Actual strategies in human induced pluripotent stem cells (hiPSCs) differentiation – perspectives and challenges

Aktualne strategie różnicowania ludzkich indukowanych pluripotencjalnych komórek macierzystych – perspektywy i wyzwania

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Summary
The application of stem cells (SCs) in regenerative medicine has recently become a rapidly growing field, holding promise for combating a number of currently incurable disorders: including diabetes, neurodegenerative, retinal and cardiac diseases, as well as muscular dystrophy. The search for alternative approaches led to the development of human induced pluripotent stem cells (hiPSCs) which have unrestricted proliferative activity and pluripotency – the capacity to differentiation into derivatives of three germ layers (meso-, ecto – and endoderm). Because hiPSCs are developed from adult human cells throughout the forced expression of pluripotency factors, they are free from the ethical concerns associated with human embryonic stem cells (hESCs), that creation involves the destruction of human embryos. Moreover, the use of hiPSCs contributes to the development of personalized medicine that exploits patient-specific cells extremely useful in autologous grafts. In the present study the methods of hiPSCs differentiation into stem cell-derived neurons, cardiomyocytes, chondrocytes and osteocytes were summarized and evaluated having regard to their most important aspects.

Streszczenie
W ostatnich latach, stosowanie komórek macierzystych w medycynie regeneracyjnej cieszy się coraz większym zainteresowaniem, ze względu na ich potencjalne wykorzystanie w stanach chorobowych, na które nie ma obecnie skutecznej metody leczenia: choroby dotyczące układ nerwowy, mięśniowy, szkieletowy. Poszukiwanie alternatywnych metod doprowadziło do rozwoju ludzkich indukowanych pluripotencjalnych komórek macierzystych (ang. human induced pluripotent stem cells, hiPSCs) z ludzkich, dojrzałych komórek jak np. fibroblastów. Komórki te posiadają nieograniczony potencjał do samoodnowy i różnicowania w pochodne trzech listków zarodkowych (ektó -, mezo – i endoderm). Ze względu na fakt, ze hiPSCs wywodzą się z ludzkich, dojrzałych komórek, ich przyszłe wykorzystanie jest wolne od kontrowersji etycznych, charakterystycznych dla ludzkich embrionalnych komórek macierzystych (ang. human embryonic stem cells, hESCs), których pozyskiwanie jest związane z niszczeniem ludzkich embrionów. Ponadto, wykorzystanie hiPSCs ma kolejną ważną zaletę, jaką jest możliwość ich wykorzystania w medycynie spersonalizowanej np. w przeszczepach autologicznych. W niniejszej pracy, zostały opisane i podsumowane aktualne metody różnicowania hiPSCs w kierunku neurony, kardiomiocyty, chondrocyty oraz osteocyty, wraz z uwzględnieniem ich najważniejszych aspektów.

Key words: cardiomyocytes, chondrocytes, differentiation, human induced pluripotent stem cells, neurons, osteocytes, reprogramming,

Słowa kluczowe: chondrocyt, kardiomiocyt, ludzkie indukowane komórki pluripotencjalne, neurony, osteocyty, reprogramowanie, różnicowanie

Introduction
Stem cells (SCs) are primary, non-specialized cells which have ability to self-renewal, reproduction and differentiation into a specialized cells forming tissue. Those cells were firstly observed in teratoma – tumor arising in the ovaries of mice. Teratomas originate from germ cells, and thus have an extensive differentiation capability which also takes place during embryogenesis. The experiments on teratomas initiated a whole series of works, which in 1981 led
to the establishment of first mouse embryonic stem cell (mESCs) lines [1, 2]. Further studies allowed to obtain the first human embryonic stem cells (hESCs) in 1998 [3]. These embryonic stem cells are pluripotent, what means, they are able to differentiate into all derivatives of the three primary germ layers (ecto-, meso – and endoderm). Because of their plasticity and potentially unlimited capacity for self-renewal, ES cell therapies have been proposed for regenerative medicine and tissue replacement in the case of injury or disease. However, due to the fact that they are obtained from an inner cell mass of blastocyst, which is related to its irreversible damage, the application of SCs in therapeutic purposes raises many ethical and moral concerns. A real breakthrough in the subject of SCs was a work of Kazutoshi Takahashi and Shinya Yamanaka in 2006. They indicated the possibility of obtaining the pluripotent cells from mouse fibroblasts by overexpression of four transcription factors (Fig. 1) [4]. From the 24 pre-selected factors inducing pluripotency in somatic cells they selected four: OCT3/4, SOX2, KLF4 and C-MYC (OSKM) also known as “Yamanaka’s factors”. The reprogrammed cells had morphology and gene expression profile similar to ESC and thus are called induced pluripotent stem cells (iPSCs). A year later, similar cells were also obtained from human fibroblasts [5]. Those experiments by Takahasi and Yamanaka not only bypassed the ethical issues about the usage of human embryos but also provided new opportunities for the development of regenerative medicine [6-9]. From that moment, numerous protocols have been developed ensuring the formation of iPSCs not only from fibroblasts, but also from any cell of the body including keratinocytes [10], amniocytes [11], pancreatic β cells [12], mature B cells [13], cord blood cells [14], melanocytes [15] and others. Moreover, patient specific iPSC cells can be genetically modified to repair the defect cells, further differentiate, and ultimately transplant to the donor. It is worth noting that autologous transplantation reduces the risk of immune rejection [16]. These methods have been successfully applied in the animal models. Gene therapy based on iPSC cells could be successfully carried out in mice suffering from sickle cell anemia [17] or hemophilia A [18]. The before-mentioned biggest obstacle in the application of hESCs is the method of obtaining them. The necessity of destroying human embryos to isolate embryonic cells prevents the high scale production of pluripotent cells for further application. Thus, the generation of iPSC cells is regarded as a milestone for future applications. Among the four reprogramming “Yamanaka factors” only OCT3/4 and SOX2 are required to initiate reprogramming and finally generate iPSC cells. Their role is the mutual regulation of the expression and activation of pluripotency genes such as NANOG, KLF4, and C-MYC are the factors that increase efficiency and accelerate the process of reprogramming [4]. C-MYC is engaged in the activation of genes that increase proliferation rate. In addition, individually or in combination with other factors, C-MYC is a major driving inducer during the initial phase of reprogramming. The exogenous C-MYC activates pluripotency markers a few days after derivation
It has been demonstrated that C-MYC together with complexes of histone acetylases induces global histone acetylation and allows for the binding of OCT3/4 and SOX2 to DNA [20]. It was revealed that KLF4 is able to inhibit the activation of NANOG and other genes characteristic for SCs. KLF4 plays a dual role in reprogramming process: it represses the function of genes characteristic for differentiated cells during the initial step and activates the pluripotency genes associated with the final stage of reprogramming [19].

