Thus, excitatory projection neurons (Watanabe et al., 2005; Eiraku et al., 2008; Gaspard et al., 2008) and cortical interneuron progenitors

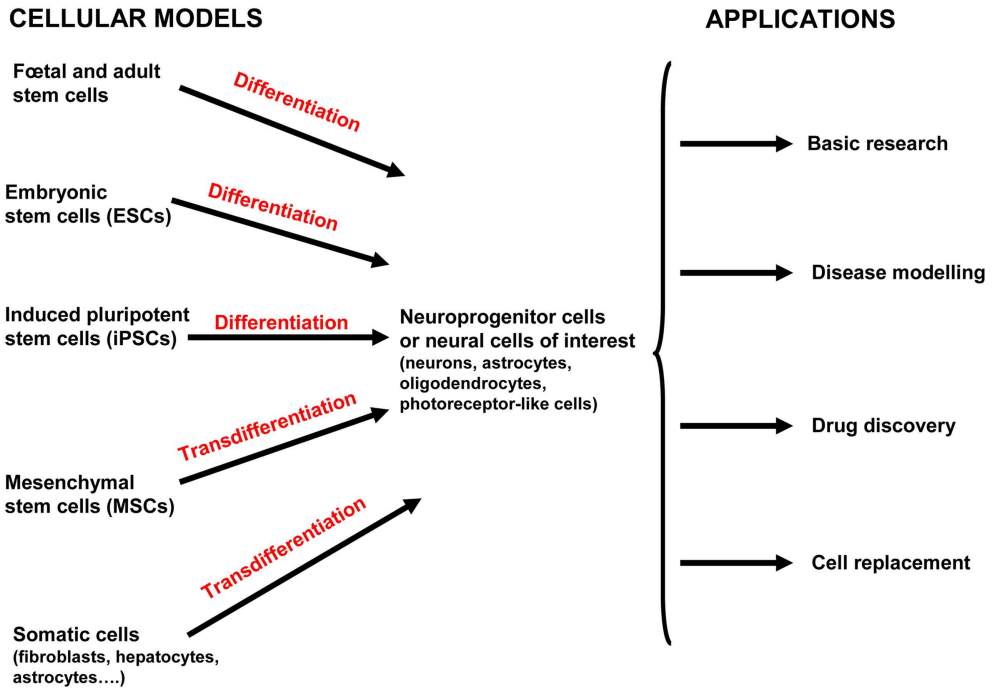

FIGURE 2 | Current and potential approaches used for human neurological disease study and therapy. Both human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs) can differentiate into neuroprogenitor cells and/or further mature into the neural cells of interest (neurons, oligodendrocytes, astrocytes, and photoreceptor-like cells). Mesenchymal stem cells (MSCs) have been isolated from various tissue including placenta, adipose tissue, lung, bone marrow, blood, and the umbilical cord (and possibly others). MSCs can be directly converted into cells of the ectodermal lineage by transdifferentiation (also called plasticity). Neural stem cells (NSCs) are isolated from fetal and adult brains of aborted fetuses and adult brains in post mortem conditions. Adult NSCs are mostly obtained from two regions of the adult brain where neurogenesis occurs: the subventricular zone of the lateral ventricle and subgranular zone of the dentate gyrus in the hippocampus. Induced neural cells (iN cells) are generated from the transdifferentiation of somatic cells from the same lineage or another one without the prior reprogramming into pluripotent cells. All these cells provide valuable model for basic developmental research, modeling diseases, high-throughput drug screening, and cell-based therapies.

(Maroof et al., 2010) have been generated from ESCs using brain development principle. When transplanted into postnatal cortex, these cortical interneuron progenitors migrated, integrated into the local circuitry and displayed morphological and electrophysiological properties of mature interneurons (Maroof et al., 2010). Therefore, hPSCs provide an unprecedented opportunity for basic research focused on neuronal activity, migration, dendritogenesis, synaptogenesis, and integration to circuitry *in vitro* or when transplanted *in vivo* (Maroof et al., 2010; Brennand et al., 2011; Kim et al., 2011a). Further understanding of the signaling pathways governing these processes in hPSCs will provide new insights into human neurodevelopment and the functional integration of transplanted cells.

MODELING HUMAN NEUROLOGICAL DISEASES

Human neurological diseases can be modeled using hESCs, essentially by two approaches. The first approach is by inducing a mutation in healthy hESCs. Perhaps the best example for this is the generation of hESCs for the modeling of Lesch–Nyhan syndrome, a disease caused by a mutation in the *HPRT1* (*hypoxanthine-guanine phosphoribosyltransferase*) gene that triggers an overproduction of uric acid, causing gout-like symptoms, and urinary stones, in addition to neurological disorders. Urbach et al. (2004) succeeded in generating a hESC-based model that recapitulates in some extent the characteristics of Lesch–Nyhan disease, by mutating the *HPRT1* gene in hESCs using homologous recombination. The second approach is through the identification of hESCs derived from embryos affected by genetic disorders during pre-implantation genetic diagnosis (PGD; Ben-Yosef et al., 2008; Stephenson et al., 2009). In this regard, hESC lines has been derived after PGD for a broad range of neurological diseases including Fragile X syndrome (FXS; Verlinsky et al., 2005; Eiges et al., 2007; Frumkin et al., 2010; Tropel et al., 2010), Spinocerebellar ataxia 2 (Tropel et al., 2010), Huntington's disease (HD; Verlinsky et al., 2005; Mateizel et al., 2006; Tropel et al., 2010), Down syndrome (DS; Biancotti et al., 2010; Sharon et al., 2011), Gaucher's syndrome (Frumkin et al., 2010), Charcot Marie Tooth disease (Mateizel et al., 2006), X-linked adrenoleukodystrophy (X-ALD; Verlinsky et al., 2005), familial amyotrophic lateral sclerosis (ALS), neurofibromatosis type 1, Patau syndrome (Biancotti et al., 2010), and possibly others (**Table 1**).

One year after the first reprogramming of human fibroblasts into hiPSCs (Takahashi et al., 2007; Ebert et al., 2009), Daley's group reported the generation of several hiPSCs from patients affected by Mendelian or complex genetic disorders including the neurological diseases Gaucher's disease, Parkinson's disease (PD), HD, DS, and Lesch–Nyhan syndrome (Park et al., 2008). Since this

Table 1 | Neurological diseases in which hPSCs (either hESCs or hiPSCs) have been derived from embryos or patients.

Disease	hPSC model used	Molecular defects associated with the disease	Phenotype reported	Reference
Angelman's syndrome (AS)	iPSC	15q11–13	*UBE3A* genomic imprinting in AS-iPSC-derived neurons	Chamberlain et al. (2010)
Alzheimer's disease (AD)	iPSC	Unknown or mutation/duplication in *APP*, *PS1*, *PS2*	High levels of amyloid-β(1-40), phospho-tau (Thr231), and active glycogen synthase kinase-3β (aGSK-3β) in AD-iPSC-derived neurons	Yagi et al. (2011), Israel et al. (2012)
Charcot Marie Tooth (CMT)	ESC	*CMT1*, *PMP22*, *GJB1*, *MPZ*, *MFN2*, *GJB1*, *GDAP1*, *NDRG1*, *HK1*, *SH3TC2*, *GDAP1*, *GJB1*, and *MPZ* (depending on the type of CMT)	Not determined	Mateizel et al. (2006)
Down syndrome (DS)	ESC and iPSC	Trisomy 21	Not determined	Park et al. (2008), Biancotti et al. (2010)
Emanuel syndrome	iPSC	Supernumerary chr 11 attached to a piece of chr 22	Not determined	Li et al. (2012)
Familial amyotrophic lateral sclerosis (ALS)	ESC and iPSC	Mutations in *SOD1*, *VAPB*, *DPP6*, *IIPR2*, *IARDBP*, *FUS*	Downregulation of VAPB expression in fibroblasts, iPSCs, and motor neurons	Verlinsky et al. (2005), Dimos et al. (2008), Mitne-Neto et al. (2011)
Familial dysautonomia (FD)	iPSC	Mutation in *JKBKAP*	Splicing, cellular migration, and neurogenesis defects in FD-iPSC-derived neurons	Lee et al. (2009)
Fragile X syndrome (FXS)	ESC and iPSC	CGG triplet repeats in *FMR1*	Reduced expresion of *FMR1* through DNA methylation and histone modification Abnormal differentiation of FXS-iPSCs into neurons (fewer and shorter neurites)	Frumkin et al. (2010), Verlinsky et al. (2005), Eiges et al. (2007), Tropel et al. (2010)
Friedriech's ataxia (FRDA)	iPSC	GAA triplet repeats in *FXN*	GAA triplet repeats in *FXN*, reduced *FXN* mRNA, defect in mismatch repair (MMR) enzymes in FRDA-iPSCs	Liu et al. (2011), Ku et al. (2010)
Huntington's disease (HD)	ESC and iPSC	CAG triplet repeats in *HTT*	Increased susceptibility to growth factor withdrawal of HD-iPSC-derived NSCs Involvement of DNA mismatch repair (MMR) machinery in CAG instability	Tropel et al. (2010), Verlinsky et al. (2005), Park et al. (2008), Mateizel et al. (2006), Zhang et al. (2010)
Lesch–Nyhan syndrome	ESC and iPSC	Mutation in *HPRT1*	Not determined	Park et al. (2008), Urbach et al. (2004)
Neurofibromatosis type 1	ESC	Point mutation in *NF1*	Not determined	Verlinsky et al. (2005)
Parkinson's disease (PD)	ESC and iPSC	Unknown or mutations in *LRRK2*, *PINK1*, *SNCA*, *PARK7*, *PRKN*, and others	Increased susceptibility to death for DA neurons derived from hESCs overexpressing the α-synudein Increased susceptibility to death for LRRK2-PD-iPSC-derived neurons when exposed to oxidative stress, proteasome inhibitor MG-132 and 6-hydroxydopamine Impairment of mitochondrial parkin recruitment and mitochondrial dysfuntion in PIMK1-PD-iPSC-derived DA neurons	Park et al. (2008), Soldner et al. (2009), Nguyen et al. (2011), Seibler et al. (2011), Devine et al. (2011), Schneider et al. (2007)
Patau syndrome	ESC and iPSC	Trisomy 13	Dramatic alterations in the expression of brain specific genes in ESC-derived EBs	Li et al. (2012), Biancotti et al. (2010)
Prader–Willi syndrome (PWS)	iPSC	15q11–13	Genomic imprinting of the imprinting center for PWS; reduced expression of the disease-associated small nucleolar RNA HBII-85/SNORD116	Chamberlain et al. (2010), Yang et al. (2010)

(Continued)

Table 1 | Continued

Disease	hPSC model used	Molecular defects associated with the disease	Phenotype reported	Reference
Retinopathies Retinitis pigmentosa	iPSC	Mutations in *RP1*, *RP9*, *PRPH2*, *RHO*, and others	Degeneration of RP-iPSC-derived rod photoreceptor cells Increase of apoptosis, oxidative stress and endoplasmic reticulum dysfunction in RP-iPSC-derived rod photoreceptor cells Identification of the cilia-related gene *male germ cell-associated kinase (MAK)* gene as a cause of RP	Jin et al. (2011), Tucker et al. (2011)
Gyrate atrophy		Mutations in *OAT*	Decline of ornithine-δ-aminotransferase activity; restored by vitamin B6 and via targeted gene repair	Meyer et al. (2011)
Rett syndrome (RTT)	iPSC	Mutation in *MECP2*	Morphological alterations of RTT-iPSC-derived neurons: fewer synapses, reduced dendritic spine density, and soma size Reduced frequency and amplitude of calcium transients and reduced frequency of spontaneous postsynaptic currents	Marchetto et al. (2010), Cheung et al. (2011), Kim et al. (2011c)
Schizophrenia (SCZD)	iPSC	Unknown	Reduced neuronal connectivity, outgrowth from soma, PSD95 dendritic protein levels in SCZD-iPSC-derived neurons Alterations of Notch signaling, cell adhesion, and slit-Robo-mediated axon guidance in SCZD-iPSC-derived neurons	Brennand et al. (2011), Chiang et al. (2011)
Spinal muscular atrophy (SMA)	iPSC	Mutation in *SMN1*	Absence of expression of SMN1, reduced number, and size of SMA-iPSC-derived motor neurons Deficit in neurite outgrowth and gem formation in SMA-iPSC-derived neurons	Ebert et al. (2009), Chang et al. (2011)
Spinocerebellar ataxia type2	ESC	CAG triplet repeats in *ATX2*	Not determined	Tropel et al. (2010)
Spinocerebellar ataxia type 3 or Machado–Joseph disease (MID)	iPSC	CAG triplet repeats in *ATX3*	Accumulation of ATX3 containing aggregates in MJD-iPSC-derived neurons, involvement of calpain, Na^+ channels, K^+ channels, ionotropic, and voltage-gated Ca^{2+} channels in the aggregate formation	Koch et al. (2011)
Warkany syndrome 2	iPSC	Trisomy 8	Not determined	Li et al. (2012)
X-linked adrenoleukodystrophy	ESC and iPSC	Mutations *in ABSCD1*	VLCFA accumulation in X-ALD-iPSC-derived oligodendrocytes; reduction of VLCFA levels in X-ALD-iPSC-derived oligodendrocytes by 4-phenylbutyrate and lovastatin	Verlinsky et al. (2005), Jang et al. (2011)

ABCD1, adenosine triphosphate-binding cassette transporter superfamily D1 member; AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; AMN, adrenomyeloneuropathy form of X-linked adrenoleukodystrophy; APP, amyloid-β precursor protein; AS, Angelman syndrome; ATXN, ataxin; CAG, cytosine-adenine-guanine; CCALD, childhood cerebral form of X-linked adrenoleukodystrophy; CMT, Charcot Marie Tooth; CNS, central nervous system; DA, dopaminergic; DS, Down syndrome; EBs, embryoid bodies; ESCs, embryonic stem cells; FD, familial dysautonomia; FMR1, fragile X mental retardation; FRDA, Friedreich's ataxia; FXD, fragile X syndrome; FXN, frataxin; HD, Huntington's disease; HPRT, hypoxanthine-guanine phosphoribosyltransferase; HTT, huntingtin; iPSCs, induced pluripotent stem cells; LRRK2, Leucine-rich repeat kinase 2; MECP2, methyl CpG-binding protein 2; MJD, Machado–Joseph disease; NPCs, neural progenitor cells; NSCs, neural stem cells; OAT, ornithine-δ-aminotransferase; PD, Parkinson's disease; PGD, pre-implantation genetic diagnosis; PINK1, PTEN-induced putative kinase 1; PS1 and PS2, presenilin 1 and presenilin 2; PSCs, pluripotent stem cells; PWS, Prader–Willi syndrome; RTT, Rett syndrome; SCZD, schizophrenia; SMA, spinal muscular atrophy; SMN1, survival motor neuron-1; SOD, superoxide dismutase; VAPB, vamp-associated protein B; VLCFA, very long chain fatty acid; X-ALD-iPSC, X-linked adrenoleukodystrophy.

first study, not a month goes by without a new article reporting the modeling of a human disease. Here, we report the principal neurodevelopmental and neurodegenerative diseases that have been modeled using hPSCs so far and the major findings regarding the pathogenesis of these diseases (**Table 1**).

Neurodegenerative diseases

Alzheimer's disease. Alzheimer's disease (AD) is the most common neurodegenerative disease. One in every eight of the population over 65 years old is estimated to have AD and 40–50% past the age of 85 may have it. AD is defined by progressive

dementia with subsequent appearance of other cognitive, behavioral, and neuropsychiatric changes that degrade independence, social abilities of the affected patient in daily life. AD is characterized by neuronal and synaptic loss associated with extracellular deposits of amyloid-β peptides in senile plaques and intraneuronal neurofibrillary tangles (NFTs) formed by hyperphosphorylated tau (a microtubule-associated protein involved in microtubule stabilization; Querfurth and LaFerla, 2010). Most of AD forms are apparently sporadic (sAD) but dominantly inherited familial forms of AD (fAD) have been also reported; including those carrying mutation or duplication of *amyloid-β precursor protein* (*APP*) gene or mutations in the *presenilin 1* and *2* genes (*PS1* and *PS2*) which encode the major component of γ-secretase enzyme that cleaves APP into amyloid-β peptides and other cleavage fragments (Israel and Goldstein, 2011). At present, the study of AD pathogenesis is limited by the lack of access to live neurons from patients and the impossibility to model the sporadic form of AD. This limitation has been recently overcome by the generation of iPSCs from patients with sAD and fAD. Yagi and colleagues were the first reporting the generation and the characterization of iPSCs derived from fAD patients with mutations in *PS1* and *PS2* (fAD-iPSCs). In this study, fAD-iPSCs-derived neurons secreted more amyloid β42 in comparison with those from healthy donor, recapitulating the molecular pathogenesis of mutant presenilins (Yagi et al., 2011). More recently, Israel et al. derived iPSCs from two patients with fAD (fAD), both caused by a duplication of the amyloid-β precursor protein gene (fAD-iPSCs), two with sAD (sAD-iPSCs) and two non-affected individuals. The most striking results from this study are that fAD-iPSC- and sAD-iPSC-derived neurons exhibited significantly higher levels of the pathological markers amyloid-β(1–40), phospho-tau(Thr231), and active glycogen synthase kinase-3β (aGSK-3β) in comparison with those derived from healthy donors. Thus, they also accumulated large RAB5-positive early endosomes. Importantly, Phospho-Tau(Thr231) and aGSK-3β levels were reduced by treatment of the cells with β-secretase inhibitors (Israel et al., 2012).

Amyotrophic lateral sclerosis. Amyotrophic lateral sclerosis (also known as Lou Gehrig's disease) is a fatal neurodegenerative disease characterized by injury and death of lower motor neurons in the brain stem and spinal cord, and of upper neurons in the motor cortex. The clinical hallmarks of ALS comprise the atrophy of skeletal muscle, eventual paralysis, respiratory failure, and death of patients within 1–5 years of disease onset. The incidence of ALS is two to three in 100,000 individuals. ALS is mostly a sporadic disease but 5–10% of cases are familial and usually of autosomal dominant inheritance. The pathogenic processes underlying ALS are multifactorial and are not completely known. In this regard, *superoxide dismutase 1* (*SOD1*), *peptidyl-peptidase 6* (*DPP6*), *inositol 1,4,5-trispohosphate receptor type 2* (*ITPR2*) and *Tar-DNA-binding protein-43* (*TARDBP*, also known as *TD43*), *fused sarcoma* (*FUS*), *vamp-associated protein B/C* (*VAPB*) have been identified as ALS susceptibility genes. Emerging evidence suggests that astrocytes and glia have an important role in the propagation of motor neuron injury in the sporadic and the familial forms of ALS (Glass et al., 2010; Ferraiuolo et al., 2011; Haidet-Phillips et al., 2011). Dimos et al. established iPSCs from an 82-year-old patient affected by a familial form of ALS with *SOD1* mutation. They showed that both normal iPSCs and ALS-iPSCs can differentiate into motor neurons but no phenotypic difference between the iPSC lines was reported (Dimos et al., 2008). New insights into the mechanisms underlying ALS degeneration have been gained when hESC-derived motor neurons were co-cultured with glial cells carrying a mutant allele of *SOD1* gene. Under these conditions, half of the hESC-derived motor neurons were lost whereas normal glial cells were not toxic. Prostaglandin and pro-inflammatory cytokines were found responsible for the toxic effect of these glial cells (Di Giorgio et al., 2008; Marchetto et al., 2010). At this step, studying glial cells derived from mutant SOD1-containing iPSCs (as the iPSC line reported in Dimos et al.) will provide crucial information on this toxicity: what render these cells toxic and what make the mutant SOD1-containing motor neurons more vulnerable than the normal ones? More recently, iPSCs were generated from ALS patients carrying mutation in *VAPB* gene, a susceptibility gene described as a rare cause of familial ALS. The protein encoded by *VAPB* gene is implicated in numerous cellular functions such as the regulation of lipid transport and homeostasis, formation of presynaptic terminal, and unfolded protein response (Ferraiuolo et al., 2011). The study did not reveal difference between the ALS-iPSCs and the normal ones in terms of their capacity to differentiate into motor neurons and regarding the intracellular distribution of VAPB protein upon basal condition and in the presence of MG-132 (a proteasome inhibitor that induces cytoplasmic inclusions of the VAPB protein). However, while the expression of VAPB protein constantly increased upon differentiation of the normal iPSCs into motor neurons, this expression remained significantly lower upon differentiation of the ALS-iPSCs (Mitne-Neto et al., 2011).

Familial dysautonomia. Familial dysautonomia (FD, Riley–Day syndrome, hereditary sensory, and autonomic neuropathy type III) is a rare neurodegenerative disease with autosomal recessive inheritance that occurs almost exclusively among individuals of Ashkenazi Jewish population. The disease affects the development and the survival of sensory, sympathetic, and some parasympathetic neurons. FD is caused by mutations in the *IKBKAP* gene, which encodes a protein called IKAP/hELP1 (IkB kinase complex associated protein). This mutation leads to a tissue-specific skipping of exon 20 of *IKBKAP* mRNA and subsequently to a reduced IKAP/hELP1 protein level in sensory and autonomic nervous systems. This protein has been shown to contribute to crucial processes within the cell such as actin cytoskeleton regulation, cell motility migration, acetylation of microtubules, and neuronal development. Recent advances have provided new insights into the underlying genetic and biochemical deficits in FD disease using iPSCs derived from patients with FD (Lee et al., 2009). Lee and colleagues derived iPSCs from three young patients affected by FD and differentiated them into neural cells. FD-iPSC-derived neural cells showed alterations in *IKBKAP* mRNA splicing, cell migration, and neurogenesis. Furthermore, the plant cytokinin kinetin corrected *IKBKAP* mRNA splicing and the neurogenesis defects but showed no effect on cell migration in these FD-iPSC-derived cells (Lee et al., 2009).

Huntington's disease. Huntington's disease is a severe late-onset autosomal dominant neurodegenerative disease that affects 5–7 in 100,000 Caucasian individuals. It is caused by CAG trinucleotide repeats in the exon 1 of the *huntingtin* (*HTT*) gene. The disease is characterized by the progressive loss of neurons, predominantly in the striatum, which leads to the typical motor, cognitive impairments, and dementia associated with the disease (Walker, 2007). Among the disease-specific iPSC lines generated in the early study of Park et al., iPSC lines were derived from a patient with HD (HD-iPSCs). DNA sequencing analysis of these HD-iPSCs confirmed the presence of 72 CAG trinucleotide repeats in one allele of *HTT* gene and 19 in the other (Park et al., 2008). Using the same HD-iPSC lines, Zhang et al. found an altered ERK activation when compared to normal iPSCs (Zhang et al., 2010), confirming previous reports (Apostol et al., 2006). Moreover, CAG trinucleotide repeats were conserved both after reprogramming of the HD-fibroblasts into HD-iPSCs and after the differentiation of the HD-iPSCs into neurons. The authors also documented their potential to differentiate into NPCs and to mature into striatal neurons but no phenotypic analysis was reported. Importantly, HD-iPSC-derived NSCs showed an increased susceptibility to growth factor withdrawal (Zhang et al., 2010). Also, HD-ESCs have been derived from embryos that harbor the mutant *HTT* allele by several groups (Mateizel et al., 2006; Niclis et al., 2009; Tropel et al., 2010; Bradley et al., 2011; Seriola et al., 2011). These HD-ESCs were pluripotent and showed the ability to differentiate into derivatives of the three germ layers *in vivo* and into NPCs *in vitro*. However, no phenotypic differences were reported (Bradley et al., 2011; Seriola et al., 2011). These studies also confirmed the presence of more than 40 CAG in these HD-ESCs (Bradley et al., 2011; Seriola et al., 2011) that remained stable upon differentiation (Seriola et al., 2011). Finally, the authors proposed that the downregulation of the proteins that form the DNA mismatch repair (MMR) machinery contributes to CAG instability in HD-iPSCs (Seriola et al., 2011). More recently, HD-iPSCs have been derived from homozygous and heterozygous HD patients. Importantly, both undifferentiated HD-iPSCs and HD-iPSC-derived neurons displayed a higher lysosomal activity compared to the normal counterparts (Camnasio et al., 2012).

