Sadašnja perspektiva primjene matičnih stanica u liječenju zatajivanja srca

Current perspective of stem cells in the treatment of heart failure

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Zatajivanje srca (ZS), kao neminovna posljedica infarkta miokarda (IM) predstavlja vodeći uzrok pobola i smrtnosti u zapadnom svijetu. Suvremeno liječenje IM ima za cilj spašavanje subletalno ozlijeđenih kardiomiocita, no ono ne može spasiti već mrtvo tkivo. Regenerativna medicina temelji se na zamjeni stanice primjenom matičnih stanica i njihovih derivata te donosi nove mogućnosti: teoretski, bilo koje tkivo bi se moglo rekonstruirati transplantacijom matičnih stanica koje se mogu bilo ugraditi u oštećeno tkivo ili lučiti ljekovite molekule koje će poboljšati oštećenu funkciju tkiva. Nakon prilično izravne i na staničnoj razini prilično jednostavno mehaničke uloge stanica miokarda, srčane matične stanice predstavljaju vrlo zanimljiv i vrlo obećavajući pristup za poboljšanje ZS. U posljednja dva desetljeća prepoznate su različite vrste matičnih stanica te su opširno ispitivane kao moguće donatorske stanice za regeneraciju tkiva/organa. Pojavile su se i ostale moguće korisne primjene derivata matičnih stanica, kao što je generacija in vitro ljudskih eksperimentalnih modela. In vitro modeli nude testiranje novih hipoteza u pogledu patofizioloških mehanizama ili testiranja novih molekula s potencijalom liječenja u kontroliranom in vitro okruženju.

Suvremeni razvoj tehnologije matičnih stanica započeo je oko 1960. godine, kada su McCulloch i Till izveli prve uspješne transplantacije matičnih stanica koštane srži na pokusnim životinjama. Samo nekoliko godina poslije izvršene su prve uspješne transplantacija koštane srži u bolesnika. Bilo je potrebno još 20 godina da se izrade protokoli za izolaciju matičnih stanica iz drugih organa, a 90-tih godina nova era genetike omogućila je napraviti određene koktele i složene medije koji su najavili revoluciju u kultivaciji matičnih stanica. Usporedo s pričom o kloniranju i rođenju ovce Dolly, novo izolirane i dobro opisane linije matičnih stanica postaju moćan alat za prve transplantacije za bolesnike, uključujući i one sa srčanom ishemijom. Početkom 21. stoljeća, a posebno u posljednjih pet godina, nastupa nova era liječenja bolesti srca: diljem svijeta pokrenuta su klinička ispitivanja s matičnim stanicama za liječenje ishemije srca koja donose obećavajuće rezultate.

U odabranom preglednom članku autori predstavljaju najnovija dostignuća u bazičnim i translacijskim istraživanjima u vezi terapije matičnim stanicama kod kardiovaskularnih

Heart failure, as the inexorable consequence of myocardial infarction is a major cause of morbidity and mortality in the western world. Current conventional therapies of myocardial infarction aim for the rescue of sublethaly injured cardiomyocytes, but they cannot rescue already dead tissue. Regenerative medicine based on the cell replacement using stem cells and their derivatives holds a new promise: theoretically, any tissue could be repaired by transplantation of stem cells which can either integrate in the damaged tissue or secrete healing molecules which in turn improve impaired tissue function. Following rather straightforward and on cellular level rather simple mechanic role of cardiac muscle cells, cardiac stem cells represent a very interesting and highly promising approach for the improvement of the failing heart. In the past two decades various types of stem cells have been identified and extensively examined as potential donor cells for tissue/organ regeneration. Other potentially useful applications for stem cell derivatives, such as generation of in vitro human experimental models have emerged as well. In vitro models, for example, offer testing of new hypotheses in regard to pathophysiological mechanisms or testing of new molecules with healing potential in controlled in vitro environment.

Stem cell technology started its modern development in 1960s, when McCulloch and Till performed first successful transplantations of bone marrow stem cells to experimental animals. Only few years after, the first successful bone marrow transplantations to the patients have been performed. It was needed 20 years more to develop protocols for isolation of stem cells from other organs and in 1990s new era of genetics allowed us to make a specific cocktails and complex media which announced a revolution in stem cell cultivation. In parallel with cloning story and birth of the sheep Dolly, newly isolated and well described stem cell lines become a powerful tool for the first transplantations to human patients, including those ones with heart ischemia. In 2000s and especially in the last five years we entered a new era in treatment of heart diseases: clinical trials with stem cells for heart ischemia are being launched all-around the world and they are yielding promising results.