Despite the significant progress in generating iPS cells, many concerns about iPS cells generation must be resolved before their application on the clinical scale. The most important issue is the safety of the method used for the reprogramming and its efficacy. Reprogramming transgene delivery methods typically are classified to viral and non-viral ones. Both of them can be further divided to integrative and non-integrative into the host genome methods as well as to DNA-, RNA- or protein-based approaches.

Direct methods of reprogramming are based on active absorption of the recombinant proteins or mRNA by the cell. The adenoviral vectors are characterized by low efficiency of reprogramming process and the limited ability to control the level of transgene expression [21]. Also, episomal vectors oriP / EBNA-based Epstein-Barr virus are characterized by a very low efficiency of reprogramming [22].

Although, the usage of Sendai virus guarantees relatively high efficiency of reprogramming, its removal from the forming iPS cells is problematic [23]. Plasmid vectors due to their inability to replicate in a cell, require multiple transfections during the process of de-differentiation [24]. The use of recombinant proteins allows obtaining iPS cells without introducing exogenous genetic material into a cell. However, proteins have a limited capacity to translocate through the cell membrane. In order to provide reprogramming factors into the cell, the protein are usually combined with the cell membrane penetration enhancers [25, 26].

Another method of generating IPS cells is the use of recombinant mRNA. This type of reprogramming is characterized by a relatively high yield, but requires several incubations with a cocktail mRNA. A significant obstacle in potential use of this protocol on a wider scale is the fact that the introduction of a foreign RNA induces defense cell systems involving antiviral response which causes high cytotoxicity of applied methods [27]. However, safer, non-integrating methods are characterized by relatively low efficiency of the reprogramming process. Integrating vectors, such as retroviral, lentiviral vectors or transposons guarantee efficient reprogramming of somatic cells. However, a transgene integrated into the genome of a cell, may cause genetic instability and malignant cell transformation. An important advantage of retroviral vectors is the spontaneous silencing of transgene expression. However, it can be reexpressed during cell differentiation [5]. The need to develop methods of obtaining secure, devoid of transgene expression iPS cells led to the creation of systems based on the excision of the integrated transgene. Several methods have been developed based on excision of the transgene or by regulating its expression. One of them involve the use of naturally occurring transposons which have the ability to translocate around the genome in a specific process – transposition. Currently, two commonly used protocols are based on transposons: piggyBAC and sleeping beauty, associated with high efficiency in reprogramming of somatic cells [28, 29]. Their removal from the genome is possible with the application of the enzyme transposase. Another system based on the excision of the integrated transgene is the Cre-loxP system, wherein the loxP site is positioned in the 3’LTR-region of the vector. During the integration loxP site is duplicated.

Figure 2. Yamanaka’s factors (OCT3/4, SOX2, KLF4, C-MYC, OSKM) play a crucial role in reprogramming process. They are responsible for induction of pluripotency state in reprogrammed adult cells [own materials source].
to 5’UTR region, which results in the integration of the transgene surrounded by two loxP sites. This enables subsequent excision with Cre recombinase [30]. The system of regulated transgene expression may also be based on the use of specific promoters, which activity can be pharmacologically regulated, for example a tetracycline-inducible promoters. The use of this system in such vectors depending on the requirements, enables or disables the transgene expression by supplementing the culture medium with doxycycline [31].

Another important issue concerning the application of iPSC cells in the clinic is the homogeneity of generated iPSC cells population. Many researchers noted, that reprogramming of mouse somatic cells into iPSC cells often generates heterogeneous population with partially and fully reprogrammed iPSC cells [32-34]. Mechanistic studies involving global analysis indicated how reprogramming factors regulate molecular changes during somatic to pluripotent state transition. Meissner et al firstly pointed out that DNA methylation has a critical role in the restablishment of pluripotency [35]. Together with many other researchers, they pointed out, that epigenetic mechanisms involved in reprogramming process play crucial role in obtaining fully reprogrammed iPSC cells which results in higher homogeneity of generated cell population [32, 36-38]. The same problem of heterogenic population occurs during differentiation process. In the recent work Loh et al defined the signals controlling the binary lineage decision. Their observation enables to block differentiation toward unwanted fates and obtain the 80-90% pure human mesodermal lineage which can be used in vivo studies [39].