Parkinson's disease. Parkinson's disease is a complex, multifactorial neurodegenerative disease of the basal ganglia and is recognized as one of the most common neurological disorders, affecting ∼1% of individuals older than 60 years. There are two major neuropathological hallmarks: the loss of pigmented dopaminergic (DA) neurons in the substantia nigra and the presence of abnormal fibrillar cytoplasmic inclusions called Lewy bodies. It is unclear why neurons degenerate in PD but it is thought to be due to a combination of genetic and environmental factors (Dawson and Dawson, 2003). Indeed, although more than 90% of PD forms seem to be sporadic, a dozen of genes have been linked to the disease (Hardy, 2010). For example, multiplications of *SNCA* gene has been described in a highly penetrant and aggressive form of PD. This defect leads to α-synuclein protein aggregates in Lewy bodies (Hardy, 2010). Similarly, a common autosomal dominant missense mutation in *Leucine-rich repeat kinase 2* (*LRRK2*) gene is correlated with a penetrance of 85% in PD patients of 70 years old (Kachergus et al., 2005). Recessive inherited *Parkin* and *PTEN-induced putative kinase 1* (*PINK1*) mutations have been also described in PD cases with slowly progressive early onset disease (Hardy, 2010). Although animal models of PD have contributed indoubtfully to our current understanding of the disease, they fail to recapitulate PD pathogenesis accurately (Chesselet and Richter, 2011). The recent development of hPSCs provides a new method to create human cell-based disease model and to investigate the disease phenotype *in vitro*. Both hESCs and hiPSCs have been used for modeling PD condition. In an early report, Schneider and colleagues established hESCs that overexpressed the α-synuclein protein. An increased susceptibility to death of these cells was shown when differentiated into DA neurons (Schneider et al., 2007). More recently, iPSCs were derived from individuals with sporadic forms of PD (PD-iPSCs). However, from these studies it remains unknown whether PD-iPSC-derived neurons display a phenotype in comparison with the normal ones under basal condition (Park et al., 2008; Soldner et al., 2009; Nguyen et al., 2011; Seibler et al., 2011). Considering that PD-iPSC lines carrying the most common PD-related mutations may be appropriate to reveal and recapitulate key phenotypes of PD, two recent PD-iPSC models have been developed. The first one has been derived from patients with mutation in *LRRK2* gene (LRRK2-PD-iPSC). Importantly, this study revealed an increased expression of the α-synuclein protein and genes involved in oxidative stress when LRRK2-PD-iPSCs were further differentiated into DA neurons. Furthermore, LRRK2-PD-iPSC-derived neurons showed an increased susceptibility to cell death in comparison with the normal ones when exposed to oxidative stress, the proteasome inhibitor MG-132, and 6-hydroxydopamine (Nguyen et al., 2011). Similarly, iPSCs were derived from a PD patient carrying a triplication of *SNCA* gene (SNCA-PD-iPSCs) and an unaffected first-degree relative. When induced to differentiate into midbrain DA neurons, those derived from SNCA-PD-iPSCs showed a twofold increase of the α-synuclein protein expression, recapitulating the cause of disease phenotype of PD patients carrying this anomaly (Devine et al., 2011). More recently, iPSCs were generated from a PD patient harboring *PINK1* mutations. Under basal condition, no differences in the differentiation potential of the PINK1-PD-iPSCs into DA neurons were found when compared with normal ones. However, PINK1-PD-iPSC-derived DA neurons showed a ∼5-fold reduction in *PINK1* mRNA levels. This study provides novel evidence for the role of *PINK1* mutations and the associated mitochondrial dysfunctions. In particular, contrary to DA neurons derived from normal iPSCs, mitochondrial depolarization of PINK1-PD-iPSC-derived DA neurons did not result in parkin protein translocation from the cytosol to mitochondria. This was accompanied by an increase of mitochondrial biogenesis as revealed by the increase of mitochondrial (mtDNA) copy number. The authors proposed that this increase could be explained by the induction of PGC-1α expression upon mitochondrial depolarization in PINK1-PD-iPSC-derived DA neurons (Seibler et al., 2011). Importantly, re-expression of parkin in PINK1-PD-iPSC-derived DA neurons corrected these defects (Seibler et al., 2011), supporting the crucial role of parkin protein in the pathogenesis of PINK1-linked PD.

Spinal muscular atrophy. Spinal muscular atrophy (SMA) is an inherited neuromuscular disorder caused by the mutation and/or deletion of the *survival motor neuron-1* (*SMN1*) gene. *SMN1* gene encodes the SMN protein, a protein found in the cytoplasm, and in nuclear bodies described as "gemini of coiled bodies" or gems. The disease is characterized by specific degeneration of alpha-motor neurons in the spinal cord, leading to muscle weakness, atrophy, and in the majority of cases, premature death. There are four forms of SMA that can be distinguished based on age of onset, pattern of muscle involvement, and inheritance pattern. Infants affected by the severe SMA (type I, Werdnig–Hofman disease) die before reaching the age of two, whereas the mild forms of the disease are characterized by relatively static muscle weakness for many years (Lunn and Wang, 2008). Ebert et al. derived iPSCs from a young boy affected by type I SMA and his unaffected mother. As expected, they confirmed the absence of SMN1 expression and the reduced presence of gems in SMA-iPSCs in comparison with the normal iPSCs. Interestingly, while no differences were found after 4 weeks of differentiation of the normal iPSCs and the SMA-iPSCs into motor neurons, SMA-iPSC-derived motor neurons were fewer and smaller than the normal ones, after 6 weeks of differentiation (Ebert et al., 2009). Treatment of the SMA-iPSCs with either valproic acid or tobramycin, two molecules that have been shown to increase SMN protein levels, efficiently increased the expression of SMN protein as well as gems in the treated cells. However, the effects of these molecules in motor neurons were not addressed. More recently, SMN protein re-expression in SMA-iPSCs restored neurite outgrowth and gem formation deficits (Chang et al., 2011). Taken together, these two studies provide the proof of principle that SMA-iPSCs can be used to model the disease and that it is possible to improve the phenotype using both pharmacological and gene correction approaches.

Spinocerebellar ataxia. Spinocerebellar ataxia is an inherited disorder of brain function with at least 28 distinct genetic forms. Patients affected by the disease experience a degeneration of the spinal cord and the cerebellum. All types of spinocerebellar ataxia are characterized by a progressive incoordination of walking and are often associated with poor coordination of hand movements, eye movements, and speech (Paulson, 2007). Machado–Joseph disease (MJD, also called spinocerebellar ataxia type 3) is the most common spinocerebellar ataxia. This neurodegenerative disease is caused by expansion of CAG triplet repeats in the *MJD1* gene (also called *ATXN3*, *ataxin-3*). The neuropathological hallmark of MJD patients is the accumulation of ATXN3 protein-containing aggregates in brain tissue; the severity of the disease is directly correlated with the amount of such aggregates. Even if the gene and the anomalies are known, the pathogenic mechanisms underlying these abnormalities remain not well understood (Costa and Paulson, 2012). ESCs have been derived from embryos that harbor the mutant *SCA2* gene (also called *ATXN2*, *ataxin-2*; Tropel et al., 2010), however to our best knowledge, no studies have been conducted using these cells. Recently, iPSCs were derived from four patients affected by MJD (MJD-iPSCs) and two related healthy donor. As expected, expansion of polyQ-coding CAG sequence in *MJD1* gene was verified in MJD-iPSCs. However, no differences were found with respect to the differentiation potentials and the functional properties between the MJD-iPSC-derived neurons and those from healthy donors. Importantly, upon repetitive stimulations with L-glutamate or *N*-methyl-D aspartate (NMDA), MJD-iPSC-derived neurons accumulated ATXN3 protein-containing aggregates whereas those from healthy donors did not. This aggregate formation was shown to involve the recruitment of other polyQ proteins (such as the TATA binding protein) and the calcium-dependent activation of caspase and calpain proteases (Koch et al., 2011).

X-linked adrenoleukodystrophy. X-linked adrenoleukodystrophy is a neurological disorder that occurs most often in males. It mainly affects the nervous system and the adrenal glands. There are three distinct types of X-ALD: a severe early onset childhood cerebral form (CCALD), an adrenomyeloneuropathy form (AMN), and a type called "Addison disease only." CCALD manifests between the age of 4 and 8 years and is characterized by attention deficit, progressive impairment of cognition, behavior, vision, and motor function that often lead to total disability within 2 years. AMN is a more slowly progressive form that manifests in adult life as progressive paraparesis, sphincter disturbances, sexual dysfunction, and often, impaired adrenocortical function. In contrast, the "Addison disease only" is a variant without neurological involvement. Female carriers present milder phenotype than males; they develop neurologic manifestations close to the AMN form with a later onset. The disorder is caused by mutations in the *adenosine triphosphate-binding cassette transporter superfamily D1 member* (*ABCD1*) gene that encodes ABCD1 protein (or ALDP), a peroxisomal protein necessary for beta-oxidation of very long chain acids (VLCFA) in the peroxisomes. As a result, elevated VLCFA levels accumulate in plasma and tissues together with the loss of axons and the demyelination in the long tracts of the spinal cord. At present, even the gene responsible for X-ALD is known, the mechanisms by which VLCFA accumulation in tissues leads to the neurological defects remain unknown (Ferrer et al., 2010). A recent study using iPSC technology opened a new avenue for the study of X-ALD pathogenesis. Jang et al. generated iPSCs from patients with CCALD (CCALD-iPSCs) and AMN (AMN-iPSCs). Both iPSCs displayed mutations in the *ABCD1* gene. Considering that the cerebral demyelination resulting from oligodendrocyte degeneration and the loss of neurons are the two major hallmarks of X-ALD, CCALD-iPSCs, and AMN-iPSCs were differentiated into neurons and oligodendrocytes. No difference was found in the differentiation potentials of CCALD-iPSCs and AMN-iPSCs when differentiated into neurons and oligodendrocytes in comparison with the normal ones (Jang et al., 2011). These results are consistent with the absence of developmental defect observed in the brain of X-ALD patients before onset of the disease (Ferrer et al., 2010). However, VLCFA levels were greater in neurons and oligodendrocytes derived from CCALD-iPSCs and AMN-iPSCs in comparison with the normal counterparts. Thus, VLCFA levels were significantly higher in CCALD-iPSC-derived oligodendrocytes compared with AMN-iPSC-derived ones recapitulating the much more severe phenotype observed in the CCALD form. Moreover, VLCFA levels in CCALD-iPSC-derived oligodendrocytes were significantly reduced by 4-phenylbutyrate and lovastatin, two compounds that upregulate the expression of *ABCD2*, a closely

related *ABCD1* gene that probably compensates the *ABCD1* gene defects. The reduction of VLCFA levels in CCALD-iPSC-derived oligodendrocytes by pharmacological approaches gives the proof of principle that these iPSCs provide a promising model not only to study the pathogenesis of the disease but also to test compounds that restore the disease phenotype (Jang et al., 2011).

Neurodevelopmental diseases

Angelman syndrome. Angelman syndrome (AS) is a neurodevelopmental disorder with an estimated incidence between 1 in 10,000 and 1 in 20,000 individuals. AS is characterized by severe mental retardation, neurological problems, absence of speech, dysmorphic facial features, microcephaly, epileptic seizures, and electroencephalogram abnormalities. It is caused by a variety of genetic abnormalities involving the chromosome 15q11–13 region (60–75%), paternal uniparental disomy (2–5%), imprinting defect (2–5%), and mutation in the *ubiquitin protein ligase E3A* (*UBE3A*) gene (10%). *UBE3A* is subjected to a tissue-specific genomic imprinting. The paternally inherited allele is repressed and the maternally one is expressed in mature neurons of the brain whereas both alleles are expressed in the remaining tissues (Van Buggenhout and Fryns, 2009). *UBE3A* imprinting is thought to be mediated by a long non-coding transcript called *UBE3A-ATS* in human. Mouse models of AS exist but differ from human condition in the timing, mechanisms, and tissue specificity of *UBE3A* repression (Leung et al., 2011). In a recent study, Chamberlain et al. established iPSC lines from two patients with AS who carried maternally inherited deletions of chromosome 15q11–q13 (AS-iPSCs). AS-iPSCs maintain the methylation imprint of the parental fibroblasts following reprogramming and after long term culture. This iPSC-based model recapitulates the tissue-specific pattern of *UBE3A* imprinting as the paternal *UBE3A* was silenced in AS-iPSC-derived neurons in contrast with the normal ones. The authors demonstrated that *UBE3A* silencing is mediated by the sudden expression of *UBE3A-ATS* during neurogenesis (Chamberlain et al., 2010). Considering the results of this study, this iPSC-based model could allow a better understanding of the mechanisms that govern genomic imprinting during human neural development in AS.

Down syndrome. Down syndrome is the most common genetic developmental disorder with an incidence of 1 in 800 live births. It is caused by a trisomy of the chromosome 21 and results in varying degree of physical and mental retardation. With respect to the mental disturbances, patients with DS show cognitive impairment, learning and memory deficits, arrest of neurogenesis, and synaptogenesis and early onset of AD (Antonarakis et al., 2004). Recently, hESCs have been identified by PGD from human embryos that carried trisomy 21 anomaly (DS-hESCs). When induced to differentiate as EBs, the DS-hESC-derived cells displayed chromatin modifications in comparison with the normal counterpart (Biancotti et al., 2010). iPSCs have been also derived from patients with DS (Park et al., 2008) but their neural differentiation potentials remain still not investigated.

Fragile X syndrome. The neurodevelopmental disorder FXS is the most common cause of intellectual disability in males and the most common single gene cause of autism. In addition to cognitive deficits, FXS patients exhibit hyperactivity, attention deficits, social difficulties, anxiety, and other autistic-like behaviors. This X-linked disorder is caused by an expansion of trinucleotide CGG repeats on the promoter region of the *fragile X mental retardation 1* (*FMR1*) gene that leads to the loss of the fragile X mental retardation protein (FMRP). The first PSCs reported for the study of FXS were derived from embryos identified by PGD (Eiges et al., 2007; Tropel et al., 2010). Eiges and colleagues established an FXS-hESC-based model for the study of the developmental events involved in the pathogenesis of the disease. The full expansion in CGG repeats was not able to inactivate the expression of *FMR1* gene in the undifferentiated FXS-hESCs. However, upon *in vivo* differentiation, FMR1 expression was significantly down-regulated through epigenetic silencing which involves DNA methylation and histone modifications (Eiges et al., 2007). More recently, FXS-iPSCs were generated from patients affected by FXS but interestingly, these cells do not confirm the differentiation dependent silencing of *FMR1* gene expression observed in FXS-hESCs (Urbach et al., 2010). Using FXS-iPSCs, Sheridan et al. (2011) provide novel evidence that the epigenetic modifications of *FMR1* gene together with the loss of FMRP expression is responsible for the abnormal differentiation and maturation of FXS-iPCs into neurons.

Friedreich's ataxia. Friedreich's ataxia (FRDA) is the most frequent hereditary ataxia, with an estimated prevalence of three to four cases per 100,000 individuals. This autosomal recessive neurodegenerative disease is characterized by progressive gait and limb ataxia, dysarthria, areflexia, loss of vibration sense, and a progressive motor weakness. GAA triplet repeat expansions within the first intron of the *frataxin* (*FXN*) gene are the most common mutations underlying FRDA. As a consequence, patients show reduced levels of a *FXN*-encoded mitochondrial protein called frataxin. The subsequent mitochondrial dysfunctions in neuronal and muscle cells lead to degeneration of nerve tissue in the spinal cord and nerves controlling muscle movement in the arms and legs. Non-neurological signs include hypertrophic cardiomyopathy and diabetes mellitus. Mouse models for FRDA and FRDA cell lines are readily available, however they do not accurately mimic the disease (Schulz et al., 2009). In two recent reports, iPSCs were successfully derived from patients with FRDA (FRDA-iPSCs). *FXN* mRNA levels were significantly reduced in the FRDA-iPSCs and FRDA-iPSC-derived EBs and NPCs. In addition, FRDA-iPSCs showed the characteristic GAA triplet repeat expansions in the *FXN* gene (Ku et al., 2010; Liu et al., 2011). The mechanistic analysis of these GAA repeat expansions revealed the involvement of the MMR enzymes MSH2 in the repeat instability observed in FRDA-iPSCs. Moreover, global mRNA expression profile analysis of FRDA-iPSCs points to a role for genes related to mitochondrial function, DNA repair, DNA damage response, cell cycle, protein modification/ubiquitination, lipid metabolism, and carbohydrates biosynthesis, confirming previous results found in FRDA patients (Ku et al., 2010). Further differentiation of FRDA-iPSCs into sensory neurons will advance the understanding of the impact of GAA repeat expansions in the dysfunction and death of the sensory neurons of the dorsal root ganglia in FRDA patients.

Prader–Willi syndrome. Prader–Willi syndrome (PWS) is a neurodevelopmental disorder caused by a deletion or disruption of genes in the proximal arm of chromosome 15 or by maternal uniparental disomy in the proximal arm of chromosome 15 (also called critical 15q11–13 region). In addition to mental retardation, PWS is characterized by reduced fetal activity, obesity, hypotonia, short stature, hypogonadotropic hypogonadism, small hands, and feet. PWS is frequently described together with AS because both are caused by genomic imprinting of the critical 15q11–13 region. The disease is due to genomic imprinting on the critical chromosomal region where the expression of genes from only one parent's chromosome is associated with silencing of those from the other parent's chromosome. The imprinting center (IC) for PWS is located in the exon 1 of the *SNURF-SNRPN* gene. This IC seems to act as a promoter for *SNURF-SNRPN* and the small nucleolar RNAs (snoRNA) *HBII-85* (also called *SNORD116*) and *HBII-52* (also called *SNORD115*) genes (deficiency of these snoRNAs is sufficient to cause PWS). As a consequence, the PWS IC of paternal origin is normally demethylated whereas the high methylation of the maternal PWS IC leads to the silencing of *SNURF-SNRPN* gene (Leung et al., 2011). Recent advances have been achieved by modeling PWS through the generation of iPSCs from individuals affected by PWS (PWS-iPSCs; Chamberlain et al., 2010; Yang et al., 2010). These PWS-iPSCs expressed markers of pluripotency, showed DNA hypomethylation of *Nanog* and *Oct4* promoters and were able to differentiate *in vivo* and *in vitro* into the three germ layers. Importantly, PWS-iPSCs maintained an appropriate methylation imprint after reprogramming. In contrast with the normal iPSCs where a methylated maternal allele and an unmethylated paternal allele was present, PWS-iPSCs showed only a methylated maternal allele (Chamberlain et al., 2010). In addition, PWS-iPSCs retained the genomic imprinting of the parental fibroblasts for PWS IC and showed a silencing of *HBII-85* gene expression (Yang et al., 2010).

Rett syndrome. Rett syndrome (RTT) is a neurological disorder caused by mutations in the X-linked gene *methyl CpG-binding protein 2* (*MECP2*). It affects almost exclusively females as young boys inheriting a mutant *MECP2* are much more severely affected and usually do not survive after infancy. It is the primary cause of severe mental retardation in girls with an incidence of ∼1 in 10,000 female births (Neul et al., 2010). Recently, RTT disease phenotype has been successfully recapitulated in RTT-iPSC-derived neurons. In particular, when RTT-iPSCs were induced to differentiate into neurons, they displayed morphological alterations such as fewer synapses, reduced dendritic spine density, and soma size (Marchetto et al., 2010; Cheung et al., 2011; Kim et al., 2011c). Thus, electrophysiological recordings revealed a decrease of the frequency and the amplitude of calcium transients together with a reduced frequency of spontaneous postsynaptic currents in RTT-iPSC-derived neurons, supporting the idea that calcium signaling is impaired in these cells (Marchetto et al., 2010). The same group provided novel evidence into the mechanisms underlying the pathogenesis of RTT disease. They found in particular that long interspersed nuclear elements-1 (LINE-1 or L1s) retrotransposition, a process that modulates gene expression through insertions, deletions, and newsplice sites, is more frequent in RTT-iPSC-derived neurons than those derived from normal healthy donors (Muotri et al., 2010).

Schizophrenia. Schizophrenia (SCZD) is a heritable developmental disorder that affects ∼0.5–1% of the population. This psychiatric disorder is characterized by psychotic symptoms (hallucinations, delusions, disorganized speech, and behavior), negative symptoms (flattened affect, avolition, and social withdrawal), and cognitive defects. Typically, patients with SCZD show decreased brain volume, aberrant neurotransmitter signaling, reduced dendritic arborization, and impaired myelination. Chiang et al. first published the generation of iPSCs from SCZD patients with a mutation in *Disrupted-in-Schizophrenia-1* (*DISC1*), a susceptibility gene that have been previously described disrupted in Finnish SCZD families (Ekelund et al., 2001). However, the neural differentiation potentials and the functional properties of these SCZD-iPSCs were not investigated in this study. Insights into the pathogenesis of SCZD have been gained by direct reprogramming of fibroblasts from patients affected by SCZD into SCZD-iPSCs and subsequent differentiation of these iPSCs into neurons (Brennand et al., 2011). SCZD-iPSC-derived neurons had reduced neuronal connectivity, reduced outgrowths from soma and reduced PSD95 dendritic protein levels. Thus, the authors not only confirmed the alteration of genes known to be involved in the pathogenesis of SCZD but also updated new altered pathways in SCZD. Importantly, these defects in neuronal connectivity and gene expression were ameliorated by the antipsychotic drug loxapine (Brennand et al., 2011). Taken together, these results support the idea that disease-specific iPSCs not only allow the investigation of the mechanisms involved in the pathogenesis but also the restoration of the defects associated with the disease.

Retinal degenerative diseases

Retinitis pigmentosa (RP) is the most common inherited human eye disease (with a worldwide prevalence of 1 case in 3000 to 1 in 7000 individuals) caused by the irreversible degeneration of rod photoreceptors. This results in night blindness and visual defects that can lead to complete blindness when the disease further affects the cone photoreceptors. The mechanisms underlying retinal degeneration are largely unknown; hundred of genes have been associated with the disease and therefore clear genotype-phenotype correlations are not possible (Ferrari et al., 2011). Recent advances in stem cell technology have led to the emergence of methods for differentiation of PSCs into multipotent retinal progenitor cells (RPCs), retinal pigment epithelium (RPE), and photoreceptor-like cells (Buchholz et al., 2009; Meyer et al., 2009, 2011; Osakada et al., 2009; Lamba and Reh, 2011). In addition, disease-specific iPSCs have been derived from patients affected by RP (RP-iPSCs) carrying mutations in *RP1*, *RP9*, *PRPH2*, or *RHO* genes (Jin et al., 2011; Tucker et al., 2011). Interestingly, in contrast with their normal counterpart, RP-iPSC-derived rod photoreceptor cells degenerated with extended culture period. The authors provide evidence that this degeneration was triggered by an increase of apoptosis, oxidative stress, and endoplasmic reticulum dysfunction in these cells (Jin et al., 2011). Importantly, the degeneration of rod photoreceptors carrying *RP9* mutations was counteracted by the antioxidant α-tocopherol but not in those

carrying *RP1*, *PRPH2*, or *RHO* mutations supporting the idea that the efficacy of the molecule depends on the genetic mutations (Jin et al., 2011). In an other study, the genetic analysis of the RP-iPSCs lead to the identification of the cilia-related gene *male germ cell-associated kinase* (*MAK*) gene as a cause of RP (Tucker et al., 2011). Similarly, iPSCs has been established from patients affected by gyrate atrophy, an autosomal recessive eye disease characterized by progressive loss of vision due to retinal degeneration. The affected iPSC-derived RPE exhibited disease-specific functional defects (such as a profound decline of ornithine-δ-aminotransferase activity) that could be restored pharmacologically using vitamin B_6 and via targeted gene repair (Meyer et al., 2011). Altogether, these studies strongly support the idea that these iPSC-based models provide a promising opportunity to identify the pathogenic mechanisms involved in retinal degeneration and give the proof of principle of functional correction of the disease phenotype using both pharmacological and gene repair approaches.

DRUG SCREENING/TOXICITY

Even if progress has been made in pharmacological treatment of some neurological diseases, most of them have minor supportive therapy to no cure available. Moreover, drug development is an incredibly expensive and time consuming process. Discovering and bringing one new drug to the public typically costs from $800 million to more than $1 billion and takes an average of 10–15 years for a pharmaceutical company. The vast majority of the candidate molecules by the current drug screening methods fails to become a drug in clinical application because of safety and efficacy issues. In other terms, current drug screening methods are insufficiently predictive for clinical toxicity and efficacy. There are many explanations accounting for this. First, the main human cellular models used for drug discovery are primary cells isolated from patient tissue and transformed cells derived from tumors or genetically modified. Even if notable insights have been gained with these cells, the limited availability and the relevance of these cells reduce their potential for drug discovery. Then, despite similarities to human patient's phenotype (Baker, 2011), mice models have several drawbacks for disease modeling and drug screening (Dibernardo and Cudkowicz, 2006; Scott et al., 2008). Perhaps the best example is the use of the transgenic mouse that overexpresses mutant superoxide (SOD), a gene found to be associated with ALS (Rosen et al., 1993). Several compounds including vitamin E and creatine were beneficial in this mouse model (Klivenyi et al., 1999) but showed no clinical improvement in humans (Desnuelle et al., 2001; Shefner et al., 2004; Aggarwal and Cudkowicz, 2008; Schnabel, 2008). Therefore, there is a real need to more accurately model human physiology. In this context, hESCs and hiPSCs provide a unique opportunity for drug discovery (**Figure 2**). In fact, after the identification of the targets involved in the pathogenesis of the disease, the next step could be the targeting of the defects using pharmacological and gene correction approaches. As a proof of concept, numerous recent studies using hESCs and hiPSCs began with target identification by choosing a biochemical mechanism involved in a disease condition, followed by the rescue of the observed defects. Defect corrections have been reported in hiPSCs with known drugs that have been previously reported beneficial in SMA (Ebert et al., 2009), FD (Lee et al., 2009), SCZD (Brennand et al., 2011), AD (Israel and Goldstein, 2011), and retinopathy (Meyer et al., 2011). By using hiPSCs, it is not only possible to confirm the interaction of the candidate molecules with the drug target, but also allows the evaluation of their efficacy by checking their activity in the neural cell of interest regarding the disease. Thereafter, the potential of the drug candidate can be assessed by rigorous screening processes which can include functional genomics and/or proteomics as well as other functional screening methods. Also, the hiPSC model offers the obvious possibility of personalized screening of molecules. By using patient specific-iPSC-differentiated cells, it could be possible to test and adapt the dose and combination of treatments to the patient. At the same time, it allows the exploration of the possible targets of patient resistance to treatments.

A critical issue for clinical translation is safety. For example, some drugs which are not aimed at targeting heart or liver have nevertheless been found to have profound toxic effects on heart muscle and hepatocyte. Cardiotoxicity and hepatotoxicity are the major forms of toxicity seen in drug development. Safety issues can be tested at earlier stage using hPSC-derived cells. Screening of the hepatotoxic and cardiotoxic effects of drugs can be evaluated by directed differentiation of the hPSCs into hepatocytes and cardiomyocytes. Similarly, due to their reliance on embryonic and differentiation pathways, hPSCs are potentially informative for embryonic development and differentiation screens (Desbordes et al., 2008). These screens may identify molecules involved in cell specification and toxicity pathways in embryonic development and differentiation of hPSCs.

REGENERATIVE MEDICINE: FROM DEVELOPMENTAL BIOLOGY TO THERAPEUTIC APPLICATIONS

Perhaps the most important potential application of hPSCs is the generation of cells and tissues that could be used for cell-based therapies (**Figure 2**). The possibility to replace lost neurons or other neural cell types and to support the remaining neural cell population by hPSC-derived cells has received considerable attention. Cell replacement may be achieved by transplantation into patients of hPSC-derived cells which have undergone differentiation and maturation *in vitro*. Preliminary research in animal models indicates that hPSC-derived cells, transplanted into a damage brain or retina, can have beneficial effects. Whether these cells can generate the neural cells of interest (neurons, glial cells, and RPCs) or stimulate the endogenous stem cells in the CNS that repopulate the damage tissue is actively under investigation. Proof of principle for such regeneration has been demonstrated for several CNS disease models. In this section, we will discuss four striking breakthroughs of PSCs in regenerative medicine.

Regarding spinal cord injury, PSC-derived cells are currently used to replace the damage area or to support axonal growth with trophic factors. Transplantation of hESC-derived neurospheres, motor neurons, or oligodendrocytes in rodent models of spinal cord injury has been shown to improve function. These hESC-derived oligodendrocytes have been shown to repopulate the site of injury and promote remyelination of the lesion (Keirstead et al., 2005; Lee et al., 2007b; Nori et al., 2011). Based on the impressive results published in animal models of spinal cord injury, the U.S. Food and Drug Administration (FDA) has approved Geron Corp.

for human clinical trials using hESC-derived oligodendrocyte progenitors (GRNOPC1) in spinal cord injury (studies registered with ClinicalTrials.gov, number NCT01217008). GRNOPC1 were administered by injection at a dose of two million cells between 7 and 14 days after injury in four patients with complete thoracic spinal cord injuries. To date, GRNOPC1 has been well tolerated with no serious adverse events observed.

Motor neuron degeneration is a pathological hallmark of motor neuron diseases such as ALS and SMA for which currently no cure exists. Recently, motor neuron replacement and protection using hPSCs has emerged as potential candidates for the treatment of motor neuron diseases. To be clinically successful, the transplanted hPSC-derived cells have to form extended axons and functional neuromuscular junctions. In rat models, spinal transplantation of hESC-derived motor neuron progenitors has resulted in partial recovery from paralysis thanks to axonal projection and muscle innervation (Harper et al., 2004; Deshpande et al., 2006; Corti et al., 2009, 2010).

Cellular therapy for PD remains quite challenging. The disease results from the degeneration of DA neurons in the substantia nigra and the subsequent loss of dopamine in the striatum. Initial studies investigated the potential of hESC-derived DA neurons in rodent models of PD. Roy et al. (2006) documented the functional engraftment of hESC-derived DA neurons together with improvement of lesion-induced behavioral deficits in a rodent model of PD. Thereafter, numerous studies supported the clinical potential of hPSCs for personalized cell therapy of PD (Tabar et al., 2008; Wernig et al., 2008b; Hargus et al., 2010; Rhee et al., 2011). The recent study of Studer and colleagues represents a major advance toward the application of hESC-derived DA neurons in clinic. They succeeded in generating DA neurons with a substantia nigra phenotype from hESCs that exhibited electrophysiological properties of substantia nigra neurons and released DA *in vitro*. Notably, these cells demonstrated *in vivo* survival and function when transplanted in three animal models of PD. In 6-hydroxy-dopamine-lesioned mice and rats, these DA neurons functionally engrafted *in vivo*, reinnervated the striatum and improved clinically relevant behavioral deficits resembling symptoms in PD patients. Importantly, the authors did not identify any neural overgrowth or tumors of the transplanted neural cells *in vivo* supporting a future hESC-based therapy for PD patients (Kriks et al., 2011).

Another important area of investigations for hPSCs is cell-based therapy for retinal degenerative diseases such as retinitis pigmentosa, gyrate atrophy, and age-related macular degeneration. The successful differentiation of hPSCs into multipotent RPCs, RPE, and photoreceptor-like cells (Buchholz et al., 2009; Meyer et al., 2009, 2011; Osakada et al., 2009; Lamba and Reh, 2011) has opened new hopes and perspectives for the therapy of retinal degenerative diseases. In the past few years, promising studies with transplantation of hPSC-derived cells in animal models of retinal degeneration have caused great excitement. In particular, hESC-derived RPE cells provided long term rescue of visual function in two rodent models of retinal degeneration, by replacing the degenerating retina (Gamm et al., 2007; Francis et al., 2009; Lu et al., 2009). Thus, the FDA has granted the permission to Advanced Cell Technology's for clinical trials using hESC-derived RPE (MA09-hRPE cells) for Stargardt macular dystrophy (SMD) and dry age-related macular degeneration (AMD; studies registered with ClinicalTrials.gov, numbers NCT01345006 and NCT01344993). A preliminary report regarding the safety and tolerability of this trial in one patient with AMD and the other with SMD showed no signs of hyperproliferation, tumorigenicity, ectopic tissue formation, or immune rejection of the hESC-derived RPE cells 4 months after transplantation (Schwartz et al., 2012).

CHALLENGES AND LIMITS

As hESCs are derived from embryos, their use for clinical application and basic research remains controversial. In addition to the obvious technical and ethical considerations about the use of hESCs, one of the major barriers for their clinical use is the challenge of immunological rejection (for a review regarding the immunological aspects of PSCs see de Rham and Villard, 2011; Preynat-Seauve and Krause, 2011). In this regard, the iPSCs provide an alternative source of autologous stem cells. Moreover, iPSCs do not require the use of human embryos or oocytes, which makes their use in basic research and in clinical application less controversial technically and ethically. Despite those advantages, significant barriers, and challenges remain unsolved in their current use in research and before their applications in clinic.

DISEASE MODELING

Pluripotent stem cells have opened a new door to study and understand human diseases. However, it is important to keep in mind that it is not possible to model all human neurological diseases *in vitro* using PSCs. The lack of hESC for some diseases that cannot be identified after PGD, account for this (see **Table 1** for the diseases in which ESCs have been derived from human embryos). This limitation concerns also iPSCs. One recent exception is cells derived from patients affected by FXS which failed to reactivate the *fragile X mental retardation 1* (*FMR1*) gene after reprogramming into iPSCs. In contrast, ESCs derived from human FXS blastocysts showed the reactivation of the *FMR1* gene (Urbach et al., 2010). This example suggests that iPSCs may not be the model of choice to study certain human genetic diseases. Moreover, even though a disease-related phenotype has been shown with iPSCs derived from patients with FD, SMA, RTT, and others (see the section "modeling human neurological diseases"), in contrast a phenotype has not been found *in vitro* using iPSCs derived from patients with PD (PD-iPSCs) and HD under basal conditions (Park et al., 2008; Soldner et al., 2009; Nguyen et al., 2011; Seibler et al., 2011). In fact, FD, SMA, and RTT manifest early in life and therefore are more prone to show the disease phenotype *in vitro* using iPSCs. Many common human neurological diseases have late-onset like AD and PD. So, a key challenge is to produce PD-iPSC-derived cells with the neuron characteristics of a 75-year-old patient affected by PD. In this regard, it remains unclear whether iPSCs retain an epigenetic memory and age-related behavior of the parental somatic cells. If yes, this could allow the modeling at least in part, of late-onset diseases. If not, iPSC-derived neural cells may not manifest the phenotype under basal conditions. Furthermore, it could be also possible to induce the age-related phenotype pharmacologically (using free radicals, molecules that induce aging, and neurodegeneration) or by gene manipulation

(mitochondrial DNA mutations). As an example, Nguyen et al. derived iPSCs from a patient with a mutation in the *LRRK2* gene, the most common cause of familial PD. Interestingly, DA neurons derived from these LRRK2-PD-iPSCs displayed a greater susceptibility to cell death when exposed to stress agents such as oxidative stress, the proteasome inhibitor MG-132, or 6-hydroxydopamine (Nguyen et al., 2011). Similarly, in DA neurons derived from PD-iPSCs harboring *PINK1* mutations, an impairment of the mitochondrial parkin recruitment has been described upon mitochondrial depolarization induced by valinomycin (Seibler et al., 2011). Neurodegeneration can also be induced by reproducing the toxic microenvironment of the dying cells. In an elegant study, neurodegeneration in ALS has been recapitulated by co-culture of hESC-derived motor neurons with glial cells carrying SOD mutations (Di Giorgio et al., 2008; Marchetto et al., 2008). Therefore, co-culture of glial cells with motor neurons derived from ALS-iPSCs carrying SOD mutations will be of great interest for the understanding of the role of the glial cells in motor neuron degeneration.

A number of potential variables must be considered when establishing an hPSC-based disease model. Regarding disease modeling and drug screening studies, the definition of a non-disease control is of crucial importance (Inoue and Yamanaka, 2011; Zhu et al., 2011). First and foremost, the genetic background of the non-disease control and the affected cells has to be identical or close in order to be sure that the differences observed in the studies are only due to the disease and not to the choice of the normal and the affected samples. In practice, most of the published articles used iPSCs from unaffected family members of the patient as controls. When this condition is not possible to achieve, control iPSCs from unrelated healthy persons together with ones from unrelated affected patients are often used to decrease the variability between the control and the affected cells and to ensure that the results are not specific for a particular control and patient. To overcome these problems isogenic controls have been recently developed using several approaches. For example, recent studies have described the possibility to obtain isogenic controls through X-chromosome inactivation as after reprogramming, iPSCs can retain an inactive X-chromosome in a non-random pattern. Taking advantage of this characteristic, several groups obtained a pair of isogenic wild-type and mutant iPSC lines. One example was the generation of a pair of isogenic normal iPSCs and mutant MECP2 expressing RTT-iPSCs (Ananiev et al., 2011; Cheung et al., 2011). Then, for monogenic diseases, isogenic controls can be generated through targeted correction of genetic point mutations. One strategy for correction is to use homologous recombination with an exogenous DNA to modify specific genomic sequences. This is referred as "genome editing" and comprises the engineered zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and oligonucleotide-directed gene editing methods (Lombardo, 2007; Miller, 2007; Moehle, 2007; Hockemeyer et al., 2011). The principal advantage of ZFNs is the ability to target any desired genomic DNA sequence with high fidelity and to induce precise gene knockouts or gene replacements by homologous recombination. This approach has been recently applied to target endogenous genes in hESCs and hiPSCs to generate isogenic disease and control cell lines (Hockemeyer et al., 2009; Soldner et al., 2009; Zou et al., 2009). The genetic corrections of the sickle cell anemia mutation (Sebastiano et al., 2011) and of the α1-antitrypsin deficiency (Yusa et al., 2011) in hiPSCs are examples of recent accomplishments using this technology.

Other factors are likely to contribute to the variability between iPSC lines such as the process of cell derivation (Lengner et al., 2010). Considering that iPSCs were found to retain epigenetic memory of their parental somatic cells and showed preferential lineage-specific differentiation (Bar-Nur et al., 2011; Kim et al., 2011b), it is important to take the same type of parental somatic cells when establishing iPSCs. Moreover, the residual expression of the viral vector (Soldner et al., 2009), the genetic alterations introduced after reprogramming (Gore et al., 2011; Hussein et al., 2011), and the protocols used for differentiation (either spontaneous differentiation into EBs or directed differentiation into neural cells of interest) may contribute to the observed variations in efficiency between iPSC clones in generating neural cells (Hu et al., 2010).

REGENERATIVE MEDICINE

The hPSC applications in regenerative medicine are an exciting and fast moving area of current studies. The recent findings are supportive of a future hPSC-based therapy for neurological diseases. Long term engraftment of hPSC-derived cells in several CNS disease models demonstrated *in vivo* survival and function of these cells together with improvement up to complete restoration of the deficits resembling the symptoms observed in human neurological diseases (Harper et al., 2004; Keirstead et al., 2005; Deshpande et al., 2006; Gamm et al., 2007; Corti et al., 2009, 2010; Francis et al., 2009; Lu et al., 2009; Kriks et al., 2011; Schwartz et al., 2012). Also, hPSCs offer the advantage to provide an inexhaustible supply of differentiated cell types compared to the other cells that have been used in clinic until now (mesenchymal stem cells, fetal, and adult stem cells). Another important advantage of hPSCs for regenerative medicine is their amenability to genetic manipulation. Gene targeting by homologous recombination in hPSCs has proven possible recently using "genome editing" techniques (Lombardo, 2007; Moehle, 2007; Hockemeyer et al., 2009; Zou et al., 2009; Sebastiano et al., 2011; Yusa et al., 2011). However, critical issues remain to be addressed. HPSCs have to be differentiated into a pure and clinical grade population of neural cells of interest regarding the disease. This purification can be achieved by selection of the differentiated cells of interest with fluorescence-activated cell sorting approaches or by limiting/blocking the growth of the undifferentiated hPSCs (by apoptosis or suicide gene induction; Bieberich et al., 2004; Fukuda et al., 2006).

The major challenge regarding PSC-based therapy is the safety of these cells when introduced into patients. In fact, the tumorigenicity of hESCs and hiPSCs is the major hurdle for their application in regenerative medicine (Blum and Benvenisty, 2009; Knoepfler, 2009). Both hPSCs have been shown to form more aggressive tumors than teratoma, the so-called teratocarcinomas (Yang et al., 2008; Blum and Benvenisty, 2009; Werbowetski-Ogilvie et al., 2009; Hovatta et al., 2010). The possible traits of these hPSCs that could induce teratocarcinomas are not completely understood. However, accumulating evidence supports that PSCs show many common similarities with tumor cells and cancer cell lines (Dreesen and Brivanlou, 2007; Knoepfler, 2009) including

high proliferation rate, high telomerase activity, and expression of oncogenes (Baker et al., 2007; Hiyama and Hiyama, 2007; Evans and Liu, 2008; Blum and Benvenisty, 2009; Ruggero, 2009; Amps et al., 2011). Several groups reported that the generation of hESCs and hiPSCs were accompanied with somatic coding mutations, copy number variations, and aberrant epigenomic reprogramming (Baker et al., 2007; Gore et al., 2011; Lister et al., 2011). Regarding hESCs, chromosomal aberrations are mostly acquired after culture adaptation over time (Baker et al., 2007). Two types of genomic aberrations can be observed in hESC culture. Transient genomic aberrations eventually appear in culture and disappear after culture passages as they are not advantageous for the hESCs (Hussein et al., 2011). In contrast, stable genomic aberrations that confer growth, self-renewal, and differentiation advantages for hESCs are often selected over time (Baker et al., 2007; Mayshar et al., 2010; Amps et al., 2011). Also, it becomes clearly apparent that genomic stability of hESCs is dependent on culture conditions such as feeder cells, culture medium, cell passaging, freezing, and thawing procedures (Lefort et al., 2009; Olariu et al., 2010). For example, passaging hESCs by "manual cutting and pasting" appears to give more stable cells with a normal karyotype than enzymatic harvesting methods (Buzzard et al., 2004; Mitalipova et al., 2005; Olariu et al., 2010). Among the aberrations observed in hESC lines, gain of chromosomes 12, 17, 20, and X are the most common changes reported (Buzzard et al., 2004; Draper et al., 2004; Maitra et al., 2005; Mitalipova et al., 2005; Spits et al., 2008; Lefort et al., 2009; Hovatta et al., 2010; Mayshar et al., 2010). Recently, the International Stem Cell Initiative analyzed 125 hESC and 11 iPSC lines from 38 laboratories worldwide for genetic changes that occur during culture in which they identified a chromosome 20 minimal amplicon conferring growth advantage (Amps et al., 2011). All these changes are of clinical importance as they have been also described in germ cell tumors and embryonal carcinoma cells (Baker et al., 2007; Blum and Benvenisty, 2009) and could explained the high malignancy of these cells after injection *in vivo*. In line with this, we identified genomic changes acquired in culture that are potentially oncogenic in four hESCs and a teratocarcinoma-like hESC. Among the altered genes, we identified those associated with leukemia translocations and those that promote tumor formation in breast and in urothelial cancers (Hovatta et al., 2010).

Regarding hiPSCs, chromosomal aberrations can originate from the somatic cell before reprogramming (after prolonged time in culture for example), be induced during the reprogramming process and after extended culture of the hiPSCs. Gore et al. investigated the genetic fidelity of 22 hiPSC lines generated by different laboratories using different reprogramming methods. Importantly, coding point mutations were found in all hiPSCs with an average of five protein-coding point mutations. More than 50% of these mutations were also present in the parental fibroblasts while the others were induced during or after the reprogramming process. The majority of these coding point mutations were enriched in genes mutated or involved in cancers (Gore et al., 2011). Moreover, the same chromosomal aberrations described for hESCs have been also reported for hiPSCs (Mayshar et al., 2010; Taapken et al., 2011). In particular, Mayshar and colleagues found that prolonged time in culture is responsible for the duplication of chromosome 12, which is the most common aberration observed in hiPSCs. This adaptation of hiPSCs to culture was associated with the increased expression of critical genes in chromosome 12 including those involved in pluripotency and cell cycle pathways such as *Nanog* and *Growth/differentiation factor 3* (*GDF3*; Mayshar et al., 2010). In line with this, we recently highlighted the crucial role of *Nanog* during reprogramming of somatic cells into hiPSCs with respect to germ cell tumor formation (Grad et al., 2011).

Accumulating evidence suggests that reprogramming of somatic cells into hiPSCs is accompanied with genetic and epigenetic changes (Gore et al., 2011; Lister et al., 2011) that may increase the tumorigenicity of these cells. The first suspects are genes used for reprogramming that are known to be oncogenes such as *klf4* and *c-myc* (Ruggero, 2009). In fact, the reactivation of *c-myc* in iPSC-derived chimeras has been shown to induce tumor formation in mice (Okita et al., 2007; Markoulaki et al., 2009). Reprogramming of somatic cells into iPSCs has been also achieved in the absence of *klf4* and *c-myc* though with a lower efficiency (Huangfu et al., 2008; Nakagawa et al., 2008; Wernig et al., 2008b). However, tumor formation has been described using only *Oct4* as reprogramming factor (Hochedlinger et al., 2005). Another potential risk for tumorigenicity concerns the use of lentiviruses and retroviruses for somatic cell reprogramming. To overcome the potential insertional mutagenesis induced by these methods and the incomplete silencing of reprogramming factor following differentiation (Ramos-Mejia et al., 2010), a number of alternative methods have been developed (see Reprogramming of Somatic Cells into a Pluripotent State). However, the efficiency of these reprogramming methods is very low, in a range of 0.001%. Recently, several groups have developed doxycycline-induced lentiviral vectors that allow their excision by Cre recombinase after cell reprogramming (Kaji et al., 2009; Soldner et al., 2009; Sommer et al., 2010). This method enables the elimination of the transgene expression with a high efficiency of reprogramming.

CONCLUSION AND FUTURE PERSPECTIVES

We believe that hPSC technology provides a promising alternative model to study the pathogenesis of human diseases as it is possible to generate cellular models for most of human diseases. It provides a unique opportunity to generate human cellular models for diseases for which a model is missing (or at least relevant human model). It also limits the use of mouse models in research and drug screening. Moreover, the new experimental finding in generating hiPSCs by reprogramming somatic cells to embryonic stem cell-like (Takahashi et al., 2007; Yu et al., 2007; Park et al., 2008) and to differentiate it into several lineages have opened the possibility to understand the pathogenesis of human diseases. *In vitro* differentiation of such cells may provide unique opportunities for regenerative medicine by generating transplantable cells without immunological rejection. Eventually, it should be possible to treat the defect associated with the disease by pharmacological and gene repair manipulation approaches before transplantation. Finally, these hiPSCs provide an interesting model for pharmacological therapies and for deciphering the molecular targets of therapy response and resistance in humans. Nevertheless, despite those advantages, several issues remain to be solved before their

clinical use such as the genomic aberrations and the tumorigenicity of these cells. Therefore, further studies are needed to address whether these cells fulfill their promise in regenerative medicine.

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Mesenchymal stem cell secreted vesicles provide novel opportunities in (stem) cell-free therapy

***Serena Rubina Baglio*[1,2]*, *D. Michiel Pegtel*[2] *and Nicola Baldini*[1]**

[1] *Laboratory for Orthopaedic Pathophysiology and Regenerative Medicine, Istituto Ortopedico Rizzoli, Bologna, Italy*
[2] *Department of Pathology, Cancer Center Amsterdam, VU University Medical Center, Amsterdam, Netherlands*

Edited by:
Anis Feki, Hopitaux Fribourgeois, Switzerland

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Petros Papagerakis, University of Michigan, USA
Konstantinos Zarbalis, University of California, Davis, USA

****Correspondence:***
Serena Rubina Baglio, Department of Pathology, Cancer Center Amsterdam, VU University Medical Center, de Boelelaan 1117, Amsterdam 1081 HV, Netherlands.
e-mail: rubinabaglio@gmail.com

Mesenchymal stem cells (MSCs) are adult multipotent cells that give rise to various cell types of the mesodermal germ layer. MSCs are of great interest in the field of regenerative medicine and cancer therapy because of their unique ability to home to damaged and cancerous tissue. These cells also regulate the immune response and contribute to reparative processes in different pathological conditions, including musculoskeletal and cardiovascular diseases. The use of MSCs for tissue repair was initially based on the hypothesis that these cells home to and differentiate within the injured tissue into specialized cells. However, it now appears that only a small proportion of transplanted MSCs actually integrate and survive in host tissues. Thus, the predominant mechanism by which MSCs participate in tissue repair seems to be related to their paracrine activity. Indeed, MSCs provide the microenvironment with a multitude of trophic and survival signals including growth factors and cytokines. Recent discoveries suggest that lipid microvesicles released by MSCs may also be important in the physiological function of these cells. Over the past few years the biological relevance of micro- and nano-vesicles released by cells in intercellular communication has been established. Alongside the conventional mediators of cell secretome, these sophisticated nanovesicles transfer proteins, lipids and, most importantly, various forms of RNAs to neighboring cells, thereby mediating a variety of biological responses. The physiological role of MSC-derived vesicles (MSC-MVs) is currently not well understood. Nevertheless, encouraging results indicate that MSC-MVs have similar protective and reparative properties as their cellular counterparts in tissue repair and possibly anti-cancer therapy. Thus, MSC-MVs represent a promising opportunity to develop novel cell-free therapy approaches that might overcome the obstacles and risks associated with the use of native or engineered stem cells.