However many obstacles occur, first clinical trial using iPSC cells started in September 2014 for treating age-related macular degeneration (AMD) [40]. The investigators at RIKEN, a research institute in Kobe, Japan, treated a single patients using retinal pigment epithelium differentiated from autologous iPSC cells derived from patient’s skin biopsy. Although these patients didn’t have any serious adverse effects, due to changes in regenerative medicine law, the trial was suspended in March 2015. The RIKEN group is now revising their trial and preparing new protocol for allogeneic cells application. The allogeneic cells will come from CiRA, the institute which is the pioneer in developing a bank of iPSC cells [41]. The use of allogeneic cells, which are haplotype matched to patient’s major human leukocyte antigens (HLAs), can omit the problem of transplant rejection and necessity to use immunosuppression. However giving patients their own cells still remains the best solution, due to costs and time of their generation, the HLA-matched bank of iPSC cells seems to be beneficial. This review presents recent advances in stem cell-based therapy and summarizes data from the literature concerning hiPSC differentiation into neural, chondrogenic, osteogenic and cardiomyogenic lineage (Fig. 3)(Table I).

**iPSC differentiation into neurons and neural progenitors**

Although iPSC technology is a novel strategy in regenerative medicine, it shows a great promise in the therapeutic use and in disease modeling. Because, iPSC technology allows for *in vitro* production of neural cells and progenitors, which are difficult to obtain via alternative methods, neurological diseases are likely to be successfully treated in the near future. Development of efficient differentiation methods is especially important in the potential use of iPSC-derived cells as patient-specific grafts [42].

**Differentiation of iPSCs into neuronal cell lines**

There are numerous efficient methods used for iPSC differentiation into neuronal cells. One of the most commonly used protocols involve iPSC growth as spheres. Firstly, iPSCs are detached from feeder layer and then placed in human neural progenitor growth medium with the addition of serum free supplement for neural cell culture – B27, basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) and heparin. After two weeks, spheres are dissociated and plated on laminin coated glass coverslips. For induction of differentiation into motor neurons, spheres are cultured in neural induction medium supplemented with retinoic acid (RA), sonic hedgehog (SHH), cyclic adenosine monophosphate (cAMP), ascorbic acid (AA), brain derived neurotrophic factor (BDNF) and glial cell-line derived neurotrophic factor (GDNF) [43]. Karumbayaram and colleagues used embryoid body (EB) formation in non-adherent conditions to differentiate hiPSCs into motor neurons. hiPSC-derived EBs were treated with RA and SHH pathway agonist and

**Figure 3.** The currently methods of hiPSC differentiation are mainly based on the formation of embryoid bodies, micromass culture, pellet culture and monolayer culture. These approaches enable obtaining stem-cell derived such as neurons, astrocytes, cardiomyocytes, chondrocytes, osteoblasts and osteocytes as well as they mimic main stages of differentiation and maturation processes taking place during embryo development (phot. Augustyniak E; own materials source).
after 5 – 7 days generated neural progenitors that stained positive-ly for POU class homeobox (BRN2), SRY (sex determining region Y)-box3 (SOX3) and Paired box 6 (PAX6). After next 8-10 days of culture with RA, SHH pathway agonists and neurotrophic factors: BDNF, ciliary neurotrophic factor (CNTF) and GDNF, EBs gave rise to motor neuron progenitors that revealed the presence of characteristic markers: NK6 homeobox 1 (NKX6.1) and Oligodendrocyte Lineage Transcription Factor 2 (OLIG2). Finally, after 3 – 5 weeks differentiated EBs were able to form mature motor neurons [44, 45]. Another effective approach to obtain neuronal stem cell-de-

Table I. The most common markers characteristic for pluripotent, neural, cardiac, chondrogenic and osteogenic cell lines.

<table>
<thead>
<tr>
<th>POSITIVE MARKERS</th>
<th>PLURIPOTENT</th>
<th>NEURAL</th>
<th>CARDIAC</th>
<th>CHONDROGENIC</th>
<th>OSTEOSTIC</th>
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<tbody>
<tr>
<td>OCT3/4, NANOG,</td>
<td>BMP-4, DESMIN, GATA-4, NXX2.5, TROPONIN T,</td>
<td>AGGRECAN, TYPE II &amp; IV</td>
<td>ALKALINE</td>
<td></td>
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<tr>
<td>SSEA-3, SSEA-4,</td>
<td>TROPONIN I, aSARCROMIC ACTIN, MYOSIN HEAVY CHAIN, MYOGLIN, CK-MB, TBX5, ATRIAL NATIONURETIC FACTOR,</td>
<td>AGGREGANTE, TYPE II</td>
<td>PHOSPHATASE, TYPE I</td>
<td></td>
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</tr>
<tr>
<td>TRA-1-60, TRA-1-81, SOX2, CD9, CD24, CD30, CD133, SALL4, STAT3, E-CADHERIN, ALKALINE PHOSPHATASE, INTEGRIN β1, INTEGRIN α6, C-MYC, KL5, LIN-28, SMAD2</td>
<td>CD15, CD44, NESTIN, O1, O4, OLIG2, NOGGIN, NOTCH1, S100B, SOX10, GFAF, GLAST, PAX6, MAP2, GALT, NeuN, βTUBULIN III</td>
<td>AGGREGANTE, TYPE II</td>
<td>II COLLAGEN, OSTERIX, OSTEOCALCIN, BONE SIALOPROTEIN 2, RUNX2, OSTEONECTIN, FIBRONECTIN, SPARC, THROMBOPOIE-TIN, BIGLYCAN</td>
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<td></td>
<td>CD15, CD44, NESTIN, O1, O4, OLIG2, NOGGIN, NOTCH1, S100B, SOX10, GFAF, GLAST, PAX6, MAP2, GALT, NeuN, βTUBULIN III</td>
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to use neural stem-derived cells that precisely recapitulate disease course taking place in human nervous system. Patient-specific cells that are burdened with neurological disease background can be differentiated into specific neural cells and further used to investigate disease mechanisms [57]. The ability of iPSC-derived cells to establish useful cellular models of neurological diseases constitute efficient platforms for developing new drugs [54]. hiPSC-derived neuronal cells recapitulate properties observed in neuronal cells in vivo. Shi and coworkers (2014) demonstrated that differentiated hiPSCs can be used to investigate the model of human cortical development. hiPSCs were differentiated into neuroepithelial cells and then towards cortical stem and progenitor cells. Further differentiation in vitro mimicked processes taking place during neurogenesis in vivo, such as acquisition of mature electrophysiological properties and formation of functional excitatory synapses [58]. The capacity of hiPSC-derived neurons to recapitulate properties of human neurons makes them great candidates for establishing neurological disease models and drug screening platforms. Yagi and colleagues (2011) proved that hiPSCs generated from patient suffering from familial Alzheimer’s disease (FAD) can be a viable source of neurons for both disease modeling and drug screening. FAD-iPSC-derived neurons showed increased secretion of amyloid β42 (Aβ42). After treatment with a γ-secretase inhibitor and with a selective Aβ42-lowering agent, Aβ42 secretion was inhibited or modulated, as expected [59]. In another study, the hiPSC-derived cortical cells capable of recapitulation of Aβ-induced synaptic patomechanisms were established [60]. The immunopurification procedure allowed to generate electrophysiologically active mature hiPSC-derived neurons susceptible to synaptotoxic effects of Aβ. The patient-specific hiPSC derived neurons were also used to elucidate disease mechanisms in Huntington’s disease (HD). Mattis et al. (2012) used fibroblasts derived from patients with different numbers of CAG repeats to generate hiPSC lines. The comparison of gene expression profile between control and HD hiPSCs revealed that these two cell lines are characterized by different properties. Some of the observations contributed to the discovery of the novel altered pathways involving matrix metalloproteinases or developmental genes. HD hiPSCs that had been differentiated into neural progenitor cells through formation of spheres displayed decreased cell-cell adhesion properties and reduced energy metabolism as well as they showed significant changes in actin cytoskeleton organization. Differentiated HD neural stem cells (NSCs) with higher number of CAG repeats were more prone to cell death and were more sensitive to the absence of BDNF [43, 61]. An and co-workers (2012) proved that hiPSCs generated from patients with HD can be genetically corrected in vitro by the replacement of the expanded CAG region with a normal repeat region using Flp-FRT recombination. Both corrected HD-hiPSCs and NSCs derived from them showed no further HD progression. Moreover, NSCs derived from corrected HD-hiPSCs were transplanted into the striatum of mouse affected by HD, wherein they have the capacity to populate the striatum and further differentiate into neurons and glial cells [62]. hiPSCs were also used as a source of cells for autologous transplantation in cynomolgus monkey (CM) Parkinson’s disease (PD) model. CM-iPSCs were differentiated into dopamine neurons using co-culture with stromal feeder cells [63]. The monkeys subjected to the autologous transplantation of dopamine neurons into putamen revealed significant improvement of motor function and activity. Cells derived from iPSCs were also used in the transplantation during therapy of spinal cord injury (SCI) in the common marmoset. In this case, secondary and tertiary neurospheres differentiated from hiPSCs, were transplanted into non-human primate model of SCI. Cell transplantation reduced the severe demyelination, increased axonal survival and regrowth as well as improved angiogenesis in the lesion epicenter. Fur-