Keywords: mesenchymal stem cell (MSC), microvesicles, exosomes, regenerative medicine, therapy

THE THERAPEUTIC POTENTIAL OF MESENCHYMAL STEM CELLS

Over the last decades, adult stem cells have been extensively studied with regard to their potential implications in regenerative medicine. The multipotent precursors of the bone marrow stroma were the first adult stem cells to be identified (Till and McCulloch, 1964; Friedenstein et al., 1970) and are still now a focus of great interest because of their ability to home to damaged sites, function in tissue repair and regeneration and modulate the immune response. As a result of their self-renewal potential and of their ability to differentiate to various phenotypes of the mesenchymal germ layer, these non-hematopoietic stromal cells are currently referred to as mesenchymal stem cells (MSCs) (Caplan, 1991).

In the bone marrow MSCs represent about the 0.01% of the mononuclear cells and provide the structural and functional support for hematopoietic stem cells (HSCs) in their niche (Johnson and Dorshkind, 1986; Pittenger et al., 1999). However, MSCs have been isolated from a variety of fetal and adult tissues including placenta, umbilical cord blood, adipose tissue (Lee et al., 2004), skeletal muscle, peripheral blood (Bosch et al., 2000; Zvaifler et al., 2000), dental pulp, and, more recently, endometrium and menstrual blood (Musina et al., 2008). Among the various sources, adipose tissue is gaining more and more interest because adipose-derived MSC are available in large amounts from liposuction procedures and thus considered major candidates for future regenerative medicine approaches (Schreml et al., 2009).

MSC CHARACTERISTICS

The identification and the characterization of MSCs have been widely discussed elsewhere (Dominici et al., 2006). The absence of known specific MSC-restricted markers and the observation that the morphology of these cells can vary from spindle to trapezoid shape depending on culture conditions and passage, render it challenging to univocally identify MSCs. For this reason, the International Society for Cellular Therapy (ISCT) established minimal requirements to designate MSCs, i.e., (1) plastic adherence, (2) expression of CD73, CD90 and CD105, and negativity for various hematopoietic markers, and (3) ability to differentiate

into mesenchymal cell types including adipocytes, chondrocytes and osteoblasts (Dominici et al., 2006).

In spite of these efforts, there is still a high need to further characterize the biology of these adult stem cells. In particular, plastic adherence does not appear an essential characteristic of MSCs, as conceived previously. Recent studies from multiple laboratories have shown the existence of non-adherent MSC (NA-MSC) subpopulations that display the same multipotent potential of adherent MSCs. Moreover, the non-adherent MSCs present the same ability to migrate to damaged tissues *in vivo* as adherent MSCs and also function in tissue repair and regeneration (Leonardi et al., 2009; Zhang et al., 2009).

The surface antigen pattern is also an aspect of MSC characterization to be carefully considered because the expression of markers changes depending on the surrounding environment, during culture and upon exogenous stimuli (Dominici et al., 2006).

Finally, concerning the multipotent potential of these cells, the existence of a subpopulation within bone marrow-derived MSCs capable of differentiating not only into the same mesodermal-lineage, but also into other lineages of the ectodermal and endodermal germ layers has been proposed, but is still strongly debated (Dezawa et al., 2004, 2005; Trzaska et al., 2007; Snykers et al., 2011).

The increasing interest around adult MSCs is further triggered by at least two additional characteristics: the immunoregulatory properties of these cells and their homing ability and specificity (**Figure 1**).

IMMUNOREGULATORY PROPERTIES OF MSCs

One of the best-described functional properties of MSCs *in vivo* is their potent effect on the immune system. Indeed, it is well-known that MSCs have the capacity to suppress the immune response (Jiang et al., 2005; Corcione et al., 2006; Casiraghi et al., 2008; Jarvinen et al., 2008; Sheng et al., 2008). However, it has also been demonstrated that they can function as antigen presenting cells (Chan et al., 2006; Stagg et al., 2006).

The latter property of MSCs has been exploited successfully in a therapeutic setting to overcome graft versus host disease after haemopoietic-stem-cell transplantation (Le Blanc et al., 2008). In addition, MSCs have been used to limit inflammation in Crohn's diseases (Garcia-Olmo et al., 2005), and to reduce autoimmune side-effects following engraftment (Christopeit et al., 2008). Numerous characteristics contribute to the immunosuppressive

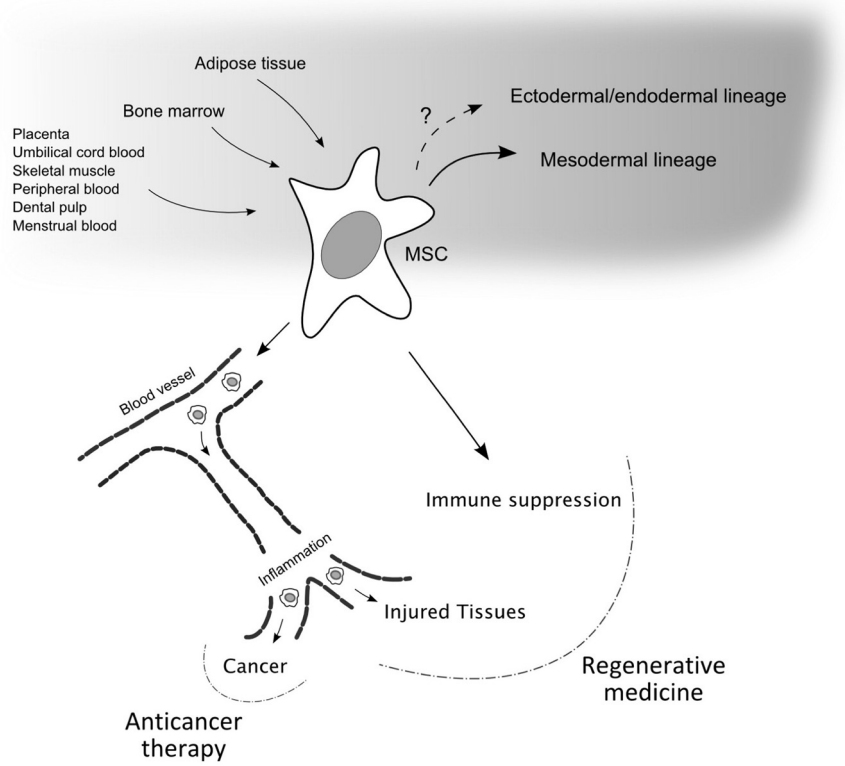

FIGURE 1 | Mesenchymal stem cell properties and relevance for therapeutic applications. Mesenchymal stem cells (MSCs) can be isolated from different sources, in particular from bone marrow and adipose tissues, and can differentiate into various cell types of the mesodermal germ layer. The possibility that these adult stem cells can differentiate in cells of the other germ layers is not excluded and is still debated. Two main properties make MSCs good candidates for therapeutic applications. First, their ability to enter the blood circulation and home to sites of inflammation, i.e., damaged and cancerous tissues, where MSCs can release a multitude of trophic factors. Second, MSCs have the ability to suppress the immune system via different mechanism. While the latter property, together with the tropism for injured sites, can be exploited in the field of regenerative medicine, the homing of MSCs, engineered to carry anti-proliferative or pro-apoptotic signals, to cancer may be important for the development of anticancer therapy approaches.

effect of MSCs. Besides being characterized by low expression of Major Histocompatibility Complex class II (MHCII) and costimulatory molecules (B7-1 and B7-2), they interfere with various pathways of the immune response by means of cell-to-cell interactions and secretion of soluble factors, including members of the transforming growth factor-β family, interleukins 6 and 10, matrix metalloproteinases (MMPs), nitric oxide and indoleamine 2,3 deoxygenase (IDO). Different studies have reported the ability of MSCs to suppress T-cell proliferation, most likely via Prostaglandin E_2 (PGE_2) production (Jarvinen et al., 2008), to induce the T regulatory cells (Casiraghi et al., 2008), and to express co-inhibitory molecules as B7-H1 on their surface upon IFN-γ treatment (Sheng et al., 2008). Moreover, MSCs can impair maturation and function of dendritic cells and inhibit the proliferation, the differentiation and the chemotaxis of B-cells *in vitro* (Aggarwal and Pittenger, 2005; Beyth et al., 2005; Jiang et al., 2005; Corcione et al., 2006).

The immune-stimulating properties of these adult stem cells have been less investigated and seem to depend on the production of pro-inflammatory cytokines (Rasmusson et al., 2007). The dual immunoregulatory function of MSCs has been proposed to be cell dose-dependent, since high numbers of MSCs suppress whereas very low numbers seem to stimulate lymphocyte proliferation (Le Blanc et al., 2003). This latter observation has important implications in the use of MSCs as cell-therapeutics, as the cell dose is critical for the *in vivo* function and may rely on factors that are not well-understood, thereby limiting widespread use in the clinic.

HOMING OF MSCs

An important distinguishing feature of MSCs compared to most other cell-types is that MSCs retain the ability to migrate to differentiated tissues. A number of telling studies have clearly demonstrated that when MSCs are systemically or locally administered, they selectively home to sites of injury and cancer (Ortiz et al., 2003; Rojas et al., 2005; Kidd et al., 2009). In pathological conditions an increase of circulating MSCs can be observed, suggesting the existence of a reservoir of mesenchymal cells that are mobilized in response to injury to target the damaged site and aid in tissue repair (Alm et al., 2010; Deng et al., 2011).

Why MSCs specifically home to these sites and what damaged and cancerous tissues have in common that attract MSCs are still open questions, but inflammation is most likely the responsible denominator. The high concentration of inflammatory chemokines released after tissue damage can indeed control the migration of MSCs, which express receptors for a number of grow factors including PDGF and IGF-1, and chemokines receptors, as CCR2, CCR3, CCR4, and CCL5 (Ponte et al., 2007). On the other hand, strong connections exist between tissue injury, chronic inflammation and cancer, as first described by Mina Bissell's group (Dolberg et al., 1985), so that tumors have been defined "wounds that do not heal" (Dvorak, 1986), where inflammatory cytokines and chemokines are produced and can drive MSC homing (Birnbaum et al., 2007; Dwyer et al., 2007; Menon et al., 2007).

The current knowledge about the mechanisms driving MSC migration and homing comes from studies on leukocytes (Butcher, 1991) and HSCs (Voermans et al., 1999). The initial adhesive interactions between circulating leukocytes and endothelial cells, called "rolling contacts," are mediated by selectins (Lawrence and Springer, 1991). Next, the activation of integrin adhesiveness by chemokines determines the formation of more firm contacts that ultimately lead to extravasation (Lewinsohn et al., 1987). Bone marrow-derived MSCs express various integrins on their surface, among which integrin α4/β1, which mediates cell-cell and cell-extracellular matrix interactions by binding to vascular cell adhesion molecule (VCAM)-1 and to the V-region of fibronectin, respectively. In damaged tissues fibronectin is deposed together with fibrin at the injured sites to stop the bleeding. The provisional matrix is then remodeled by macrophages and fibroblasts, determining an increase in V region-exposing fibronectin, which, in turn, allows MSCs to adhere and transmigrate into the extracellular matrix (Valenick et al., 2005). Among the chemotactic chemokines involved in MSC homing, stromal cell-derived factor 1 (SDF-1) seems to play an important role. Although only low levels of the SDF-1 receptor, CXCR4, are present on the surface of MSCs, high intracellular levels of the receptor have been detected and seem to function as a reservoir. Indeed intracellular CXCR4 can be translocated to the membrane upon chemokine stimulation, thus contributing to the migration of MSCs (Wang et al., 2001; Wynn et al., 2004). Moreover, MSCs are able to secrete different metalloproteinases, including MMP-2 and MT1-MMP, which degrade the extracellular matrix barriers and allow extravasation and subendothelial migration (Ries et al., 2007).

The precise mechanisms driving MSC homing are still unclear, but represent a very attractive subject of investigation because of their implications in the therapeutic applications of these cells, as both reparative effectors and vectors of specific signals.

MSCs IN REGENERATIVE MEDICINE

The unique characteristics of MSCs, such as their multipotency, immunological properties, homing and effects on tissue repair, raised expectations on the possibility to exploit these cells for therapeutic approaches. Indeed, MSCs are readily isolated from bone marrow and fat tissue (Lee et al., 2004), and can be administered to patients in an autologous manner, thus preventing rejection by the immune system.

MSCs have been extensively studied and already clinically tested for their role in bone repair and regeneration. Allogeneic MSCs have been used for the treatment of bone disorders as osteogenesis imperfecta (Horwitz et al., 2002; Le Blanc et al., 2005; Otsuru et al., 2012). For bone tissue engineering applications, these cells are used in combination with "scaffolds" that are designed to allow cell adhesion, survival and growth and that are even functionalized to provide cells with pro-osteogenic stimuli (Warnke et al., 2004; Marcacci et al., 2007). The advantage of using mesenchymal osteogenic precursors relies not only on the ability of these cells to differentiate into osteoblasts, but also on their capacity to provide trophic signals as growth factors and cytokines to the damaged tissues, thereby accelerating the regeneration process (Ciapetti et al., 2012).

Apart from bone-repair MSCs are also used to treat cardiovascular diseases. In particular acute myocardial infarction has

been an important area of study to exploit MSC-based therapies. Cell death due to ischemia leads to decreased contractility of the heart. The general lack of an effective intrinsic mechanism to repair such damage prompted researchers to investigate both *in vitro* and *in vivo* the ability of MSCs to differentiate into cardiomyocytes (Toma et al., 2002; Wang et al., 2006). However, as mentioned before, there is currently no clear consensus if MSCs have the ability to differentiate into cardiomyocytes and, if so, by what signals. Experiments conducted by intravenously injecting MSCs in rodents showed that the majority of cells are "trapped" in the lungs (Schrepfer et al., 2007; Fischer et al., 2009). Moreover, only a small percentage of MSCs administered in swines using different delivery approaches is retained in the heart 2 weeks after transplantation (Freyman et al., 2006). For these reasons, it is believed that the positive effects of MSCs on damaged heart, may not be solely due to their ability to differentiate into cardiomyocytes. Instead, the release of trophic factors together with the suppression of inflammation may also be responsible for the healing effects of MSCs.

MSCs are also used for the treatment of neuronal injury and neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's diseases. In this case, the reparative potential could depend on the ability of MSCs to locally secrete high amounts of brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF), indeed *in vitro* experiments have shown that the expression of these factors increases when MSCs are exposed to injured brain extracts (Chen et al., 2002). Moreover, the ability of MSCs to modulate the immune response might be crucial for neurodegenerative diseases characterized by chronic inflammation (Lee et al., 2010). However, different studies have also suggested the trans-differentiation of bone marrow-derived MSCs into neuronal-like cells under specific induction *in vitro* (Tondreau et al., 2008; Trzaska and Rameshwar, 2011).

Finally, MSCs are able to reverse acute kidney injury in mouse models. Also in this case the precise mechanisms by which MSCs protect from tissue damage is not understood. While initial studies demonstrated that trans-differentiation of the administered MSCs into tubular epithelium cells was responsible for the structural and functional repair of the kidney (Morigi et al., 2004), following experimental evidences revealed that only 2.0–2.5% of MSCs were actually engrafted (Herrera et al., 2007). Therefore, as suggested by additional studies in rodents, the release of factors that can regulate the immune response and have trophic, pro-angiogenic and mitogenic activities is the most accepted mechanism of action of MSCs in kidney repair (Tögel et al., 2005; Semedo et al., 2009).

MSCs IN ANTICANCER THERAPY

While the potential of using MSCs in regerative medicine is releatively well-established, the use of MSCs in anticancer therapy is receiving increasing attention. Because MSCs have a clear capacity to home specifically to tumor sites in humans, they could be used as specialized delivery vehicles for targeted anticancer drugs or gene-therapy (Kidd et al., 2009, 2010; Loebinger et al., 2009; Sasportas et al., 2009; Yang et al., 2009). Nonetheless, this putative approach raises many (safety) questions because, although MSCs have intrinsic anti-tumorigenic activities, they also hold pro-tumorigenic properties, as suppressing the immune response and expressing growth factors and pro-angiogenic molecules that can aid in the formation of cancer stem cell niches (Roorda et al., 2009). Grisendi et al. already designed a novel cancer therapy strategy relying on the use of adipose-derived mesenchymal progenitors (AD-MSCs) as cellular vectors of a pro-apoptotic signal, i.e., tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). When injected intravenously or subcutaneously into mice, TRAIL-transduced AD-MSCs were able to localize into tumors and mediate tumor cell apoptosis without apparent toxicities to normal tissues (Grisendi et al., 2010). Whether this strategy may also be suitable to eradicate human tumors awaits to be studied.

LIMITATIONS OF STEM CELL THERAPY

The use of stem cells for the therapy of human diseases raised several concerns in the past decade that proved a challenging objective to overcome. The result of the interaction between adult stem cells and target microenvironment needs to be further investigated before we can rule out potential risks for human health and obtain effective approaches for regenerative medicine.

Some of the challenges concerning transplanted MSCs are immune-mediated rejection, senescence-induced genetic instability or loss of function, and limited cell survival (Lim et al., 2011). Besides these issues, the major problem in using MSCs for clinical applications is the possibility of malignant transformation. The production of a sufficient amount of MSCs for clinical use requires a consistent *in vitro* expansion, which can lead to spontaneous transformation of the cells (Rubio et al., 2008). The exact mechanisms of MSC transformation are not completely understood, but c-myc upregulation, p-16 repression and increased telomerase activity seem to be involved. Furthermore, genetic manipulations of MSCs for the treatment of different diseases can *per se* increase the oncogenic potential of the cells, either because the transgene may be tumorigenic or because it might cause disruptions in the genome. MSCs have been found in a number of tumors including gastric adenocarcinoma (Xu et al., 2011), lipoma (Lin et al., 2007) and osteosarcoma (Brune et al., 2011), strongly suggesting their involvement in tumor development, and, importantly, various studies indicate these cells as potential sources of tumor associated fibroblasts (TAFs) (Kidd et al., 2012).

In the light of these observations, the choice of translating the potential of MSCs to the clinic should be cautiously considered.

MSC RELEASED VESICLES AS A NOVEL APPROACH OF CELL-FREE THERAPY

In spite of the multipotent and self-renewal potential of MSCs and beyond the somewhat controversial ability of these cells to trans-differentiate into lineages of other germ layers, MCS have clear beneficial effects in the reparative processes of injured tissues. Experimental studies showed that only a small proportion of MSCs, locally or systemically administered, will actually be incorporated into injured tissues (Rosario et al., 1997; Li et al., 2008), indicating that the beneficial effects in tissue repair and

regeneration is more likely indirect and depends on the paracrine activity of MSCs and not on their engraftment.

This intriguing hypothesis opens novel therapeutic perspectives aimed at the development of cell-free strategies based on the use of MSC secretome as a safe and potentially more advantageous alternative to cell-therapy approaches. While the soluble secretome of MSCs is partly characterized (Parekkadan et al., 2007; Lee et al., 2010; Roche et al., 2012), it seems unlikely that specific cytokines and growth factors alone give MSCs their remarkable healing abilities.

EXOSOMES AND MICROVESICLES

Besides the long-time notion of growth factors and cytokines being an important part of the cellular secretome, it now appears that most, if not all cells, secrete large amounts of micro- and nano-vesicles, either constitutively or upon activation signals. The biochemical composition, the complex biogenesis of these vesicles and, in particular, their physiological role have only partially been unraveled. Yet, their potential as mediators of cell communication has not gone unnoticed, since these vesicles have remarkable features, including the ability to transfer proteins and functional genetic material such as RNA to other cells (Ratajczak et al., 2006; Valadi et al., 2007; Skog et al., 2008; Pegtel et al., 2010).

In particular exosomes have received much attention as these are a subclass of (nano)vesicles (50–100 nm) that are derived from specialized intracellular compartments known as late endosomes or Multi-vesicular bodies (MVBs). Many other types of vesicles exist that presumably derive from the plasma membrane and consensus has been reached to collectively name these extracellular membrane vesicles. Exosomes are released from most cells constitutively, but following activation their release is significantly increased. They were first implicated in reticulocyte maturation and later shown to have an important role in immune responses. More recently exosomes have been found in different biological fluids such as urine, plasma, malignant and pleural effusions of ascites and synovial fluid, and, because of their specific content, have been proposed as suitable biomarkers of different diseases (Skog et al., 2008; Nilsson et al., 2009). The biogenesis of exosomes involves the formation of intraluminal vesicles (ILV) by inward budding of the limiting membrane of MVBs. It is presumed, although many molecular details are lacking, that MVBs fuse with the plasma membrane to release the ILVs as exosomes (**Figure 2**). Once secreted exosomes can either be taken up by target cells localized in proximity of the cell of origin or travel to more distant sites through the blood and possibly other biological fluids. Théry et al. (2006) provided a detailed description of the most recognized procedures to isolate and characterize exosomes from cell supernatant and bodily fluids. The development and use of standardized protocols is critical because other kinds of vesicles as well as membrane fragments are normally present in the starting material and can contaminate exosome preparations. Mechanistically, exosomes, but also other types of microvesicles, can operate in a multitude of ways since they can be considered as complex vectors that can hold essentially all known biological molecules and likely the solutes that are present in the parental cells. These molecules include, but are not restricted to, proteins (both ubiquitous and cell-specific), mRNAs, microRNAs (miRNAs) and lipid molecules.

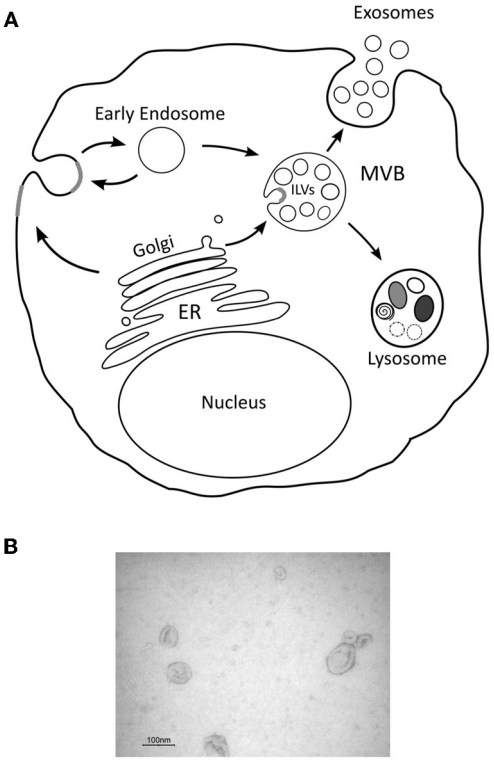

FIGURE 2 | Schematic representation of exosome biogenesis. Intraluminal vesicles (ILVs) are generated by the inward budding of the limiting membrane of a subgroup of late endosomes called multivesicular bodies (MVBs). MVBs can be directed towards the cell periphery and, after fusion with the plasma membrane, release their content in the extracellular space. Secreted ILVs, now called "exosomes," are then taken up by target cells **(A)**. Electron microscopy picture of exosomes isolated by differential ultracentrifugation **(B)**.