<table>
<thead>
<tr>
<th>Author</th>
<th>iPSC origin</th>
<th>Cell type into which iPSC differentiate</th>
<th>Model of neurological disease</th>
<th>The most important conclusions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yagi T et. al [49]</td>
<td>Fibroblasts from FAD patients</td>
<td>Neurons</td>
<td>Familial Alzheimer’s disease</td>
<td>Neurons from FAD iPSCs shown increased Aβ42 secretion</td>
</tr>
<tr>
<td>Nieweg K et. al [50]</td>
<td>Commercially available human iPSC lines (8/25 and DF6–9–9T.B)</td>
<td>GABAergic and glutamatergic neurons</td>
<td>Alzheimer’s disease</td>
<td>Neurons shown Aβ-induced patomechanisms</td>
</tr>
<tr>
<td>Mattis VB et. al [51]</td>
<td>Fibroblasts from HD patients</td>
<td>Neural progenitor cells, neural stem cells, striatal neurons</td>
<td>Huntington’s disease</td>
<td>HD associated changes in HD neurons correlate with number of CAG repeats</td>
</tr>
<tr>
<td>An MC et. al [52]</td>
<td>Fibroblasts from HD patients</td>
<td>Neural stem cells</td>
<td>Huntington’s disease</td>
<td>Neurons from genetically corrected iPSCs populated striatum and differentiated after transplantation</td>
</tr>
<tr>
<td>Hallett PJ et. al [53]</td>
<td>Fibroblasts from cynomolgus monkey</td>
<td>Midbrain dopamine neurons</td>
<td>Parkinson’s disease</td>
<td>Neural cell transplantation improved motor functions and activity</td>
</tr>
<tr>
<td>Kobayashi Y et. al [54]</td>
<td>Commercially available human iPSC lines (20187)</td>
<td>Secondary and tertiary neurospheres</td>
<td>Spinal cord injury</td>
<td>Neurosphere transplantation improved angiogenesis, axonal survival, myelination and functional recovery</td>
</tr>
</tbody>
</table>

Abbreviations: iPSC – induced pluripotent stem cells; FAD – familial Alzheimer’s disease; GABA – γ-aminobutyric acid; Aβ – amyloid-β; HD – Huntington’s disease
The use of differentiation process in obtaining human cardiomyocytes-like cells

hiPSC-derived cardiomyocytes (hiPSC-CMs) are considered as candidates for the emerging field – regenerative medicine involving personalized medicine (e.g. autologous cardiomyocytes) and patient/disease-specific in vitro models of human cardiac tissue. The capacity to differentiate hiPSCs into cardiomyocytes was advanced by the observation that three-dimensional ESC aggregates named EBs could give rise to spontaneously contracting cells, which possess cardiomyocyte-specific markers [65]. There are several effective methods of obtaining hiPSC-CMs that will be summarized in this part of study. HiPSCs were differentiated into cardiomyocytes throughout EBs for 10 days. This process proceeded spontaneously for 30-50 days on gelatin-coated plates [66]. One of the less complicated and cost-effective method of hiPSCs differentiation into cardiomyocytes is the formation of EBs and their further culture in suspension for two weeks. This approach eliminates the addition of expensive exogenous GFs and the need for transfer of EBs on gelatin-coated plates as well as this protocol is not time-consuming. Zwi and co-workers (2009) proved that hiPSCs have the ability to differentiate into cardiomyocytes via EBs without the additional GFs. They demonstrated that during the first stage of differentiation, the level of pluripotency markers such as OCT4, NANOG abruptly decreased as well as primitive streak and cardiomesoderm markers (Brachury, mesoderm posterior bHLH transcription factor: 1-MESP1) increased [67]. Then, the presence of cardiac-specific transcription factors and finally cardiac-specific structural genes (sarcomeric related proteins and ion channels) was observed. These cells possess characteristics assigned to mature cardiomyocytes such as the presence of homeobox protein NKX2.5 (NKX2.5), GATA binding protein 4 (GATA4), atrial natriuretic peptide as well as they can be used in in vitro pharmacological tests, particularly cardiac electrophysiological studies and drug screening [68]. EBs formation and culture them in EB20 medium (DMEM containing 20% FBS, 1 mM non-essential amino acids, 1 mM l-glutamine, 0.1 mM β-mercaptoethanol, 100 units/ml penicillin, 100 µg/ml streptomycin) is the most common method of obtaining hiPSC-CMs [69, 70]. Another approach of hiPSCs differentiation into cardiomyocytes was represented by Liang et al. (2013), who obtained hiPSC-CMs via EBs in suspension culture [71]. They implemented sequential administration of following GFs: BMP-4, bFGF, activin A, Dickkopf-related protein 1 (DKK1) and vascular endothelial growth factor (VEGF) for generation of hiPSC-MCs from healthy subjects and patients with hereditary long QT syndrome, hypertrophic cardiomyopathy as well as dilated cardiomyopathy patients. In that study, hiPSC-MCs were used in evaluation of drug-induced cardiotoxicity. Healthy and diseased individuals reveal different susceptibilities to cardiotoxic drugs what confirms that disease-specific hiPSC-CMs may be used in effective prediction of adverse drug response. Kim and collaborators (2015) established the following protocol for obtaining hiPSC-derived cardiomyocytes via EBs in suspension culture: days 0–1, BMP4 (5 ng/ml); days 1–4, BMP4 (10 ng/ml), bFGF (5 ng/ml) and Activin (1.5 ng/ml); days 4–20, DKK1 (150 ng/ml) and VEGF (10 ng/ml) for the investigation of mechanism responsible for spontaneous contractions of hiPSC-CMs [72]. Cardiac differentiation through attached on 0.1% gelatin EBs, can be strongly enhanced by the addition of 100 µM ascorbic acid during the whole differentiation process, which lasted approximately 20 days. These cells were successfully utilized in the investigation of hypertrophic cardiomyopathy characteristic for Friedreich’s ataxia (FRDA) disease [73]. Another approach involves the creation of nearly pure hiPSC-CTMs population. The hiPSC-CMs with high cardiomyocyte purity can be selected throughout the expression of blasticidin resistance under the control of the cardiac myosin heavy chain 6 (MYH6) promoter. The hiPSC-CMs with high purity display cellular electrophysiological properties that are similar to mature cardiomyocytes. In addition, these cells can be easily expanded suggesting the susceptibility to high-throughput assays as well as they can be effectively cryopreserved [74].

Recently, small number of protocols involve differentiation into cardiomyogenic lineage through monolayer culture. Jung and colleagues (2016) established efficient method for obtaining hiPSC-CMs via complex monolayer differentiation protocol on Matrigel-coated plates. They characterized the ontogeny of downstream pathways of β-AR signaling in hiPSC-CMs. In those cells cardiac functionality matured significantly earlier than features involving cardiac remodeling and toxicity. Those data contribute to conclusion that hiPSC-CMs should be studied and investigated when components of β-AR signaling reflecting cardiac remodeling and toxicity are fully mature [75]. Another direct differentiation protocol included application of activin A and BMP-4 under serum-free, monolayer culture conditions. Furthermore, the use of T3 promoted maturation of hiPSC-CMs: they exhibited a larger cells size, longer sarcomeres length, enhanced calcium handling properties and increased maximal mitochondrial respiration capacity [76]. Currently, there is reduced demand for differentiation of hiPSCs into hiPSC-CMs on a laboratory scale. The autonomous differentiating process is time – and cost-consuming. Furthermore, the utility of obtained cells should be validated: the increase of desirable ectodermal markers, decrease of pluripotency markers, functionality in in vitro and in vivo models. Thus, researchers reach for fully defined and available cell lines, e.g. Guo and co-workers (2011) used iCell® cardiomyocytes in estimating the risk of drug-induced proarrhythmia [77]. Unlike primary culture systems of human cardiomyocytes, iCell® cardiomyocytes express cardiac contractile proteins, major functional ion channels and reflect spontaneous mechanical and electrical activities assigned to mature cardiomyocytes and are very useful in human-based cardiotoxicity testing models in vitro [78].
activities that are remarkably similar to adult cardiomyocytes, but also have many features required for advanced studies of cardiac ion transporters (Na/K pump, Na/Ca exchange currents, Na/H exchange and Cl/OH exchange). Fine and colleagues (2013) demonstrated that these cells relatively easily undergo molecular manipulations like overexpression and knockdown of transporters and regulatory proteins [79]. This characteristic makes them very attractive culture system for studies involving regulatory proteins and long-term regulatory pathways influencing the expression and function of membrane transporters.

The most relevant properties of hiPSC-CMs
Cardiomyocytes differentiated from hiPSCs exhibit features characteristic for in vivo cardiac myocytes: functional ion channels, receptors, transporters, syncytial and contractile activities as well as electrophysiological and biochemical responses upon exposure to environmental stimuli. Furthermore, Citro and co-workers (2014) who proved less mature cells than those of adult hearts [84]. A comprehensive study of ARVC [70]. Latest literature data indicate that application of pluripotent SCs is an interesting and promising tool that may result in significant decreased number of MSCs [94, 95]. Often strongly limited because of the age and condition of patients that are frequently limited because of the age and condition of patients.

Thus, the treatment of degenerative diseases related to the joint degeneration and long-term regulatory pathways influencing the expression and function of membrane transporters.

Induced pluripotent stem cells in cartilage and bone repair.

The another application of SC based therapies involves the treatment of muscle-skeletal diseases concerning bones and joints. In the near future, currently incurable diseases such as osteoarthritis, osteoporosis will constitute a great burden, particularly in developed countries [88, 89]. Cartilage has reduced regeneration capacity, due to its avascular nature and small number of functional cells associated with the dense extracellular matrix (ECM). Thus, the treatment of diseases related to the joint degeneration is extremely challenging. The current techniques of cartilage regeneration such as autologous chondrocyte implantation (ACI) or microfracture very often result in the formation of undesirable fibrocartilage instead of characteristic hyaline cartilage [90, 91]. In turn, bones have the capacity to regenerate itself, when the damages or depletion are relatively small. In patients suffering from huge trauma such as bone tumor resection, osteoporosis, bone infections, the self-regenerative process is not sufficient and thus, commercial available scaffolds are necessary to improve recovery of patients [89, 92, 93]. The capacity to regenerate fractures in elder’s people limbs may be decreased because of the presence of osteoporosis, which is responsible for the reduction of natural bone scaffold. Those issues constitute a challenging area of regenerative medicine, because currently there is no direct treatment reflecting natural microenvironment of tissue. MSCs seems to be a suitable source of cells for SC based therapies, due to their increased proliferation capacity and ability to differentiate into chondrocytes or osteoblast. However, their potential application is often strongly limited because of the age and condition of patients that may result in significant decreased number of MSCs [94, 95].

Thus, the pluripotent SCs are an interesting and promising tool in treatment of degenerative diseases related to the bones and
cartilage [96]. They are an unlimited source of cells with self-renewal properties, which enable to cultivate them to appropriate amount of cells and subsequently differentiate them into desired lineage [97]. There are many studies involving chondrogenic and osteogenic differentiation of pluripotent SCs. There are many issues, which need to be verified before future application of stem cell-derived chondrocytes and/or osteoblasts. Functionality similar to donor tissues, complete integration to the donor site, recovery of three dimensional structure of implemented cells is required. Moreover, these cells should be obtained with the use of efficient, cost-effective and not time-consuming protocols. Thus, further research involving differentiated chondro – or osteocytes from SCs is needed.