Given the multiplicity of signals carried by these vesicles through the horizontal transfer of functional RNAs and proteins, their implication in various diseases and especially in cancer is being intensively investigated. It is becoming more and more evident that cancer cells exploit exosome-mediated signaling to modify their microenvironment, but also to exert systemic functions. Indeed exosomes can promote the formation of pre-metastatic niches, thereby optimising the conditions for tumor spreading (Hood et al., 2011). Moreover, the amount and the content of exosomes consistently vary based on the microenvironmental conditions, and particularly when cells are subjected to stress factors (Parolini et al., 2009; Hedlund et al., 2011; Lv et al., 2012). For instance, the acidic extracellular pH associated with the aggressiveness and chemoresistance of various solid tumors (Simon et al., 1993; Mahoney et al., 2003; Nishisho et al., 2011) is able to increase exosome release and uptake (Parolini et al., 2009).

On the other hand, the sophisticated make up of exosomes, which strongly suggests an important role in cell-cell communication, opens novel perspectives in exploiting these vesicles in

therapeutic settings. Exosomes might be isolated from cells that hold promising therapeutic applications, as MSCs in regenerative medicine, and systemically or locally administered to mimic the effect of the parental cell. Whether MSC-derived exosomes retain the homing properties of the cells of origin is still largely unknown and is an important question to be answered, although *in vivo* studies have shown beneficial effects of intravenously injected exosomes in tissue repair. Moreover, exosomes can be used as targeted delivery vehicles of therapeutic miRNAs. Alvarez-Erviti et al. (2011) succeeded in delivering functional siRNA to the mouse brain by systemically injecting targeted exosomes. To confer tissue-specificity to exosomes the authors engineered low immunogenic cells to express an exosomal membrane protein, Lamp2b, fused to the neuron-specific RVG peptide. Exosomes were then isolated and loaded with exogenous siRNAs by electroporation. Considering the reparative, immune suppressive and homing properties of MSCs, the use of exosomes derived from these cells modified to express high levels of specific miRNAs could also be considered, once ascertained that the miRNAs of interest are actually enriched in the exosomal compartment. In case the tropism of exosomes would not reflect that of MSCs or if different targeting would be required, exosomes bearing tissue-specific receptor on their surface could be engineered (Alvarez-Erviti et al., 2011), or local administration might be considered (**Figure 3**).

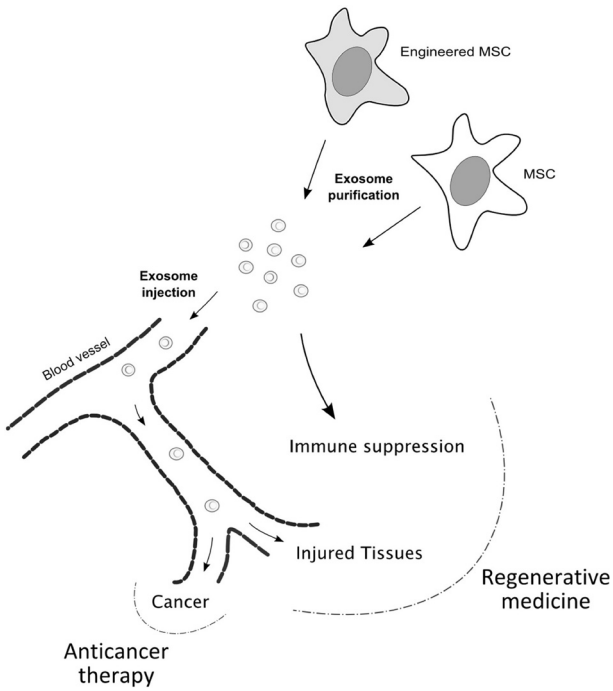

FIGURE 3 | Proposed model for therapeutic applications of MSC-derived exosomes. MSC-derived exosomes may be used instead of MSCs in regenerative medicine and anticancer therapy, since they seem to hold the beneficial properties of the parental cells. MSC-derived exosomes might retain the homing ability displayed by MSCs towards sites of inflammation and function in tissue repair, also by modulating the immune response. Concerning anticancer therapy, exosomes derived from engineered MSCs might be used to mediate anti-proliferative or pro-apoptotic effects. Moreover, whenever the tropism of exosomes would not be retained, MSCs may be modified to confer specific targeting to exosomes.

CHARACTERIZATION OF MSC-RELEASED VESICLES

Despite the interest raised by MSC-derived microvesicles for their potential role in physiological and pathological conditions, and for their possible applications in the treatment of various diseases, only few studies have been conducted on the (specific) RNA and protein content of these vesicles.

The presence of selected miRNAs within MSC-derived microvesicles has been proposed by Collino et al. (2010). In these studies a comparative miRNA profiling was performed with arrays using bone marrow and tissue specific (liver) MSCs and their respective microvesicles. The authors found that some miRNAs were present both in microvesicles and in the cells of origin. However, some miRNAs appeared to have been selectively sorted into the MVs, as these were not detectable in the cells, while, on the contrary, others were present in the cells but not in the MVs. These observations support the existence of a mechanism that controls the sorting of miRNAs in MSC vesicles. Nevertheless, without accurate quantitation by either deep-sequencing techniques and/or quantitative RT-PCR in combination with functional experiments, the biological relevance of these findings remain unclear. The only way to explain that miRNAs are present in exosomes and not in the producing cells is that the mature RNA species are rapidly secreted, having no chance to accumulate within the cytoplasm with the risk of being degraded. Specific miRNAs might be produced by these cells only for the purpose of cell–cell communication, without ever reaching a critical level in the cell of origin to exert a function, being repressing the translation of particular target mRNAs. Although this is possible in theory, no experimental data is available. In contrast, the sorting of specific signaling molecules (proteins) into exosomes does seem to have a clear effect on the producing cells (Chairoungdua et al., 2010; Verweij et al., 2011) and may also be of importance *in vivo* (Al-Nedawi et al., 2009; Peinado et al., 2012). However, it does appear that microRNAs may be specifically transported into cells for specialized functions while the target cells seems to lack these microRNAs (Rader and Parmacek, 2012).

Based on the Gene Ontology analysis, the predicted and validated targets of the miRNAs detected in both MSCs and MSC-MVs are related to development, cell survival and differentiation, while some MSC MV-enriched miRNAs were more associated with the regulation of the immune system. Moreover, microvesicle-derived miRNAs transferred to recipient cells were able to suppress specific targets, thus confirming the functionality of these mediators in cell-to-cell communication (Collino et al., 2010).

Besides the genetic content of MVs, the proteome may be equally important. Only one study has been conducted so far that characterized the protein composition of MSC-derived MVs in more detail. By characterizing the content of bone marrow MSC-derived MVs, Kim et al. identified 730 proteins, among which mediators controlling self-renewal and differentiation. Interestingly, their analysis revealed a number of surface markers

such as PDGFRB, EGFR, and PLAUR, signaling molecules of RAS-MAPK, RHO, and CDC42 pathways, cell adhesion molecules and additional MSC antigens that support a possible role for such vesicles in tissue repair (Kim et al., 2012). Based on these results, it appears that MSC-MVs hold many of the characteristics of the MSCs themselves, and may be important for the function of these adult stem cells *in vivo* besides the classic secreted factors.

MSC-RELEASED VESICLES IN REGENERATIVE MEDICINE AND CANCER

While the predominant role of MSC paracrine activity in tissue repair has already been established, whether MSC-MVs also have a role remains to be studied.

The protective paracrine activity of MSCs in kidney injury fostered several studies into the potential contribution of MSC-derived microvesicles in renal repair. Microvesicles released by MSCs protect against renal injury in the mouse remnant kidney model, support renal repair in ischemia/reperfusion-induced acute kidney injury (AKI), and protect from lethal cisplatin-induced AKI, most likely by inhibiting apoptosis of tubular epithelial cells (Gatti et al., 2011; Bruno et al., 2012; He et al., 2012).

In animal models of intervertebral disc (IVD) degeneration MSCs have been demonstrated able to restore the normal disc structure. Since IVD degeneration seems to depend on alterations of nucleus pulposus (NP) cells, Strassburg et al. (2012) investigated the interactions between MSCs and degenerate NP cells and found that the two cell types primarily communicate via an extensive direct transfer of membrane components and via microvesicles.

The potential use of MSC-MVs for the treatment of cardiovascular diseases has recently been reviewed by Lai et al. (2011). The authors previously demonstrated the therapeutic activity of MVs isolated from embryonic stem cell-derived MSCs (ESC-MSCs) in a mouse model of myocardial ischemia/reperfusion (Lai et al., 2010). They suggest that the secretion of protective exosomes is a general property and perhaps a predominant function of MSCs, probably related to the supporting role of the stromal cells. Considering the limitations and costs related to the use of embryonic stem cells and the high amount of cells required for MV production, the same group also generated MYC-immortalized ESC-MSCs and demonstrated that MVs derived from these cells still display their original cardioprotective activity (Chen et al., 2011).

If the beneficial and protective effects of MSC-MVs in tissue repair have been reported in different pathological conditions, their use for cancer therapy needs careful consideration.

Bone marrow MSC-derived exosomes have been shown to support tumor growth and angiogenesis in a mouse xenograft model of gastric carcinoma, and the pro-angiogenic effect has been ascribed to the increase of VEGF expression in tumor cells (Zhu et al., 2012). This evidence is not completely unexpected since MSCs have been reported to have various tumor promoting functions (Roorda et al., 2009), and highlights once more that it is mandatory to be cautious when evaluating the risks related to the use of engineered MSCs or MSC-derived exosomes in anticancer therapy. Finally, Kyo Won Lee's group demonstrated that both breast and ovarian cancer cells (Cho et al., 2011, 2012) can condition AD-MSCs to generate tumor associated myofibrobasts. It might be interesting to investigate whether, in turn, MSC-derived myofibroblast-like cells, or pre-conditioned MSCs exploit the same mechanism to further support tumor development.

CONCLUDING REMARKS

While the use of MSCs in regenerative medicine and anti-cancer treatment raised high expectations, concerns about safety and tight regulations hampered their practical use in clinical settings. However, the use of MSC-derived secretome and, in particular, of the vesicles released by these cells may have many advantages compared to a cell-based approach.

The increasing interest around this strategy of intercellular crosstalk adopted by MSCs relies on the ability of these vesicles to condition and reprogram the surrounding microenvironment, thereby influencing a variety of biological responses, in particular in injured tissues and cancer.

MVs function primarily in cell–cell communication, which is, as discussed above, highly relevant in the biology of MSCs. The significant contribution of MSC paracrine activity, rather than their ability to differentiate, to the reparative process has already been established. It will now be of importance to decipher the exact role of MSC secretome and vesicles, since it is likely that in parallel to soluble factors as growth factors and cytokines, MVs strongly contribute to the paracrine effects of these cells. Indeed MVs present a complex composition that mirrors that of the parental cells and seems to have similar properties *in vivo*.

Therefore, the use of MSC secretome-derived vesicles represents an interesting alternative for tissue repair that might overcome the limitations and risks associated to cell-therapy approaches. Concerning the potential applications for cancer therapy, exosomes released by opportunely engineered MSCs may still retain the ability to home to tumor site and, at the same time, mediate anti-proliferative or pro-apoptotic effects, relieving the concerns related to the genetic manipulation of stem cells for cell-therapy.

Evidently, crucial questions need to be answered before the objective described above can be satisfactorily fulfilled. First, it is necessary to verify to which extent MSC micro- and nano-vesicles contribute to the beneficial effects mediated by MSCs; second, the content of these vesicles, in terms of proteins and, in particular, small RNAs, needs to be thoroughly characterized; and, finally, it is essential to uncover the effect of the genetic content of MSC-MVs on recipients cells and determine which cellular pathways may be affected.

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Identification of displaced endometrial glands and embryonic duct remnants in female fetal reproductive tract: possible pathogenetic role in endometriotic and pelvic neoplastic processes

Jean Bouquet de Jolinière[1]*, Jean Marc Ayoubi[2], Guy Lesec[3], Pierre Validire[4], Alexandre Goguin[5], Luca Gianaroli[6], Jean Bernard Dubuisson[1], Anis Feki[1] and Jean Gogusev[5]*

[1] *Department of Gynecology, Fribourg Hospital, Fribourg, Switzerland*
[2] *Department of Gynecology, Foch Hospital, Suresnes, France*
[3] *SIPATH-Institut de Cytologie et d'Anatomie Pathologiques, Clermont Ferrand, France*
[4] *Department of Pathology, Institute Mutualiste Montsouris, Paris, France*
[5] *INSERM U567 Hôpital Necker, Paris, France*
[6] *Reproductive Medicine Unit, Società Italiana di Studi di Medicina della Riproduzione, Bologna, Italy*

Edited by:
Thimios Mitsiadis, University of Zurich, Switzerland

Reviewed by:
Gianpaolo Papaccio, Second University of Naples, Italy
Daniel Graf, University of Zurich, Switzerland
Francesco De Francesco, Second University of Naples, Italy

****Correspondence:***
Jean Bouquet de Jolinière, Service de Gynecologie Obstetrique, Hôpital Cantonal HFR Fribourg, Chemin des Pensionnats 2-6, 1708 Fribourg, Switzerland.
e-mail: bouquetdejolinierej@h-fr.ch;
Jean Gogusev, Cochin Institut, INSERM U1016, CNRS 8104, Université Paris Descartes 24, Rue du Fb Saint Jacques, 75014 Paris, France.
e-mail: gogusev@necker.fr

Background: Recent findings strongly promoted the hypothesis that common pelvic gynecological diseases including endometriosis and ovarian neoplasia may develop *de novo* from ectopic endometrial-like glands and/or embryonic epithelial remnants. To verify the frequency, the anatomical localization and the phenotype of misplaced endometrial tissue along the fetal female reproductive tract, histological and immunohistochemical analyses of uteri, fallopian tubes, and uterosacral ligaments were performed. **Methods:** Reproductive organs were collected from seven female fetuses at autopsy, five of them from gestational ages between 18 and 26 weeks and two fetuses with gestational ages of 33 and 36 weeks deceased of placental anomalies. Serial sections from areas containing ectopic glands and embryonic duct residues were analyzed by histological and immunohistochemical procedures. **Results:** Numerous ectopic endometrial glands and stroma were detected in the myometrium in two fetuses with low levels of expression of estrogen receptor-alpha (ER-α) and progesterone receptors (PR). The embryonic ducts were localized in the uterine broad and ovarian ligaments and under the fallopian tube serosa in six fetuses. Low levels of steroid receptors expression were found in the embryonic residues, whereas the carcinoembryonic antigen (CEA) and the tumor marker Ca 125 were not detected. The embryonic residues stromal component strongly expressed the CD 10 and vimentin proteins. **Conclusion:** The anatomical and the immunohistochemical features of the ectopic organoid structures identified in fetal female reproductive tract suggest that endometriotic as well as neoplastic disease in adult women may develop on the basis of misplaced endometrial glands and/or embryonic cell remnants.

Keywords: fetus, endometriosis, neoplastic process, ectopic glands, immunohistochemistry

INTRODUCTION

Endometriosis is a heterogeneous gynecological disease clinically characterized by the presence of different anatomo-clinical subtypes (Giudice, 2010). The most frequently proposed pathogenetic mechanism is tubal regurgitation during menstrual cycle, which however cannot explain all clinical forms of this disease (Sampson, 1927; Bulun, 2009). Indeed, occurrence of endometriosis was described in patients with Rokitansky–Kuster–Hauser syndrome who does not have functioning endometrial tissue (Acien, 1986; Cho et al., 2009) as well as in male patients with endometriosis of the prostate, bladder, and the abdominal wall (Schrodt et al., 1980; Beckman et al., 1985; Martin and Hauck, 1985). In this regard, the theory of transformation of the vestigial tissue of Müllerian or Wolfian origin and the coelomic metaplasia theory can explain the origin of distinct entities of endometriotic lesions as well as development of particular types of ovarian neoplasms (Ridley, 1968; Suginami, 1991; Varma et al., 2004; Mandai et al., 2009; Wei et al., 2011). In the same context, a recent study has proposed the fetal origin of endometriosis, that could develop on the basis of altered migration of primitive endometrial tissue during embryogenesis (Signorile and Baldi, 2010). These authors assessed that the incidence of the dislocated embryonic structures in fetuses is similar to that of endometriosis occurring in the adult female population (Signorile and Baldi, 2010). In the same direction, relationship between endometriosis and malignancies arising in gonadal and extragonadal endometrial implants become supported by several clinical pathologic and molecular investigations (Brinton et al., 1997; Vercellini et al., 2000; Varma et al., 2004; Prowse et al., 2006;

Wei et al., 2011). These studies suggested that histogenetically, endometriosis represents an important site of origin of ovarian and other pelvic malignancies (Vercellini et al., 2000; Mandai et al., 2009; Wei et al., 2011). It was described that such neoplasms are constituted of clear epithelial cells and tend to be detected in earlier stages, their prognosis being more favorable (McMeekin et al., 1995; Wei et al., 2011). In addition, embryonic duct remnants were often microscopically observed adjacent to ovarian tumors as well as close to pelvic endometriotic lesions suggesting a pathogenetic relationship (Rudgers and Scully, 1988; Mai et al., 1998; Dubeau, 2008; Nissenblatt, 2011).

In the present study, we evaluated the incidence and the anatomical location of displaced endometrial tissue in the reproductive tract in seven female fetuses. Serial sectioning of the reproductive organs was realized followed by immunohistochemical analysis of tissue areas containing ectopic glands and embryonic cell rests. It was observed that the anatomical and the phenotypic features of the mislocated tissue components recall some pathological characteristics of both benign and malignant gynecological conditions.

MATERIAL AND METHODS

TISSUE PREPARATION

Reproductive organs from a series of seven human female fetuses at different gestational ages ranging from 18 to 36 weeks were obtained at autopsy. All together, the causes of fetal death were placental pathology in two samples, cardiac malformations in two cases and voluntary abortions in three. The reproductive organs were carefully dissected, fixed in buffered formaldehyde, and included in paraffin.

Between 200 and 400 serial sections with thickness of 5–7 mm from each paraffin block containing uteri, fallopian tubes, ovaries, and uterosacral ligaments were prepared and stained by hematoxylin and eosin (H&E). To ascertain tissue sections containing ectopic endometrial glands and/or embryonic duct remnants, every sixth slide was separately stained and microscopically analyzed.

ANTIBODIES

The following antibodies were employed; rabbit anti estrogen receptor-alpha (ER-α), (cat N° sc-54, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); monoclonal mouse anti human progesterone receptor, clone PR 636 (Dako Laboratories Glostrup, Denmark); monoclonal anti human CA125, clone M11 CA125 (Dako); monoclonal anti human CD10 clone 56C6 (Dako); monoclonal anti human carcino-embryonic antigen (CEA) clone II-7 (Dako); rabbit anti human alpha-1-foetoprotein (Dako); monoclonal anti human epithelial membrane antigen (EMA) clone E29 (Dako); mouse anti human Cytokeratin 7 (clone RCK 105, cat N° sc-23876, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and monoclonal mouse anti-vimentin clone VIM 3B (Dako).

IMMUNOHISTOCHEMISTRY

Five micrometres thick sections were deparaffinized in xylene, rehydrated through graded alcohol series, and washed in PBS. For antigen retrieval, the slides were immerged in citrate buffer (pH = 6.0) during 25 min at 96°C essentially as described (Hsu et al., 1981). After brief wash in PBS, the appropriate dilution of the primary antibodies was applied on slides during 1 h. Immunoreactivity was revealed using the avidin-biotin complex method (LSAB2 System HRP, Dako, Denmark) with 3,3′-diaminobenzidine tetra hydrochloride (DAB) as a chromogen. After checking the staining intensity, the sections were washed in water and counterstained with Harris hematoxylin (Sigma-Aldrich Chimie Sarl, Saint-Quentin Fallavier, France). The slides were then dehydrated in ascending grades of ethanol and after clearing the sections with xylene mount and they were covered with DPX mountant (Merck Chimie SAS, Fontenay sous Bois, France). Negative controls consisted of replacement of the primary antibodies with non-immune mouse or rabbit serum or buffer alone. The extent and the intensity of the staining were determined by the objective observer procedure. Epithelial staining intensity was graded on a 0–3 scale, where 0, no staining was assessed with anti-rabbit secondary antibody alone; 1, weak; 2, moderate; and 3, intense staining. The percentage of immunoreactive cells was obtained by counting the number of stained cells from a total of 200 cells at magnification of ×20, composing the ectopic endometrial glands and stroma or embryonic duct structures and stroma of each case and from each location.

RESULTS

Tissue sections of the reproductive organs from all fetuses stained by hematoxylin an eosin showed normal anatomical morphology and histological structure with asymmetrical uterine endometrial branching invaginations, while inflammatory or fibrotic areas or hemorrhage were not detected. In the fetuses of the lower gestational age, the uterine central cavities appeared lined by columnar epithelial cells and were devoid of differentiated glandular structures. The uterine cavity of the 33 and 36 weeks old fetuses was lined by epithelial cells forming rare immature glandular structures of different sizes. Remarkably, ectopic foci of glandular structures surrounded by a densely distributed stromal cells were found in serial sections of the uterine myometrium in two fetuses, with gestational ages of 25 weeks and of 36 weeks (**Figure 1**). The histological structure of the ectopic glands predominantly showed a single layer of columnar cells similar to the endometrial epithelial lining with basal nuclei and mucin containing cytoplasm. Some of the misplaced glands of larger size showed focal cytological atypia but mitotic figures were not observed. Histological appearance of the ectopic glands distributed in the uterine wall of the 36-week-old fetus is shown in **Figure 2A**. Of note, distinct large foci of epithelial cellular elements forming tubular gland like structures were present in the ovarian hilus in one fetus with gestational age of 23 weeks (not shown).

The second form of ectopic structures observed were the embryonic tubular duct formations located bilaterally in the broad uterine and ovarian ligaments in five tissue samples and under the fallopian tube serosa in one fetus, some of them being surrounded by a dense endometrial-like stroma (**Figure 1**; **Figures 2B–D**). In general, the embryonic structures present in the uterine and ovarian ligaments histologically appeared as discontinuous segments of tortuous ducts surrounded by a rich vascular network. Each duct remnant exhibited a lumen lined by cuboidal cells, surrounded by a layer of mesenchymal stroma like component. Interestingly, in

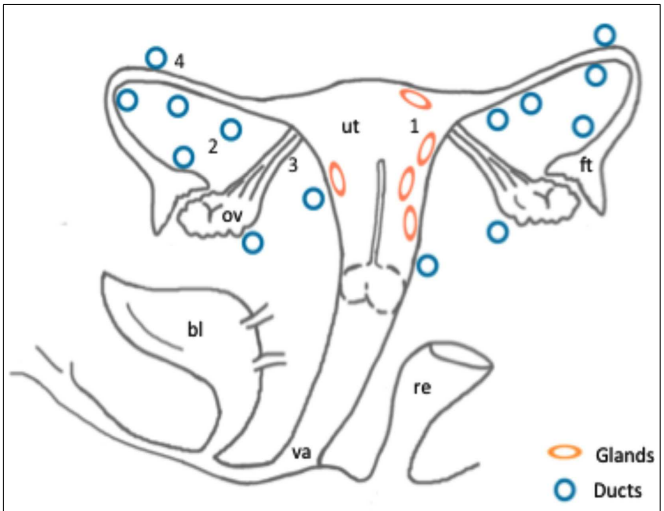

FIGURE 1 | Schematic representation of the anatomical localization of ectopic endometrial glands and embryonic duct remnants along the reproductive tract of six female fetuses. Areas of the distribution of ectopic glands in the myometrium (1) in two fetuses: (ellipses). Distribution of embryonic ducts in the uterine broad ligaments (2), ovarian ligaments (3), and under the fallopian tube serosa (4) in a total of six fetuses: (circles). Abbreviations: ut, uterus; bl, bladder; va, vagina; ov, ovary; ft, fallopian tube; re, rectum.

the 36 weeks old fetus, both embryonic ducts located in the broad ligament and ectopic glands embedded in the myometrium were simultaneously observed.

By immunohistochemistry, various levels of several marker antigens were detected in both the ectopic uterine glands and embryonic ducts. As a rule, a higher level of the EMA, PR, and ER-α molecules were detected in cells lining the uterine cavity with a mean of 64, 53, and 21% of labeled elements respectively. A lower percentage of immunoreactivity for EMA (23%), PR (14%), and ER-α (6%) was revealed in the ectopic glandular structures localized in the uterine wall. A strong expression of CD10 (41.3%) but lower level of vimentin specific immunolabeling (20%) was observed in the stromal component surrounding the displaced glands. Representative illustrations of PR expression in orthotopic endometrial cells as well as the expression of PR, ER-α, and CD10 in the uterine ectopic glands and stroma are shown in **Figures 3A–D**.

Concerning the embryonic remnants, consistent EMA immunostaining ranging between 32 and 53% was detected in the duct lining cells, while lower level of expression in duct cells of both ER-α (10–23%) and PR (13–44%) molecules was found in all samples. The stromal-like component surrounding the duct residues contained between 1 and 3% of immunoreactive cells for ER-α and between 4 and 13% of immunoreactive cells for PR. Approximately 2–11% of the ductal cells and 1–3% of the stormal cells expressed cytokeratin 7, while CEA and CA125

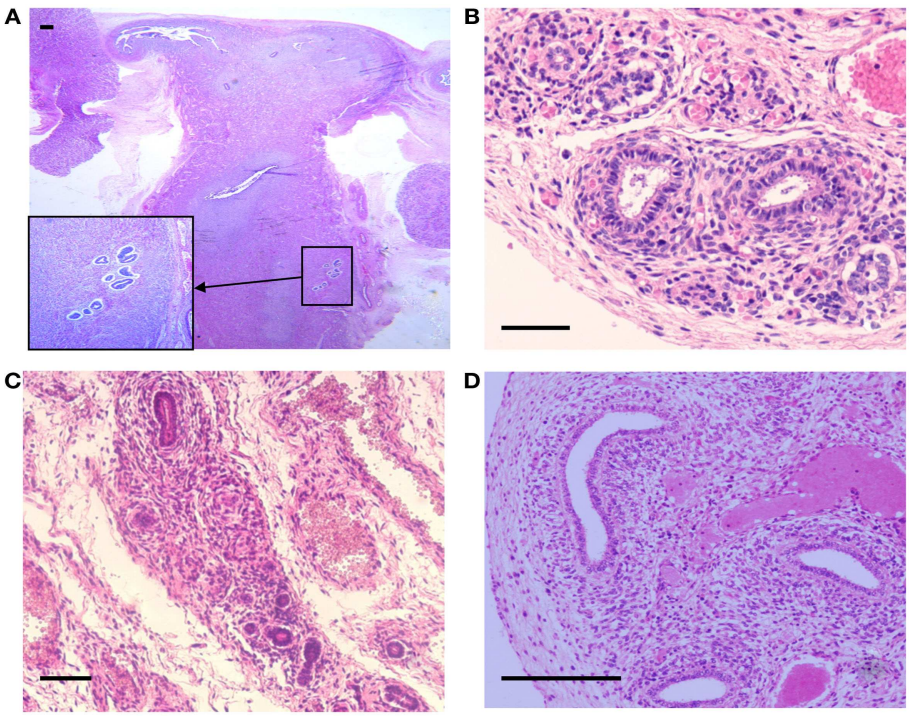

FIGURE 2 | Hematoxylin and Eosin stained sections with areas of ectopic endometrial glands and embryonic ducts. Histological appearance of ectopic glands and stroma observed (insert at higher magnification) in fetal uterine wall **(A)**. Presence of embryonic ducts located in the broad ligament **(B)**, under the fallopian tube serosa **(C)**, and ducts located in the ovarian ligament **(D)**. Note presence of a stromal component surrounding the duct residues in **(A–D)**. Scale bars, 100 μm.

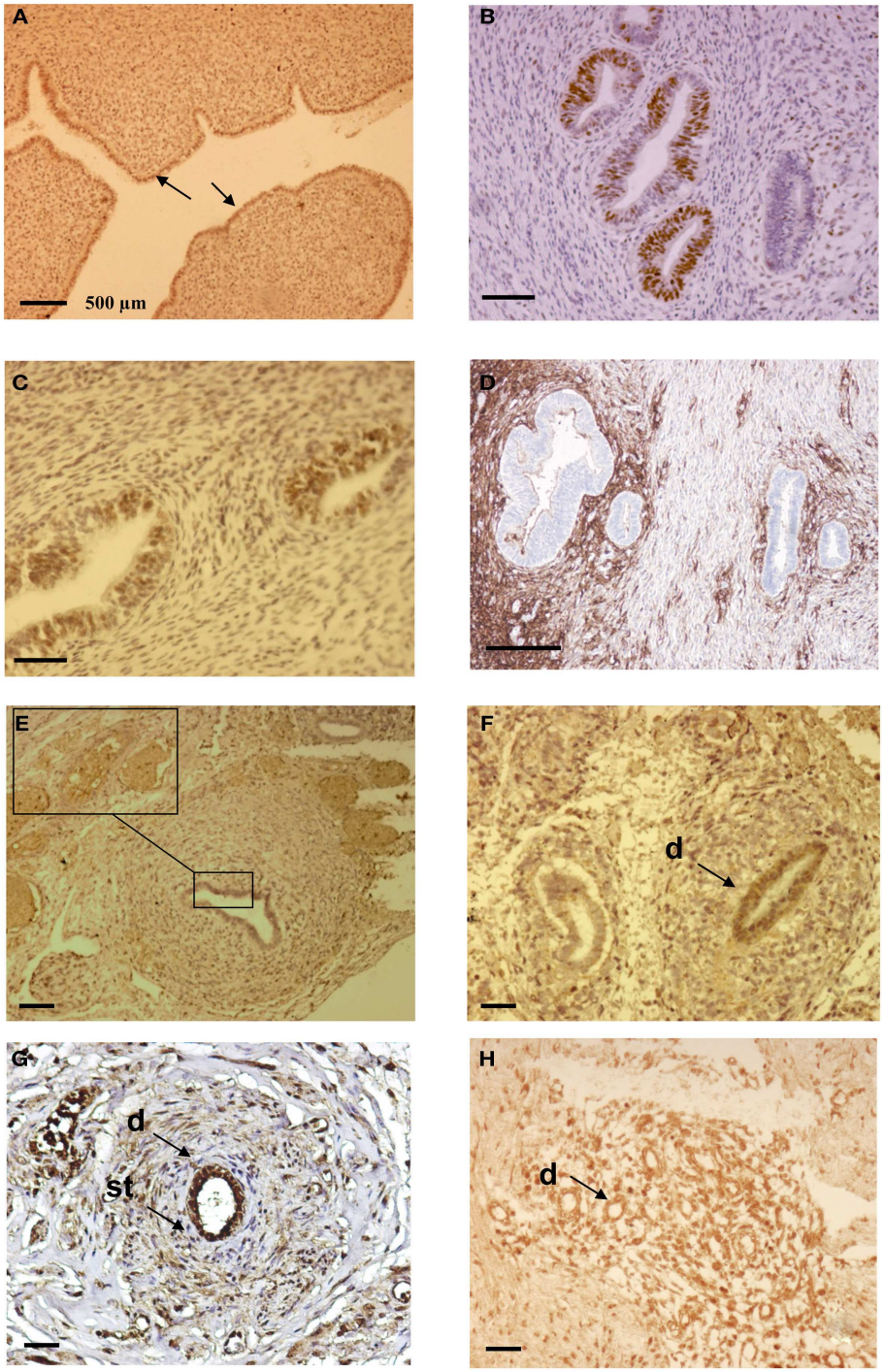

FIGURE 3 | Immunohistochemical analysis of ectopic endometrial glands and embryonic ducts in fetal reproductive tract. Immunolabeling with anti PR antibody of uterine cavity wall lining cells [**(A)**, arrows]. Immunostaining of the fetal ectopic glands located in the uterine myometrium with anti PR **(B)**, anti ER-α **(C)**, and anti CD10 antibodies **(D)**. Expression of the PR in embryonic duct located in the ovarian ligament; insert at higher magnification **(E)**, expression of ER-α in a duct located under the fallopian tube serosa **(F)**, and expression of alpha-1-fetoprotein **(G)** and CD10 molecules **(H)**. D = duct; St = stromal layer. Scale bars; 500 µm in **(A)** and 100 µm in **(B–H)**.

molecules were not detected. Using anti alpha-1-fetoprotein antibody, close to 42% of the epithelial and duct surrounding mesenchymal cells were intensely immunoreactive on sections from two fetuses. Representative illustrations of the embryonic duct residues labeled with anti ER-α, anti PR, anti alpha-1-fetoprotein and anti CD10 antibodies are shown in **Figures 3E–H**. The mean

Table 1 | Summary of the percentages of immunoreactive cells in embryonic ducts and the surrounding stroma present in various locations of the fetal reproductive tract.

Gestational age (weeks)	EMA*		Cytokeratin 7*		ER*		PR*		Vimentin*		CD10*	
	D	St	D	St	D	St	D	St	D	St	D	St
18	32	–	2	–	10	–	19	11	35	4	2	55
19	52	–	–	–	12	–	21	6	26	9	–	38
20	38	–	–	3	18	3	13	8	34	13	–	42
21	43	–	–	–	13	–	19	4	22	20	–	39
22	46	–	–	–	23	–	31	13	25	6	–	44
32	0	0	0	–	0	0	0	0	0	0	0	0
36	53	1	11	1	16	1	44	9	53	32	4	64
Mean value**	264	1	13	4	92	4	147	51	195	84	6	282

*D, embryonic duct; St, Stromal layer; *, percentage of cells expressing the indicated marker; mean value** [represents a ratio of the number of counted cells (200) divided by the number of immunolabeled cells].*

values of the percentage of imunoreactive embryonic duct lining cells and stroma against EMA, Cytokeratin 7, ER-α, PR, vimentin and CD10 antigens are reported in **Table 1**.

DISCUSSION

In this study we show presence of misplaced endometrial glands and embryonic duct-like remnants in the reproductive organs in six of seven examined female fetuses. The phenotypic features of the ectopic glands in the myometrium of two fetuses indicate particularly weak expression of PR and ER-α steroid hormone receptors in comparison to their high level of expression in cells lining the endometrial cavity. In these two cases, moderate level of PR expression was also revealed in the cell nuclei of the stromal component, while ER-α receptors were not found. Overall, the presented findings are in accordance with other studies describing the levels of ER-α and PR in misplaced glands in patients with both endometriosis and adenomyosis (Van der Walt et al., 1986; Ferenczy, 1998; Bulun et al., 2010). In fact, inconsistent results concerning the pattern of steroid hormones receptor expression in fetal female genital tissues were reported by several studies (Glatstein and Yeh, 1995; Brandenberger et al., 1997). For example, in adult patients, decreased expression of both ER-α and PR were reported in seven cases of ovarian endometriomas in comparison to their consistent levels in the autologous endometrial cells (Tamaya et al., 1979). This is in agreement with other observations indicating that adenomyotic nodules located in the vaginal fornix and the rectovaginal septum do not express PR and ER-α suggesting that they may originate from undifferentiated Müllerian residues (Nisolle and Donnez, 1997; Donnez et al., 2003). Another study reported equal levels of expression of ER and PR receptors in both autologous endometrium and the adenomyotic lesions in adult patients (Ferenczy, 1998).

Concerning the foci of embryonic duct remnants observed, moderate expression of PR and low levels of ER-α were uniformly revealed in the epithelial cells in all cases. Histologically, some ducts appeared dysplastic and surrounded by a dense cellular stromal layer consistently expressing vimentin and CD10 molecules. Of interest, these tubular structures in most of the samples did not clearly express cytokeratin 7, and Ca 125 protein molecules. This might be related to particular phenotypic features of coelomic metaplastic cell rests at given gestational age (Batt and Smith, 1989; Fujii, 1991). Comparatively, consistent level of expression of both estrogen receptor and CA 125 antigens was described in displaced organoid structures in fetal rectovaginal septum, the Douglas pouch, the rectal tube, and at the posterior wall of fetal uteri (Signorile et al., 2009).

In spite of the limited number of studied cases, the findings of ectopic glands and/or embryonic ducts in the reproductive tract of female fetuses is a remarkable phenomenon that could be referred to the theory of involvement of Müllerian or Wolfian cell rests in the pathogenesis of both endometriosis and particular pelvic malignancies (Fujii, 1991; Redwine, 1998, 2003; Leiserowitz et al., 2003; Batt et al., 2007; Signorile et al., 2009; Wei et al., 2011). At present it is considered that a multitude of ovarian, adnexal, and pelvic masses originate from the secondary Müllerian system. On the other hand, it is well known that the coelomic epithelial cells and the accompanying mesenchymal component referred as secondary Müllerian system, have the potential to differentiate toward a Müllerian-directed epithelium and stroma (Fujii, 1991; Mai et al., 1997). Thus, it has been hypothesized that aberrant migration of Müllerian ducts could cause spreading of embryonic cells along the migratory pathway during fetal organogenesis with potential to induce lesions including both endometriosis and ovarian neoplasms (Fujii, 1991; Redwine, 1998; Varma et al., 2004; Batt et al., 2007; Mandai et al., 2009; Signorile et al., 2009). Our data are in relation with another study that described presence of endometriotic foci adjacent to the embryonic duct remnants of coelomic origin involving ovaries and fallopian tubes in three among 18 adult patients (Mai et al., 1998). Phenotypically, the epithelial cells of the duct remnants appeared transformed and showed a diffuse but weak immunoreactivity for estrogen receptor (Mai et al., 1998). The findings of embryonic ducts spread in broad ligaments and under the fallopian tube serosa in our series could be related to studies describing occurrence of endometriomas as well as neoplasms of a Müllerian origin in these locations (Zacharia and O'Neill, 2006; Handa et al., 2007; Wei et al., 2011). Finally, the aberrant and consistent expression of alpha-1-fetoprotein revealed

in the embryonic ducts in two fetuses is in accordance with other studies that reported *de novo* expression of this protein in ovarian malignancy arising from endometriosis (Horiuchi et al., 1998; Certin et al., 2007; Takahashi et al., 2011).

In conclusion, the presented data support the theory that at least some subtypes of endometriotic and gynecological neoplastic lesions may be related to anomalies occurring during the embryogenesis. These data stand in relationship with the embryological origin of certain pelvic malignancies based on the metaplastic potentiality of the secondary Müllerian system. The observed frequency of displaced embryonic structures, also suggests a complex pathogenetic mechanism in the development of endometriosis-associated neoplasms including genetic, hormonal, and/or environmental events. Consequently, further studies of endometriotic and neoplastic lesions should include novel embryonic cellular phenotypic markers, that could provide important diagnostic and predictive information to guide clinical decision making.

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Regenerative strategies for craniofacial disorders

Catharine B. Garland*[1,3] *and Jason H. Pomerantz*[1,2,3,4]

[1] *Department of Surgery, Division of Plastic and Reconstructive Surgery, University of California San Francisco, San Francisco, CA, USA*
[2] *Department of Orofacial Sciences, University of California San Francisco, San Francisco, CA, USA*
[3] *Craniofacial and Mesenchymal Biology Program, University of California San Francisco, San Francisco, CA, USA*
[4] *Eli and Edythe Broad Center of Regeneration Medicine and Stem Cell Research, University of California San Francisco, San Francisco, CA, USA*

Edited by:
Anis Feki, Hopitaux Fribourgeois, Switzerland

Reviewed by:
Daniel Graf, University of Zurich, Switzerland
Catherine Chaussain, Université Paris Descartes Paris Cité, France
Riccardo D'Aquino, Teslab S.r.l., Italy

***Correspondence:**
Jason H. Pomerantz, Division of Plastic and Reconstructive Surgery, Department of Surgery, University of California San Francisco, 505 Parnassus Avenue, Room M-593, San Francisco, CA 94143, USA.
e-mail: jason.pomerantz@ucsfmedctr.org

Craniofacial disorders present markedly complicated problems in reconstruction because of the complex interactions of the multiple, simultaneously affected tissues. Regenerative medicine holds promise for new strategies to improve treatment of these disorders. This review addresses current areas of unmet need in craniofacial reconstruction and emphasizes how craniofacial tissues differ from their analogs elsewhere in the body. We present a problem-based approach to illustrate current treatment strategies for various craniofacial disorders, to highlight areas of need, and to suggest regenerative strategies for craniofacial bone, fat, muscle, nerve, and skin. For some tissues, current approaches offer excellent reconstructive solutions using autologous tissue or prosthetic materials. Thus, new "regenerative" approaches would need to offer major advantages in order to be adopted. In other tissues, the unmet need is great, and we suggest the greatest regenerative need is for muscle, skin, and nerve. The advent of composite facial tissue transplantation and the development of regenerative medicine are each likely to add important new paradigms to our treatment of craniofacial disorders.

Keywords: regeneration, craniofacial, stem cell, satellite cell, fat transfer, facial nerve

INTRODUCTION

As the field of regeneration biology progresses, new strategies will develop for treating craniofacial disorders. Craniofacial disorders are unique in that they affect multiple tissues simultaneously and occur across the full spectrum of patient age and development. Treatments for craniofacial disorders have advanced remarkably over the previous century. However, as with all reconstructive approaches, these treatments remain imperfect. The hurdles remaining are generally related to improving our ability to faithfully "replace like with like" and to minimize and eliminate treatment-associated morbidity. The nascent field of regenerative medicine offers promise to achieve some of these goals.

Regeneration is a specific process, different from "healing." For the purpose of this review, "regeneration" refers to the replacement of human cells or tissues by like cells, reestablishing the original form and function (Mason and Dunnill, 2008). The term regeneration refers to different mechanisms in different tissues. Regeneration may involve the proliferation and differentiation of stem cells within tissue (Greenow and Clarke, 2012; King and Newmark, 2012). For example, this type of regeneration occurs in skin after partial thickness burn injury. Regeneration can also refer to distinct processes, such as axonal regeneration in peripheral nerves, that does not involve direct cell division and proliferation, but are reliant upon supporting cells for regeneration to occur (Zochodne, 2012). In contrast to regeneration, the healing of injuries in humans replaces injured tissue with a collagen-dense scar. In healing, the resultant tissue differs from the native tissue in gross and histologic appearance, strength and stiffness, and function (e.g., scarred muscle has diminished contractility, and scarred skin has diminished sensation and sweating).

A number of evolutionary hypotheses exist as to why adult mammals heal by scar formation after injury, while certain other vertebrates such as salamanders have a remarkable ability to regenerate solid tissues, including complete limbs, without scar. In mammals, including humans, significant regenerative capacity exists primarily during fetal and perinatal development. One hypothesis is that healing by scar formation conferred an evolutionary survival advantage in mammals. The rapid deposition of fibrotic tissue to seal a wound prevents infection and protects vital structures, but also may actively prevent regeneration (Brockes and Kumar, 2008; Gurtner et al., 2008). In support of this, inhibiting the fibrotic response after spinal cord injury in mice permits axonal regeneration (Stichel et al., 1999; Klapka and Muller, 2006). Another proposed teleological hypothesis is that mammals have evolved more stringent negative regulation of cellular growth control as part of advanced tumor suppressor mechanisms. This could confer a survival advantage at the expense of regenerative capacity (Blau and Pomerantz, 2011). A logical extension of such observations is that regenerative capacity may have been lost in higher organisms in favor of tissue stability, avoidance of infection, cancer prevention, and longevity. However, an alternate hypothesis is that regeneration was not lost as mammals evolved, but rather that regeneration separately evolved in certain species. Some evidence supporting this theory is the discovery of specific proteins involved in regeneration that are unique to salamanders (Garza-Garcia et al., 2010). It is possible that each of these hypotheses is partly true. A better understanding of the regenerative mechanisms of both lower vertebrates and developmentally immature mammals may inform our approaches to mammalian regeneration.

Regenerative medicine has emerged as "the process of creating living, functional tissues to repair or replace tissue or organ function lost due to age, disease, damage, or congenital defects" (http://report.nih.gov/NIHfactsheets/ViewFactSheet.aspx?csid=62). Plastic and reconstructive surgery, and the craniofacial subspecialty, is an old field of medicine with an almost identical focus: repairing or reconstructing defects of form and function in diverse tissues and patients (American Board of Plastic Surgery, 2012). The purpose of this review is to discuss where novel approaches to treat craniofacial conditions are most needed. This review will examine how regenerative strategies may improve upon current reconstructive practices. We consider the spectrum of craniofacial disorders and how craniofacial tissues differ from their analogs elsewhere in the body in function and embryologic origin. We then present a problem-based approach to illustrate current strategies for treatment, as well as what we consider the most critical regenerative goals for craniofacial bone, fat, muscle, nerve, and skin. Regenerative strategies for teeth, cartilage, salivary glands, and sensory organs contributing to sight, hearing, smell, and taste, all of importance to craniofacial medicine, are beyond the scope of this review and are addressed in other excellent reviews (For teeth, see: Mao et al., 2006; Huang et al., 2009; Yildirim et al., 2011; Machado et al., 2012; for auricular cartilage, see: Bichara et al., 2012; for salivary glands, see: Kagami et al., 2008; for retina, see: Lamba et al., 2008; Singh and MacLaren, 2011; for inner ear, see: De Felipe et al., 2011; Okano and Kelley, 2012; for olfactory, see: Goldstein and Lane, 2004; Costanzo and Yagi, 2011; for taste, see: Miura and Barlow, 2010). In some cases, our current treatments and innate healing responses provide adequate solutions. In other craniofacial disorders, regenerative medicine may lead to improved tissue appearance and function, and decreased morbidity.

THE COMPLEX FUNCTIONS OF CRANIOFACIAL TISSUES IN HEALTH AND DISEASE

The face has a remarkably complex function in humans. The tissues of the face receive and transmit tremendous amounts of information each day. The cranial nerves receive information from all five senses. Muscles of the face respond to stimuli with complex expressions, and are responsible for rapid movements of the eyes and forceful movements of the jaw in mastication. In addition, bones of the skull protect the brain and orbits. Facial appearance is a fundamental component of individuality. Craniofacial disorders lead to abnormalities in a wide range of patients and tissues that disrupt these functions (**Table 1**). These problems can be physically, emotionally, and socially disabling. Given the complex nature of craniofacial function and disease, recreating these tissues is a daunting task. Even the most sophisticated of our current approaches do not fully reproduce the fine complex function and form that is the hallmark of craniofacial anatomy and physiology. Newer regenerative approaches may offer paradigm changes toward this goal. In developing regenerative strategies, the tissues must be considered individually, as well as in combination with each other. Some endogenous tissue repair mechanisms may provide solutions for regenerating tissues. In other cases, true regeneration may not be necessary to achieve an excellent outcome.

THE UNIQUE EMBRYOLOGIC ORIGINS OF CRANIOFACIAL TISSUES AND THE ROLE OF NEURAL CREST CELLS

One reason that craniofacial disorders manifest differently from disorders in the trunk and extremities may relate to the distinct embryologic origins of the craniofacial tissues. They subsequently have distinct gene expression patterns and physiology. Understanding these differences may be important for inducing regeneration of craniofacial tissues. Studies of regeneration across phyla suggest that reactivation of developmental signaling pathways is a common theme (reviewed in Sanchez Alvarado and Tsonis, 2006). Therefore, regeneration might be expected to recapitulate the complex interactions of the ectoderm, mesoderm, and endoderm that form the pharyngeal arches, as well as the generation of critical structures by cranial neural crest cells (**Figure 1**).

Cranial neural crest cells have unique features and play a critical role in the development of the face and head (Le Lièvre and Le Douarin, 1975; Gitton et al., 2010; Grevellec and Tucker, 2010; Cordero et al., 2011; Le Douarin and Dupin, 2012). The dentin-secreting odontoblasts of teeth are exclusively derived from cranial neural crest cells (Lumsden, 1988). While other populations of neural crest cells also contribute to neurons, ganglia, and pigment cells, only cranial neural crest cells are able to form cartilage and bone (Couly et al., 2002; Le Douarin et al., 2007; reviewed in Hall and Gillis, 2012). Most bones of the body are derived from the mesoderm and ossify by endochondral ossification. In contrast, the bones of the face and much of the cranial vault originate from neural crest cells and undergo intramembranous ossification during development (Couly et al., 1993; Jiang et al., 2002; Levi et al., 2012). Cranial neural crest cells are also the primary contributor to fibro adipogenic progenitor cells in the face, whereas fibro adipogenic progenitor cells are of mesodermal origin in the trunk (Lemos et al., 2012). Fibro adipogenic progenitor cells give rise to adipocytes, contribute to fibrofatty infiltration in tissues, and some reports suggest that they may function in concert with muscle precursor cells to facilitate muscle differentiation after injury (Joe et al., 2010).