Cartilage and bone development
During embryo development three germ layers: mesoderm, endoderm and ectoderm are present. The musculoskeletal and hematopoietic tissues have an origin in mesenchyme, which is mature form of mesoderm. Because bone and cartilage have the common origin, the currently differentiation protocols are mostly based on receiving mesodermal population of cells [98]. During the first stage of chondro – and osteogenesis, the cells originated from condensed mesenchymal cells express the N-cadherin, neuronal adhesion molecule 1 (NCAM1) Tenascin-C (TNC), SRY (sex determining region Y)-box 9 (SOX9) and type II collagen (COL2A1) [99]. This process enables the formation of cartilage-like nodules. Then, the deposition of proteoglycans caused by increased expression of aggrecan (ACAN) proceed [100]. The maturation of chondrocytes is accompanied by hypertrophy: chondrocytes growth, enlarge and stop producing ECM proteins characteristic for hyaline cartilage (first of all type II collagen). The type I collagen (COL1A2), type X collagen (COL10A1), Runt-related transcription factor 2 (RUNX2) and matrix metalloproteinase 13 (MMP13) expression is also increased, what results in the final maturation of chondrocytes [99, 101].

After the mineralization of the cartilage, ECM serves as suitable scaffold for formation of the bones in the process called endochondral ossification. The remaining ECM is infiltrated by blood vessels and populated by bone marrow mesenchymal cells. RUNX2 and Osterix are one of the crucial genes responsible for differentiation of mesenchyme into osteoblasts and further osteoblasts [102]. The increased level of RUNX2 contributes to synthesis of type I collagen – one of component of bones, osteopontin (OPN) and osteocalcin (OCN). Differentiation and maturation of osteoblasts is maintained by high expression of OPN and bone sialoprotein (BSP). Further deposition of inorganic and organic component leads to the formation of osteocytes [103-105].

iPSC differentiation into chondrocyte-like cells
For cartilage treatment, many approaches involving different populations of SCs: MSCs and hESCs were established. They include SC differentiation protocols based on micromasses, high density pellets, monolayer, EBs or combined various techniques, i.e. monolayer with high density pellets or EBs combined with monolayer and micromasses. In the case of iPSC, the confirmation of efficiency and real usefulness of established protocols is still widely investigated. Above-mentioned techniques of differentiation are crucial, because they reflect and mimic biology of formation of cartilage tissue during embryo development and reconstruct three dimensional (3D) structures of tissues. It was revealed, that 3D systems of in vitro culture of chondrocytes improves their properties and decrease likelihood of undesirable dedifferentiation process characterized by decreased production of type II collagen, aggrecan with simultaneous increased production of type I, X collagen and the presence of hypertrophic phenotype [106]. The basic pro-chondrogenic medium consists of insulin – transferrin – selenium (ITS), dexamethasone, L-aspartic acid (both responsible for increased expression of ECM in cartilage), L-proline (basic component of cartilage cells), sodium pyruvate, non-essential amino acids. Furthermore, medium is supplemented with defined pro-chondrogenic GFs, that mostly are members of TGF-β and Bone Morphogenic Proteins (BMP) family [96]. In order to confirm the presence of chondrocyte-like cells after differentiation, the gene expression of chondrocyte-related genes must be evaluated. SOX-trio (SOX9, SOX6, SOX5), COL2A1, ACAN genes are a key factors in the regulation of ECM cartilage. The cartilage function strongly depends on the appropriate deposition of ECM compounds, therefore immunohistochemistry techniques are very useful to detect specific compounds of fibrous components. For cartilage, the alcian blue (intense blue), Safranin-O (intense red), toluidine blue (intense purple) stains are routinely performed. They enable the detection of polysaccharides responsible for the appropriate biomechanical functions of cartilaginous tissues. Yang and co-workers (2012) implemented differentiation protocol of pluripotent SCs derived from keratinocytes (KiPSCs), which had been previously established by Oldershaw (2010) on hESC lines (HUES1, HUES8 and HUES9) [107, 108]. That protocol was executed onto matrigel – coated plates in the presence of growth defined medium mTeSR daily supplemented with other GFs. KiPSCs were differentiated for 14 days and the whole chondrogenic process was divided into two stages. Firstly, the mesendoderm was obtained by culture of cells with Wingless-type MMTV integration site family member 3A (WNT3A), Activin A, BMP-4, fibroblast growth factor 2 (FGF-2) and follistatin. During the second stage, the chondrogenesis was induced by growth/differentiation factor 5 (GDF5) belonging to the TGF-β family. It resulted in the high expression of SOX9 and COL2A1. Although, the expression of pluripotency markers had lower level in comparison with pluripotent SCs, their presence was clearly noticeable [108]. Nejadnik and colleagues (2015) induced mesenchymal-like cells from hiPSCs using 4 week-monolayer culture. HiPSC-derived mesenchymal cells were differentiated into chondrocyte-like cells for 3 weeks in pellet culture using chondrogenic medium supplemented with TGF-β3 (10 ng/ml). That approach resulted in the formation of chondrocyte – like cells, what was confirmed by immunohistochemistry and gene expression analysis of markers characteristic for chondrocytes. The appropriate long culture period ensured significant decrease of pluripotency markers and resulted in non-teratoma regeneration of full-thickness of cartilage in rat knee [109].
Ko and colleagues (2014) established protocol that effectiveness was confirmed onto rat in vivo model. Firstly, the hiPSC cells were differentiated for 10 days via EBs. After that, EBs were dissociated and differentiated in micromass in chondrogenic medium supplemented with TGF-β3 (10 ng/ml) for 21 days. Those obtained cells revealed the characteristic features of hyaline cartilage. The gene expression of SOX9 and COL2A1 was elevated in differentiated cells demonstrating the obtaining of chondrocyte-like cells [23]. Moreover, the expression of genes (COL10A1, COL1A2 and RUNX2) responsible for hypertrophic cartilage was at very low level [110, 111].