Since neural crest cells exert a major influence on craniofacial development, are they also mediators of healing potential and disease? If this were the case, one implication would be that engineered or transplanted tissue replacements would either need to be derived from neural crest sources, or be able to derive the phenotypes and perform the functions of cranial neural crest derivatives. The following examples illustrate these considerations. In the skull, it is possible that the unique origin and ossification of craniofacial bones from neural crest cells may be optimized for the massive skull growth occurring in infancy (Jiang et al., 2002). It has also been suggested that the origin of different skull bones influences their healing potential. For example, frontal bone derived from neural crest cells regenerated to fill a defect more rapidly than parietal bone derived from paraxial mesoderm in both juvenile and adult mice (Quarto et al., 2010). In contrast to the differences seen in bone, fibro adipogenic progenitor cells from both the face and trunk appear to exhibit a similar phenotype, differentiation potential, and response to muscle damage despite differences in gene expression (Lemos et al., 2012). Finally, the preference for certain diseases to uniquely affect the face may be attributed to defects

Table 1 | Examples of craniofacial disorders and corresponding unmet "regenerative" needs.

Disease	Tissue defects	Current strategies	Regenerative need
CONGENITAL			
Craniosynostosis	Early bony suture fusion, aberrant skull growth if untreated	Successful bone regeneration after surgery if treated before age one	Promoting complete regeneration of the skull after surgery in all cases
Cleft lip/palate	Deficiency of palatal fusion including bone, muscle, and mucosa	Staged surgical repairs	Mucosa, without scarring that limits bone growth and causes maxillary deficiency
	Secondary deformities from inadequate growth after surgical intervention	Alveolar bone grafting	Elimination of bone graft donor site morbidity
Craniofacial microsomia	Deficient bone and soft tissue development of the face	Distraction osteogenesis Fat grafting Free tissue transfer	Multiple structures are hypoplastic: bone, muscle, skin, cartilage, nerve Achieving normal appearance
Microtia	Deficient and abnormal ear cartilage formation	Reconstruction with rib graft or alloplastic material	A functional reproduction of a normal ear without requiring a rib graft, and with less scarring
Moebius	Bilateral facial paralysis due to underdevelopment of cranial nerves	Free tissue transfer	Cranial nerve generation, or regeneration Development of target muscles
TRAUMATIC			
Burn	Need for full skin coverage	Split-thickness skin grafting	Regenerated complete skin organ (epidermis, dermis, and appendages)
	Secondary deformities associated with scar contracture and loss of cartilaginous support	Fat and skin grafting to contractures	Supple, well-vascularized skin replacement with underlying cartilage framework
Fractures	Bone gaps occasionally present due to trauma, malunion, or non-union	Fixation Bone grafts	Regeneration of large defects
Soft tissue atrophy or tissue loss due to injury	May affect fat, muscle, skin, cartilages, mucosa, or nerves	Fat grafting Free tissue transfer Skin grafting Face transplantation	"Composite tissue" regeneration to replace subtle and complex form and function
ONCOLOGIC			
Oropharyngeal or other facial cancers	Bone, soft tissue, muscle, and nerve may be radically resected	Free tissue transfer	"Composite tissue" regeneration to replace subtle and complex form and function
Radiation	Negatively affects skin and soft tissue elasticity and healing; causes osteoradionecrosis	Fat grafting Bone grafting	Skin regeneration Bone regeneration
IDIOPATHIC			
Bell's palsy	Facial nerve paralysis Secondary muscle denervation and atrophy	Micro-neurovascular free muscle transfer	Nerve and muscle regeneration to achieve complex function of multiple muscles
Parry-Romberg/ progressive hemifacial atrophy	Progressive loss of soft tissue, nerve, muscle	Fat grafting	Fat regeneration Nerve and muscle regeneration
AGING			
	Fat atrophy Loss of skin elasticity Changes in skin pigmentation	Fat grafting Skin resurfacing	Rejuvenation of skin quality Rejuvenation of fat quantity and location

in cranial neural crest cell number or function. Neural crest cells have been directly associated with several craniofacial malformations. Treacher Collins syndrome (OMIM 154500), characterized by facial bone hypoplasia, ear deformities, and colobomas of the eyelids, is caused by mutations in *TCOF1* that results in a decrease in the number of neural crest cells (Trainor, 2010). In CHARGE syndrome (OMIM 214800), mutations in *CHD7* are implicated in affecting neural crest cell migration. Dysfunction of neural crest cell migration is also implicated in Waardenburg syndrome, type 2D (OMIM 608890) and Mowat–Wilson syndrome (OMIM

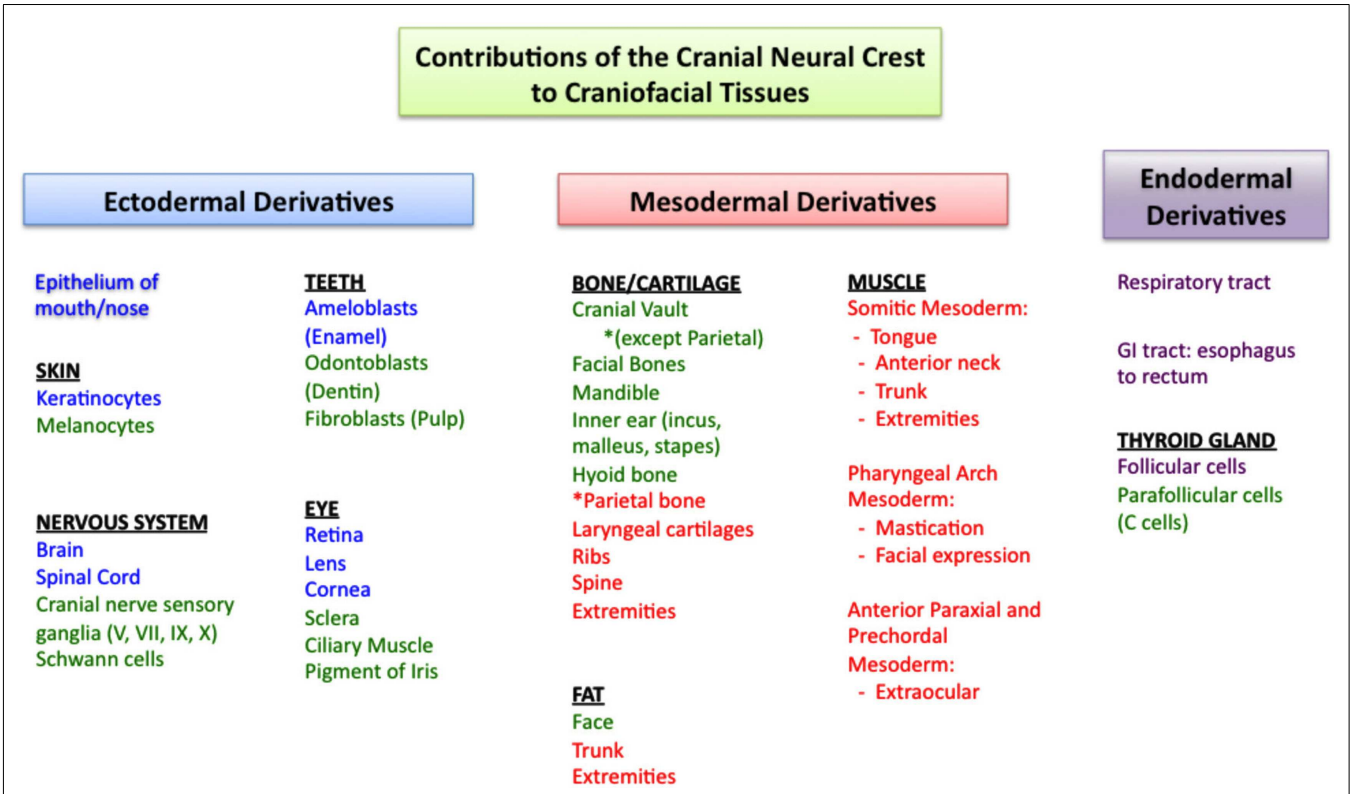

FIGURE 1 | Cranial neural crest cells have unique contributions to tissues of the face and head. Ectodermal derivatives are in blue, mesodermal derivatives in red, and endodermal derivatives in purple. In green are the components of these tissues that develop primarily from cranial neural crest cells. In the face and head, cranial neural crest cells contribute to bone, cartilage, and fat, while this is not the case in the trunk and extremities. While all of the craniofacial muscles arise from the mesoderm (red), note that different muscle groups develop from different regions of the mesoderm. References to the developmental origins of the structures in this figure are located throughout the manuscript.

235730; reviewed in Cordero et al., 2011). Neural crest cells may also be involved in fat dystrophies that uniquely affect the face, such as congenital infiltrating lipomatosis (Chen et al., 2002). Other lipodystrophies such as Dunnigan–Kobberling syndrome (OMIM 151660) affect the trunk and extremities, but spare the face. These conditions highlight that a somatic mutation results in distinct phenotypes among craniofacial and body tissues and reinforces the notion that faithful generation or engineering of craniofacial structures may require unique building blocks.

DIFFERENT SETS OF CRANIOFACIAL MUSCLES ARISE FROM DIFFERENT REGIONS OF MESODERM

Like other muscles, craniofacial muscles are also derived from mesoderm, however, groups of craniofacial muscles arise from distinct regions of mesoderm (reviewed in Noden and Francis-West, 2006; **Figure 1**). Somitic mesoderm forms much of the muscle of the trunk and extremities, but in the face, only the muscles of the tongue and anterior neck are derived from the somites. In contrast, the muscles of mastication and facial expression arise from pharyngeal arch mesoderm, where they develop in close association with the neural crest-derived bones and tendons (Grenier et al., 2009). Finally, extraocular muscles arise from anterior paraxial and prechordal mesoderm (Noden and Francis-West, 2006; Sambasivan et al., 2009). The differences in embryologic origin of face muscles and body muscles are accompanied by differences in the signaling molecules that trigger muscle differentiation in these locations (Sambasivan et al., 2009; reviewed in Kelly, 2010).

Similarly, satellite cells, the tissue-resident muscle stem cells, have different gene expression patterns and characteristics in the face compared with the body. For example, in the trunk, satellite cells express Pax7 and Pax3 (Relaix et al., 2005). However, only Pax7 is expressed in the muscles of the face (Harel et al., 2009; Otto et al., 2009; Kelly, 2010). Satellite cell frequency in muscle fibers also differs. Extraocular, laryngeal, and masseteric muscles have a greater frequency of satellite cells than other skeletal muscles (McLoon et al., 2007). Furthermore, uninjured extraocular and laryngeal muscles contain significant populations of activated satellite cells under normal conditions. These muscles have a high level of basal regenerative activity, and are resistant to the myotoxicity of local anesthetics (Kalhovde et al., 2005; McLoon et al., 2007). Determining whether these differences in satellite cells are intrinsic and how they contribute to regenerative potential is unclear, however. In one comparison of satellite cells between the masseter and limb, there was no difference with regards to myogenic potential *in vitro* (Grefte et al., 2012). Another study showed that masseteric satellite cells differentiated more slowly, but contributed to limb muscle regeneration *in vivo* (Ono et al., 2010). Limb satellite cells have not been studied

in models of facial muscle injury and so the prospects for using limb muscle stem cells to regenerate facial muscles are not yet defined.

Assessment of regenerative potential from satellite cells must include analysis of both the satellite cell proliferative response and the regenerated muscle fiber type and function. Skeletal muscles and craniofacial muscles differ in the myosin isoforms that they express. The muscle fibers of the face express embryonic and neonatal myosin in addition to adult myosin isoforms. Occasionally, facial muscles express multiple myosin isoforms within a single muscle fiber, which has not been observed in other muscles (Stal, 1994; Porter, 2002). Distinct myosin isoforms and a greater number of mitochondria in craniofacial muscle cells may contribute to the resistance to fatigue that craniofacial muscles exhibit. Assuming these unique characteristics of craniofacial muscles are important to their structure or function, recreating these nuances using body muscle stem cells may not be straightforward.

Finally, craniofacial muscles exhibit different susceptibility to pathological conditions. In diseases such as amyotrophic lateral sclerosis (OMIM 105400), the extraocular muscles are not affected. Other craniofacial muscles such as the masseter are affected less severely than body skeletal muscles (Valdez et al., 2012). In contrast, diseases such as myasthenia gravis (OMIM 254200), oculopharyngeal muscular dystrophy (OMIM 164300), and chronic progressive external ophthalmoplegia (OMIM 157640) preferentially affect the extraocular and facial muscles (Benveniste et al., 2005; Greaves et al., 2010).

With regards to regenerative strategies for muscles, the importance of the differences between craniofacial and body muscles in developmental origins, satellite cells, and contractile elements is unclear. The phenotypic differences between extraocular, masseteric, and limb skeletal muscle may be important for regenerating muscle for craniofacial diseases. It is unknown whether satellite cells from the same muscle subset are required to achieve the same phenotype, or whether transplanted satellite cells will adopt the phenotype of their new environment. The answers to these questions could have critical implications for the treatment of muscle-group specific dystrophies. For example, if satellite cells retain adequate intrinsic plasticity, one potential regenerative strategy would be to use autologous transplantation of cells from unaffected or less affected muscle groups to more severely affected muscles. Similarly, satellite cells could be harvested from expendable muscles of the body to regenerate craniofacial muscle defects, with the goal of achieving function in addition to form.

Innervation to the different groups of facial muscles is by the cranial nerves, which have a highly conserved organization among vertebrates. Unlike spinal motor neurons, stemming from columns along the spine, cranial motor neurons extend from discrete nuclei in the midbrain and hindbrain (reviewed in Gilland and Baker, 2005; Guthrie, 2007). Each cranial motor nerve innervates a large number of distinct muscles, many of which can be controlled individually. Some cranial nerves are strictly efferent motor neurons, including cranial nerves III, IV, and VI to the extraocular muscles and cranial nerve XII controlling tongue movement. Other cranial nerves are mixed with motor and sensory components. These "branchiomeric" nerves have sensory ganglia that are formed by contributions from neural crest cells (**Figure 1**), and motor components that extend to striated muscles as well as to parasympathetic ganglia (cranial nerves III, VII, IX, and X), and the mechanosensory hair cells of the inner ear (cranial nerve VIII; reviewed in Guthrie, 2007). Also unique in craniofacial nervous system development is the development of the sensory organs from the cranial placodes (reviewed in Streit, 2004; Schlosser, 2006). Despite the unique organization and development of the cranial nerves, however, they appear to be functionally similar to other peripheral nerves of the body and there is no known difference in their regenerative capacity.

In the following sections, we present clinical vignettes to illustrate typical craniofacial disorders and how regenerative approaches may be applied in order to treat the conditions.

TREATMENT OPTIONS FOR CRANIOFACIAL BONE RECONSTRUCTION DEPEND ON THE CHARACTERISTICS OF THE DEFECT AND THE PATIENT'S AGE

CLINICAL VIGNETTES

A boy presented with a large post-operative cranial defect after treatment for coronal suture craniosynostosis (**Figure 2**). Although defects of this size usually are replaced by regenerated bone in infants, the chance of regenerating this type of defect is low in children older than 2 years. This patient was 3-years-old, and he therefore required reconstruction with prosthetic material.

Another infant with multiple suture synostosis had elevated intracranial pressure due to premature closure of the cranial

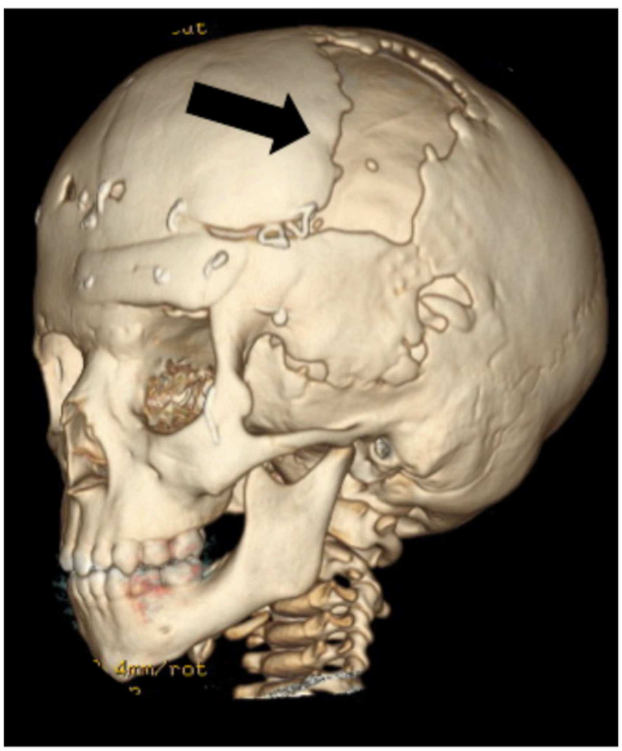

FIGURE 2 | A computed tomography scan demonstrates a large defect (arrow) in the left frontoparietal skull of a 3-year-old boy. This required reconstruction with alloplastic materials or large bone grafts.

sutures (**Figure 3A**). To increase the size of the posterior cranial vault and decrease the intracranial pressure, the child was treated with distraction osteogenesis. After creating osteotomies and placing a distraction device, the occipital bone was gradually advanced posteriorly and new bone gradually regenerated to fill the defect. Regeneration is extensive, but calcification incomplete, after 4 months (**Figure 3B**).

Craniofacial bones are responsible for bearing the forces associated with mastication, supporting the structures of the face, and protecting the brain and orbits. In adult mammals, bony defects of a critical-size will not regenerate normally and typically require reconstruction. Critical-size bony defects [8 mm in rats (Takagi and Urist, 1982), 15 mm in rabbits (Dodde et al., 2000), and 30 mm in sheep (Reichert et al., 2009)] will not regenerate over the lifetime of the adult animal. Reconstruction of such skull defects typically requires alloplastic materials or bone grafting.

In contrast to adults, infants successfully regenerate bone in large cranial defects. One common example in which this occurs is after surgical treatment for craniosynostosis. In this operation, large bony gaps are created to expand the skull and permit brain growth. Remarkably, when cranial vault expansion is performed before one year of age, normal cranial bone is regenerated to fill the large iatrogenic defects. In the clinical scenario described in **Figure 2**, the patient required alloplastic reconstruction given his relatively advanced age of 3 years. The mechanisms behind age-related differences in skull regenerative capacity remain poorly understood. Cranial bone regeneration is thought to occur by osteoinduction from the underlying dura (Hobar et al., 1993). Therefore, identifying how dural signaling changes after infancy would presumably shed light on how the regenerative capacity of cranial bone changes with age. Currently available treatments to replace bone meet the functional requirements of cranial bone. However, regenerative strategies could theoretically improve outcomes in certain scenarios by preventing the need to use prosthetic materials or obviating complications such as failed bone grafts, infection, and donor site morbidity. One theoretically attractive avenue would be to restore the mechanisms that allow full regeneration of cranial bone in infants and apply these principles to older patients (Wan et al., 2008).

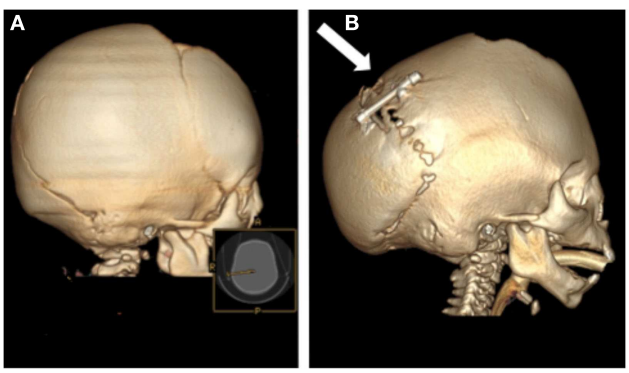

FIGURE 3 | Computed tomography scans of an infant with multiple suture synostosis preoperatively (A) and 4 months after distraction of the posterior cranial vault (B). The distraction footplates have been gradually separated by a distance of 25 mm and evidence of calcified bony regenerate is present between the footplates (arrow).

A highly effective treatment for craniofacial bony defects and deficiencies is distraction osteogenesis, which induces bone generation (reviewed in McCarthy et al., 2001). In this procedure, an osteotomy is made in the area of desired bone generation, and early fracture healing leads to callous formation. The fibrovascular matrix that comprises the callus is then lengthened by gradual mechanical strain in the desired direction of growth. Osteoblasts secrete osteoid to fill the bony gap, and after complete mineralization and bony remodeling, the histology of this new bone resembles that of normal bone (Alman et al., 2011). Strength of the bone after distraction osteogenesis, however, was approximately forty percent less than normal bone when studied in the mandible (Schwarz et al., 2010). This technique, first developed in long bones, has been effectively used to lengthen the mandible in cases of mandibular hypoplasia (Ow and Cheung, 2008), or expand the cranial vault in cases of complex craniosynostosis (**Figure 3**; Taylor et al., 2012). While distraction osteogenesis was initially developed for linear vectors of growth, strategies are being developed to apply distraction to the complex shapes of the facial skeleton, via use of multi-directional distraction devices (Schendel, 2011).

Distraction and bone grafting offer very effective treatments for craniofacial bony deficiencies. In fact, one may argue whether additional "regenerative" approaches are warranted. The strongest argument in favor of developing new approaches relates to the morbidity of the current solutions, which can be considerable with distraction and procedures that involve harvesting of bone grafts. Another important issue of relevance is the need for approaches that produce bone that will grow with the patient. Such advances would eliminate the need to delay or repeat treatments.

Additional strategies for regenerating bone include the use of growth factors or stem cells. Bone morphogenetic proteins (BMPs) have enhanced effective osteogenesis and improved healing potential in critical-size calvarial defects (Sato and Urist, 1985; Lindholm et al., 1988). BMPs were approved for use in the US in 2004, with approved indications including tibial fractures, sinus augmentations, alveolar ridge augmentations, and lumbar spinal fusions. The complication rate associated with the use of BMPs has recently called into question the use of BMP, however (Williams et al., 2011). This highlights the complexity associated with "targeted molecular" approaches to induce bone formation. Other growth factors such as transforming growth factor-β (TGF-β), and platelet-derived growth factor (PDGF) may also contribute to improved regeneration potential in the appropriate environment (Schilephake, 2002). Given the unique embryologic origins of cranial bone, it is reasonable to assume that the response of cranial osteoblasts to particular growth factors could differ from the response of long bone osteoblasts. Furthermore, the importance of mechanical forces in bone healing may play a large role in the healing potential of bone given the unique processes by which cranial and axial skeletal bone form (i.e., endochondral vs. intramembranous ossification). These unique characteristics of cranial bone compared with the axial skeleton must be taken into account as sophisticated

methods of inducing bone regeneration are investigated and developed.

With regards to cell-based approaches, both bone marrow-derived mesenchymal stem cells and adipose-derived mesenchymal stem cells have been demonstrated to form bone *in vitro* (Jaiswal et al., 1997; Zuk et al., 2001; Dragoo et al., 2003; Hicok et al., 2004) and *in vivo* when delivered in conjunction with scaffolds. Some studies demonstrated that the regenerated bone was histologically comparable to surrounding bone (Cowan et al., 2004; reviewed in Zuk, 2008). A variety of precursor cells can be differentiated into osteogenic cells *in vitro* (reviewed in Mao et al., 2006; Seong et al., 2010). Current research also attempts to further understand how different combinations of scaffolds, cells, and growth factors may improve bony regeneration of craniofacial structures. Despite the large body of basic science evidence supporting some of these strategies, few have made their way into common clinical practice. Clinical trials are underway investigating the use of particular bone marrow fractions to induce or enhance alveolar bone regeneration with subsequent dental implants (Kaigler et al., 2012; ClinicalTrials.gov ID NCT01616953). However, because many successful clinical tools are already available, morbidity is acceptable, and outcomes are generally good, these new approaches would need to present much improved function, safety, cost, and decreased morbidity in order to be widely adopted.

STABLE RESTORATION OF FACIAL CONTOUR BY TRANSPLANTATION OF ADIPOSE TISSUE

CLINICAL VIGNETTE

A man with HIV lipodystrophy presented with severe hollowing in the cheeks due to atrophy of the malar fat pads (**Figure 4A**). Another man with a remote history of trauma to the right side of his face developed progressive soft tissue atrophy leading to severe facial asymmetry (**Figure 4C**). Both men underwent several sessions of autologous fat grafting to restore more normal volume and contour to their faces (**Figures 4B,D**).