**iPSC differentiation into osteoblast-like cells**

Although, osteoblast and osteocytes significantly differ from chondrocytes, the formation of these cell populations in vitro from SCs is based on very similar protocols. The combination of EBs formation and monolayer culture of pluripotent SCs are the most often used methods. The obtaining induced pluripotent stem cells derived mesenchymal stem cells (iPSC-MSC), is closely related to the first stages (e.g. formation of mesenchyme) of chondro – and osteogenesis naturally occurring in vivo during embryo development. In the majority of protocols, medium used for osteogenic differentiation is relatively similar to chondrogenic one. The well-defined osteogenic medium is consisted of L-ascorbic acid, dexamethasone (both cause synthesis of type II collagen, but concentration of dexamethasone is higher in osteogenic medium, than in chondrogenic one) and β-glycerophosphate (activates alkaline phosphatase, triggering the deposition of minerals into the bones). However, in some protocols the addition of 1,25 – dihydroxyvitamin D or GFs such as TGF-β and BMP family members remarkably improved osteogenic process in vitro [112-116]. In the osteoblast – like cells, the RUNX2 and Osterix – crucial regulators of mesenchyme differentiation into osteoblasts are widely evaluated. The presence of COL1A1, due to its high amount in the bone environment, is also commonly analysed to confirm the obtaining of osteoblast-like cells. Finally, in order to prove the maturation of osteoblasts accompanied by the organic and inorganic components, the alkaline phosphatase (ALP), OCN, BSP, and OPN gene expression are assessed. To demonstrate the desirable properties of obtained stem cell-derived osteoblasts and osteocytes, the immunohistochemistry analysis, such as staining alkaline phosphatase activity (intense dark blue colour observed in highly ALP active cells) and Alizarin red staining (intense red) is required.

The interesting results were obtained by Yamamoto et al. (2015) who carried out the direct induction of fibroblast cells into osteoblasts by transduction with the combination of four factors (ROXL): RUNX2, Osterix (both characteristic for osteogenic cells) OCT4 and L-MYC. That approach enabled avoiding the additional steps such as derivation of pluripotent SCs by transduction with Yamanaka factors and then differentiation them into desired cell lineage and this assures the direct receiving of osteoblast – cell like cells. The gene expression analysis (microarray analysis) and Alizarin Red S staining indicated high correlations between ROXL-derived and primary osteoblasts indicating the close similarity of these two cell populations. Finally, the use of osteoblast-like cells resulted in complete repair of femoral diaphysis in NOC/SCID mice. [117]. Tang and collaborators (2014) received osteoblasts from reprogrammed adult bone marrow CD34+ cells to hiPSCs. Then, the formed EB were differentiated into mesenchymal stem cell like lineage. For induction of osteogenic differentiation, the osteogenic medium with addition of 1,25 – dihydroxyvitamin was applied. During differentiation of hiPSC-MSC, the calcium phosphate cements (CPC) as a scaffold was also used. After differentiation, the cells cultivated on CPC in osteogenic medium indicated high deposition of minerals and the ALP, RUNX2, COL1A2, OCN high expression level. [114, 115].

Another approach involved receiving the osteoblast-like cells from 7 days EBs and subsequent 2 weeks monolayer culture. The exposition of cells to TGF-β, IGF-1 and FGF-2 improved osteogenic differentiation of hiPSCs. The selection of pure population of osteogenic cells based on expression of tissue-nonspecific alkaline phosphatase (TNAP) led to obtaining high amount of cells with an osteogenic potential. Further propagation of those derived cells in osteogenic medium for 40 days led to increased expression of genes related to osteoblasts such as OSX, RUNX2, COL1A1, OCN and BSP. Alizarin red staining and alkaline phosphatase activity confirmed osteogenic potential of obtaining cells [118].

**Conclusions**

The application of stem cells (SCs) in regenerative medicine has grown rapidly in recent years due to the enormous potential of these cells to treat a variety of disorders. Although numerous types of SCs exhibit differentiation potential, the development of human induced pluripotent stem cells (hiPSCs) is considered a turning point in tissue engineering. They have capacity to unrestricted self-renewal and capacity to develop into derivatives of all three primary germ layer (pluripotency). These properties make them a promising candidate for obtaining patient-specific neurons, cardiomyo-, chondro – and osteocytes. Nevertheless, the hiPSC – based therapy is a novel strategy and thus the further and intense research is required.

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**References**


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