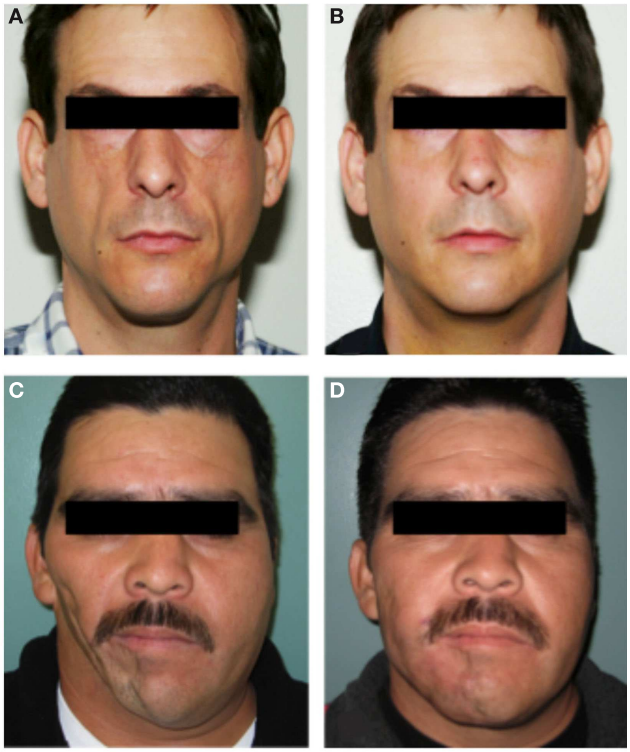

FIGURE 4 | Autologous fat transfer to treat facial soft tissue deficiency. A man with severe HIV lipodystrophy [**(A)**, preoperative photo] underwent serial fat grafting to both malar regions [**(B)**, post-operative photo]. This restored normal facial contour and a more youthful appearance. **(C)** Preoperative photo of a patient with post-traumatic soft tissue atrophy on the right side of his face had long lasting improvements in facial symmetry after several sessions of fat grafting from the abdomen to the right cheek and jaw region [**(D)**, post-operative photo). In both cases lipoaspirate was processed by brief centrifugation and passage through a syringe. The cells within the lipoaspirate were not altered or enriched for specific cell types. Multiple injections of very small quantities of fat were used in each treatment.

Fat grafting for both reconstruction and rejuvenation of the aging face has increased in popularity in the last 10 years, but fat grafting has been used in various forms for many decades. Autologous fat grafting has a number of theoretical and observed advantages that are rapidly making it the preferred approach for facial augmentation. The use of autologous living tissue has the following benefits: incorporation of a living graft into the surrounding tissues, minimal chance of infection, and a natural appearance and feel that is distinctly better than implants and most fillers. Remarkably, grafted fat not only creates volume, but its integration as a living tissue can result in beneficial interactions with surrounding tissues. For example, grafting of fat into an area of contracted, and/or irradiated, skin results in softening, improvements in elasticity, and increased health of the overlying and surrounding skin (Klinger et al., 2008; Mojallal et al., 2009; Phulpin et al., 2009). The mechanism by which grafted fat improves the quality of adjacent skin is unknown, but may involve improved vascularization or secreted paracrine factor effects. In contrast, prosthetic materials or fillers can have undesirable interactions with the surrounding tissue. At best, these materials are relatively inert. However, as a foreign material, fillers are susceptible to causing inflammatory reactions, allergies, or infection (reviewed in Hirsch and Stier, 2008).

Another challenge with soft tissue augmentation relates to the duration of the augmentation effect. Implants can be permanent in the absence of complications. However, implants may also require repositioning or replacement over time and are susceptible to capsular contracture. Synthetic or natural fillers are temporary and typically last only several months. These fillers require repeated treatments and considerable associated financial cost. Long-term studies have now shown that autologous fat grafting can last decades or longer (reviewed in Coleman, 2006a,b), offering another major benefit over the impermanence of synthetic or natural fillers. At present, the most important issue facing the wide adoption of fat grafting, however, is the wide variability in techniques and results among different practitioners. After fat grafting, the retention of fat volume ranges from 20 to 90% in various studies (reviewed in Wetterau et al., 2012). Furthermore, the biology of

fat grafting with regards to the stem cell sources of adipocytes, how engraftment occurs, and the factors that influence graft retention are not yet fully understood (Bucky and Percec, 2008).

Adipose tissue contains a robust source of adipose stem cells, and has a high rate of endogenous turnover. Approximately 50% of adipocytes in the body are replaced every 8 years (Spalding et al., 2008), although this has not been studied in craniofacial fat specifically. Preadipocytes are capable of self-renewal and differentiation into white adipose tissue, but are committed to a single cell fate prenatally or in the early postnatal period (Tang et al., 2008). In addition to preadipocytes, the stromovascular fraction of lipoaspirates contains cell populations capable of differentiation into fat, bone, muscle, and cartilage *in vitro* (Zuk, 2008). However, the precise relationship of these cells to committed preadipocytes is not entirely clear (reviewed in Cawthorn et al., 2012). Some clinicians advocate the isolation of these cells in the stromovascular fraction to augment the lipoaspirate in fat grafting (Yoshimura et al., 2009), based on the notion that adipose-derived mesenchymal stem cells in the stromovascular fraction secrete angiogenic growth factors, which may increase graft survival. Other growth factors such as insulin, insulin-like growth factor-1 (IGF-1; Yuksel et al., 2000), and platelet-rich plasma (Nakamura et al., 2010; Pires Fraga et al., 2010) have also been added to fat grafts to improve retention, with greater final fat graft weight, and vascularization as compared with untreated grafts in animal models.

Clinically, currently available fat grafting strategies are very successful for the treatment of contour deformities from lipodystrophies and rejuvenation of the aging face. The observed stability of fat transfer over the long-term strongly suggests that fat regeneration occurs within the graft, with continued differentiation of adipocytes from preadipocytes and normal fat turnover. This normal tissue homeostasis involving the continuous generation of new fat cells is evidence of the existence of a tissue-resident stem cell for fat. It follows that current fat transfer techniques are, in fact, transferring adipose stem cells along with adipocytes and other cell types. Fat grafting, therefore, largely fits the definition of regenerative medicine. Augmenting a fat graft with particular purified cell fractions or growth factors may hold promise for improving predictability and retention, although clear superiority of these techniques compared with traditional methods has not been shown. Clinical trials are underway to more critically evaluate whether concentrating the stromovascular fraction in lipoaspirates will be better than traditional methods in treating post-traumatic soft tissue deformities of the face (ClinicalTrials.gov ID NCT01564524).

The cellular mechanisms contributing to lipodystrophies and aging are not fully understood, however overcoming the gaps in knowledge about fat biology and pathology would potentially allow direct regeneration of fat without grafting from other sites. Currently adipose precursor cells have been shown to form fat *in vitro* (Kim et al., 2007; Wu et al., 2012), but direct fat differentiation *in vivo* has not been reported. A clinical trial to assess efficacy of adipose-derived stem cell injections in progressive hemifacial atrophy (ClinicalTrials.gov ID NCT01309061) is in progress. At present, however, the indications for fat grafting continue to expand and clinical results continue to improve.

PERMANENT DIPLOPIA AFTER INJURY TO AN EXTRAOCULAR MUSCLE

CLINICAL VIGNETTE

An elderly man suffered an orbital roof fracture that caused entrapment of his left superior rectus muscle. This injury left him with diplopia (double vision) and an inability to look upward with his left eye (**Figure 5**). Despite release of the muscle from the fracture fragments, the injury to the muscle was permanent due to muscle fibrosis.

While reasonably good techniques exist for replacing bone and fat, regenerating, repairing, or replacing functional muscle remains a significant challenge. Although muscle transfers (called flaps) have vastly improved our ability to treat a variety of tissue defects over the past three to four decades, there remain major limitations in the function that can be achieved. Strategies for addressing the loss of fine muscle function, including the critical functions of many small muscles of the face and head, are lacking. Myoblasts, or muscle precursor cells, have been injected into injured muscle in animal models and in patients with Duchenne muscular dystrophy to promote muscle regeneration (Rando and Blau, 1994; Miller et al., 1997). However, myoblasts exhibit relatively poor survival and engraftment into the host tissue, and studies in mice now clearly demonstrate the superiority of muscle stem cells (satellite cells) in terms of their ability to engraft and regenerate muscle. Satellite cells in the muscle, bone marrow-derived mesenchymal stem cells, and adipose-derived mesenchymal stem cells have all been shown have myogenic properties *in vitro* (Wakitani et al., 1995; Zuk et al., 2001; Muguruma et al., 2003; Di Rocco et al., 2006). However, mesenchymal stem cells have not demonstrated successful regeneration *in vivo* (reviewed in Otto et al., 2009). Only satellite cells have truly fulfilled the criteria of a stem cell for muscle.

Therefore, satellite cells currently show the most promise in translational applications for functional muscle regeneration. These adult muscle stem cells are capable of robust self-renewal, differentiation into myoblasts, and formation of mature skeletal muscle fibers in response to injury (Bischoff, 1986; Zammit et al., 2006; Cosgrove et al., 2009). Transplantation of intact single myofibers into injured muscle leads to satellite cell renewal and myofiber regeneration (Collins et al., 2005; Hall et al., 2010). Single, prospectively isolated muscle stem cells have been transplanted into mouse muscle, demonstrating self-renewal, expansion, and

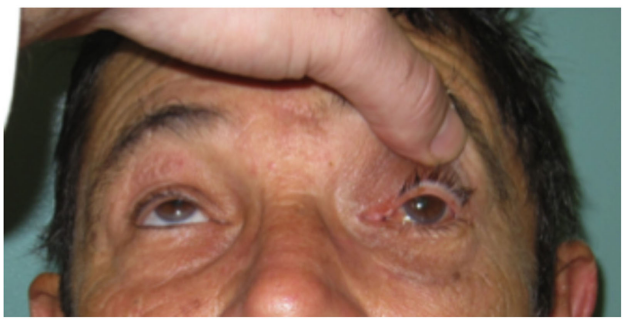

FIGURE 5 | This man suffers from the inability to look upward with his left eye after permanent injury to the left superior rectus muscle.

differentiation into functional muscle fibers *in vivo* (Cerletti et al., 2008; Sacco et al., 2008). Muscle stem cell transplantation has resulted in correction of dystrophic phenotypes in mdx mice (Sacco et al., 2010). Major remaining challenges include the translation of mouse satellite cell biology to humans, and overcoming additional hurdles such as correction of genetic defects and *ex vivo* satellite cell expansion.

As demonstrated by the example of extraocular muscle injury (**Figure 5**), regeneration of craniofacial muscle is an area of great clinical need. Ideal treatments might involve transplantation of autologous satellite cells from an area of excess to an area of need. Before it becomes a clinical reality, we must better understand the differences between satellite cell populations. Are they capable of regenerating only their native muscle phenotype? Or is it possible that limb satellite cells could effectively regenerate extraocular muscle?

REANIMATING THE FACE: REGENERATIVE STRATEGIES FOR NERVE AND MUSCLE IN FACIAL PARALYSIS

CLINICAL VIGNETTE

A girl with congenital right-sided facial paralysis was treated with an innervated muscle flap to restore a functional smile (**Figure 6**). This procedure involved free gracilis muscle micro-neurovascular transfer to the face. The muscle was innervated by the ipsilateral nerve to the masseter. With clenching of the teeth, the gracilis muscle would contract and elevate the oral commissure to recreate a natural symmetric smile.

Injury to the facial nerve leads to two tissue problems. First, injury to the nerve leads to denervation of the target muscles. Second, denervation of the muscles over the long-term leads to muscular atrophy and loss of function (Kobayashi et al., 1997). After injury, axons degenerate in response to denervation (Sunderland and Bradley, 1950). Peripheral nerves are unable to regenerate the cell body, but axons are able to regrow from existing cells at a rate of 1 mm/day in humans. For this reason, in cases of nerve transection or other injury that will not recover on its own, current treatments aim to restore continuity of the nerve sheath and guide axonal regeneration, either by primary nerve repair or nerve grafting (reviewed in Siemionow et al., 2010). Successful reinnervation can occur, but is dependent upon the location of the injury and the timing of the repair. Improving axon growth in both acute and chronic nerve injury is critical to improving the functional potential of regenerating peripheral nerves.

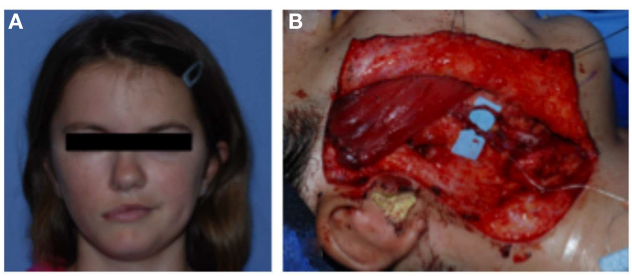

FIGURE 6 | (A) A girl with congenital right-sided facial paralysis demonstrates asymmetry with smiling. She was treated with free gracilis muscle transfer. **(B)** The muscle is inset to the zygoma and the oral commissure. The new vascular supply to the muscle is shown on the blue background. The muscle was innervated by the nerve to masseter (not shown).

Schwann cells play an important role in supporting axon growth. They closely accompany the axons as they grow. Schwann cells migrate distally from the zone of injury ahead of the regenerating axon, as if clearing a path for the axon. Schwann cells also secrete laminin, fibronectin, and other factors that facilitate axonal growth (reviewed in Zochodne, 2012). However, denervation of the nerve stump leads to loss of Schwann cells (Sulaiman et al., 2002), and limits regeneration. Therefore, one approach to supporting peripheral nerve regeneration is to transplant Schwann cells. Schwann cells have been expanded in culture and transplanted to chronically denervated rat tibial nerves. These cultured Schwann cells increased axonal regeneration and muscle reinnervation (Walsh et al., 2010). Similarly, adipose-derived stem cells have been differentiated into a Schwann cell phenotype for this purpose (Kingham et al., 2007), and demonstrated myelination and improved nerve regeneration after transplantation distal to a sciatic nerve injury in a rat (Tomita et al., 2012). While these approaches need to be further refined and verified with regards to functional outcomes, Schwann cell transplants may prove successful for peripheral nerve regeneration (reviewed in Walsh and Midha, 2009).

Several challenges must be overcome in peripheral nerve axonal regeneration. First, the rate of growth remains very slow. Finding ways to accelerate the axonal growth rate would decrease the amount of degeneration that both the peripheral nerve axon and the target muscle experience. It is known that advanced age can slow both axonal regeneration (reviewed in Verdu et al., 2000) and collateral sprouting (Kovacic et al., 2010), however there are presently no known mechanisms for accelerating axonal growth. Second, there are innate inhibitory interactions that occur at the regenerating axon (reviewed in Zochodne, 2012). Studying how to overcome these inhibitory pathways to promote axonal growth will also be important in optimizing peripheral nerve regeneration. Second, an additional major challenge is to accurately control the direction of axonal growth. This is a critical problem, noted in particular after inflammatory injury to the facial nerve, as occurs in Bell's palsy. In patients with Bell's palsy, synkinesis, or abnormal simultaneous muscle movement, can occur due to aberrant regeneration of the nerve axons. Using either physical or molecular guides to ensure an axon reaches its appropriate target would have tremendous clinical implications.

In addition to the problems associated with axonal regrowth are the subsequent deficits caused by target muscle atrophy. After denervation, muscle mass and contractile force rapidly decrease. The rapid loss stabilizes at approximately 4 months, with the muscle retaining only 25% of its mass and less than 0.1% of its maximum contractile force (reviewed in Carlson, 2004). In the early period after denervation, satellite cells are activated, proliferate, and form new muscle. However, these fibers are morphologically abnormal, small in size, and do not have satellite cells associated with them (Borisov et al., 2005). Finally, after prolonged denervation, the overall number of satellite cells present in the muscle decreases, and the capillary bed degenerates (Borisov et al., 2000;

Jejurikar et al., 2002). This phenotype is not surprising given the known dependence of developing muscle on neural input for proper formation and gene expression (Betz et al., 1980; Harris et al., 1989; Crews and Wigston, 1990; Fredette and Landmesser, 1991; Fernandes and Keshishian, 1998). Moreover, limb regeneration in amphibians, including regeneration of the limb muscles, requires innervation (Brockes, 1984, 1987). Therefore, neuromuscular intercellular communication is a prime example of the complex interplay of different tissues, requiring precise orchestration for proper formation during development and in regeneration.

The changes occurring in muscles after denervation further limit the restoration of function, even after axonal regeneration occurs. Preventing the maladaptive changes associated with denervation and reinnervating target muscles more quickly are the major challenges confronting regenerative peripheral nerve biology. For patients with both acute and chronic facial nerve injuries, developing these regenerative strategies may allow for more natural facial function than our current reconstructive treatments are capable of providing.

REGENERATION OF SKIN AFTER BURN INJURY

CLINICAL VIGNETTE

A 22-year-old man involved in an automobile accident suffered full thickness burns to nearly the entire face, scalp, and both upper extremities. His treatment required multiple operations, first for debridement and cadaveric skin grafting to prepare a suitable wound bed prior to autologous grafting (**Figure 7**). He subsequently had full and split-thickness skin grafts to the face. The scalp had exposed bone and required grafting in two stages: first with artificial dermis (Integra, Integra Life Sciences), then with autologous skin.

An ideal skin replacement in the face would be thin, pliable, similar in color, and texture to surrounding skin, have rapid and reliable engraftment, contain all the components of the skin organ, and undergo minimal contracture and scarring. Autologous skin is the best option currently available, and can be used in several forms. Local skin flaps may cover relatively small defects and provide a good match of skin color and texture. They also do not contract significantly. For larger defects, tissue expansion is successful for increasing the amount of skin available for local rearrangement, but it typically requires two operations separated by several months to recruit adequate skin. It also requires an adjacent donor site with healthy unscarred skin. In more complex cases with both skin and soft tissue deficiency, free tissue transfer of skin with its underlying muscle or fascia will provide excellent coverage. In craniofacial reconstruction, skin flaps often come from a remote location, and reconstruction suffers from poor color and texture match. Finally, in cases such as the burn patient (**Figure 7**), full or split-thickness skin grafting is the most commonly used strategy for achieving massive amounts of skin coverage. Skin may be harvested from local or remote donor sites and is versatile with regards to its use on both large and small defects. The limitations of autologous skin grafting include donor site availability, donor site morbidity, graft loss, lack of certain dermal elements (sweat glands and hair follicles), and scar contracture. Large burns require multiple operations and serial grafts to finally achieve wound closure, and patients are usually left with significant deformities.

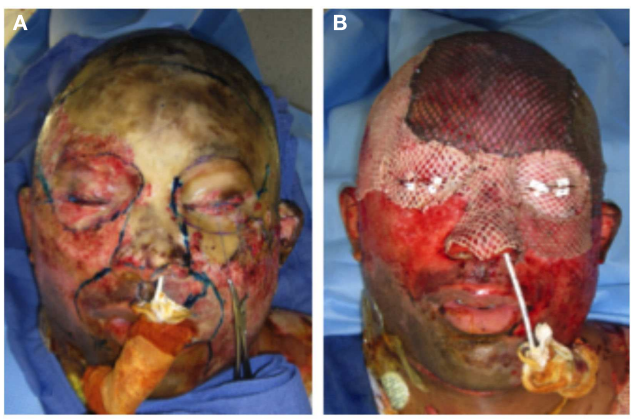

FIGURE 7 | A young man with full thickness burns of the face and scalp (A) prior to debridement and (B) after cadaveric skin graft placement. He required multiple operations prior to final skin grafting.

Partial thickness burns retain the components of the skin organ that are responsible for regeneration. The skin has a robust source of stem cells located in the basal layer of the epidermis, the bulge of the hair follicles, and the base of sebaceous glands (Fuchs and Nowak, 2008). Each of these stem cell compartments is capable of forming new epidermis. Alternatives to autologous skin grafts using cells derived from skin stem cells are cultured autologous epidermis and autologous cell suspensions. Cultured autologous epidermis has the advantage that small biopsies may be expanded for large amounts of graftable epidermis. However, these grafts contain an abnormally layered epidermis and, most importantly, lack a dermis. Without a dermis, graft take decreases and scar formation increases, making cultured autologous skin grafts inferior to autologous skin grafts. This is because these grafts lack the elastic properties of a dermal component, resulting in a much more fragile construct, prone to sloughing (Pham et al., 2007). Autologous cell suspensions have potential to improve outcomes with regards to skin quality, color, and rate of healing when used in partial thickness burns (Wood et al., 2012). However, autologous cell suspensions cannot be used to treat more complex full thickness burns for the same reasons detailed above for altered epidermis.

Currently, the most significant hurdle for skin regeneration is the regeneration of the dermis in full thickness and deep partial thickness burns. The dermis is home to the stem cells residing in the hair follicles and sebaceous glands. Loss of the dermis results in an inability to regenerate. Furthermore, the dermis is responsible for the stability of the graft and native skin, elasticity of the skin and prevention of contracture, and important cell-extracellular matrix interactions that are necessary for healing and homeostasis. Without dermis, it is not possible to obtain a stable skin construct that will resist contracture, trauma, and infection.

To address this problem, multiple approaches have been used to engineer artificial dermal matrices. Thus far, collagen-based matrices appear to have better cellular integration than synthetic polymers (reviewed in Widgerow, 2012). Artificial dermis is successful in improving contour and graft take onto bone, cartilage, or tendon (reviewed in Yannas et al., 2011). However, artificial

dermis requires the use of autologous skin grafting with some native dermis present in the graft, and does not appear to improve long-term contracture or healing (Philandrianos et al., 2012). A dermal matrix that also contains keratinocytes or basal stem cells and is capable of resurfacing large wounds in one step has yet to be developed. Developing mechanisms for regenerating dermis, or engineering and culturing full thickness skin for grafting, will dramatically change acute burn care. In patients with large areas of full thickness burn in sensitive areas of the face, regenerated skin and dermis could provide greatly improved functional and cosmetic outcomes and allow for treatment to be completed with fewer surgical interventions. Like other tissues, skin is complex, is comprised of multiple cell types, is vascularized by blood vessels, and is innervated. Skin injuries have a great capacity to heal, but the drawbacks of healing are most evident in injuries to the skin of the face. Scarring, deformities, and loss of function are the norm and approaches to "replace like with like" are needed.

In addition to the acute need for skin coverage to prevent infection and fluid loss, burn patients suffer from distinct long-term deformities, such as scar contracture that require additional operations for release. Fat grafting is one strategy that has also been used to soften and improve the quality of scars in burn patients. The molecular mechanisms are unclear, but fat grafting increases the vascularity of the scar and alters its collagen content (Klinger et al., 2008).

THE FUTURE OF REGENERATIVE STRATEGIES IN CRANIOFACIAL DISEASES

Two general strategies are emerging as future solutions to craniofacial reconstructive challenges: regenerative approaches discussed in this manuscript and composite tissue transplantation. Composite tissue transplantation has been making inroads in recent years, with the first successful face transplant in 2005 (Devauchelle et al., 2006). At least 18 have been performed worldwide since, including several full face transplants (Pomahac et al., 2012). Facial allotransplantation holds great promise with regards to the restoration of form and function superior to that of traditional reconstructive techniques. One clear advantage of facial allotransplantation is that the complex tissues of the face are fully and normally formed prior to transplantation. Current disadvantages include the need for life-long immunosuppression, with the risks of developing life-threatening infections, and unclear functional integration and cosmetic appearance of the graft. Some facial muscle function and sensation has been documented with facial allotransplantation, but normalization of sensation, expression, and function has yet to be demonstrated with long-term follow up.

Regenerative medicine approaches to regenerate individual functional tissues based on developmental mechanisms may ultimately lead to clinical composite tissue regeneration. A theoretical advantage of this approach is the achievement of fully integrated, complex, functional tissue that is truly "self"-derived. Many unanswered questions exist at this point, including whether function, appearance, and sensation may be better achieved through regeneration of native structures rather than reinnervation of a transplant. The regeneration of complex facial structures also requires precision and specificity. Directing the regeneration of cells such that they proliferate in the appropriate locations at appropriate times, and reach terminal differentiation when the organ is fully regenerated will be challenging. Regeneration strategies will need to develop hand in hand with tissue engineering strategies that allow us to build the components of the face precisely.

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