In the selected review article the authors are presenting the recent advances in basic and translational research concer-

bolesti[1]. U posljednjih nekoliko godina opsežna bazična istraživanja su dovela do brze implementacije znanstvenih laboratorijskih dostignuća u klinička istraživanja po principu dvosmjernog povezivanja laboratorija i bolesničke postelje. Ovo je prepoznatljivo baš u primjeni istraživanja matičnih stanica u kliničkoj kardiologiji. Nekoliko kliničkih istraživanja koja se bave transplantacijom autolognih mononukleara iz koštane srži i njezinih podskupina (matična stanica odraslih, ASC), npr. hematopoetskih matičnih stanica, mezenhimalnih matičnih stanica i endotelnih progenitorskih stanica kod bolesti srca dostigle su fazu I i II te najnoviji razvoj predstavlja napredak u fazu III. Nejasno je kakvu dobrobit donose presađene matične stanice jer one mogu dovesti do novih kardiomiocita kada se presađuju životinjskim modelima, a mogu se otkriti samo do 8 tjedana nakon transplantacije. Stoga se vjeruje da presađene matične stanice stimuliraju proliferaciju i diferencijaciju srčanih matičnih stanica domaćina, a ne postaju značajan izvor novih kardiomiocita. Druga strategija je izvršena u bazičnim istraživanjima gdje su matične stanice *in vitro* bile prvo diferencirane u kardiomiocite, a potom su transplantirane u srce životnjskogh modela čija je funkcija bila oslabljena[2]. Ovaj pristup je pokazao bolje ishode od transplantacije nediferenciranih matičnih stanica. Međutim, transplantacija diferenciranih kardiomiocita trenutno nije u središtu kliničkih ispitivanja. Osim suspenzije pojedinačnih stanica, testira se i transplantacija prethodno formiranih 3D zakrpa tkiva koja se sastoji od izvanstaničnog matriksa, progenitorskih srčanih stanica i vaskularnih elemenata na životinjskim modelima s promjenjivim uspjehom i aritmogenosti kao glavnom preprekom[3].

Najčešće se matične stanice klasificiraju prema njihovom podrijetlu kao embrionalne matične stanice (ESC) i ASC. ESC su izolirane od rane blastociste, a imaju sposobnost neodređenog samoobnavljanja ili diferencijacije u derivate svih triju zametnih slojeva, uključujući kardiomiocite[4]. Iako ljudski ESC imaju najveću mogućnost diferencijacije te se stoga smatraju zlatnim standardom u biologiji matičnih stanica, njihova uporaba je opstruirana mnogim pitanjima, uključujući etička pitanja, učinkovite derivacijske protokole, optimalne metode uzgoja, funkcionalnu sposobnost izvedenih kardiomiocita, imunološko odbacivanje i tumorogenost.

ASC obuhvaćaju skupinu od nekoliko tipova matičnih stanica koje se nalaze u ljudskim tkivima i organima. One se razlikuju u svojoj sposobnosti diferencijacije, npr. matične stanice koštane srži se smatraju pluripotentnim jer se može razviti nekoliko različitih vrsta krvnih stanica, dok se rezidentne matične stanice tkiva smatraju unipotentnim i mogu se diferencirati u samo jednu vrstu stanice. Iako imaju upitnu diferencijaciju potencijalni ASC-i nisu popraćeni moralnim pitanjima i pitanjimaa vezanih za imunogenost i odbacivanje presatka. Ipak, ASC-i su samo skupina matičnih stanica koje se koriste u kliničkim ispitivanjima za rekonstrukciju srca sa zastojem koji su dosegli fazu III kliničkih ispitivanja[1]. Prikazana je sigurnost i primjenjivost skupine ASC u terapiji matičnim stanicama kod infarkta miokarda, a izvješća o regeratornim sposobnostima su manje dosljedna.

Značajan napredak je učinjen razvojem induciranih pluripotentnih matičnih stanica (iPS) za koje je Yamanaka dobio Nobelovu nagradu 2012. godine[5]. iPS stanice su genetski proizvedene stanične linije generirane uvođenjem pluripotentnih čimbenika (npr. Oct4, NANOG, Sox2, Lin28 itd.) u već terminalno diferencirane stanice kao što su fibroblasti. Te stanice predstavljaju obnovljivi izvor autolognih stanica koje mogu nastati od pojedinog bolesnika. Dakle, ljudske iPS stanice su lako dostupne i nisu ograničene etičkim pitanjima i mogućim imunogenim opterećenjem. Međutim, potencijal diferenciranja iPS stanica je još uvijek u fazi opsežnih istraživanja i mnoge studije se trenutno bave njihovom

ning the cell therapy in cardiovascular disease1. In the recent years the extensive basic research efforts has lead to fast translation from bench to bedside that is recognizable in the application of stem cell research in clinical cardiology. Several clinical trials which are testing transplantation of autologous bone marrow mononuclear cells and its subgroups (adult stem cells, ASCs), e.g. hematopoietic stem cells, mesenchymal stem cells and endothelial progenitor cells in cardiac disease have reached the phase I and II and the most recent development is advancement to phase III. It is unclear what benefit carry engrafted stem cells since they can give rise to new cardiomyocytes when transplanted to animal models, but they can be detected only up to 8 weeks following the transplantation. It is therefore believed that engrafted stem cells stimulate proliferation and differentiation of resident cardiac stem cells rather than becoming the significant source of new cardiomyocytes. Another strategy has been taken in basic research where stem cells were first i*n vitro* differentiated into cardiomyocytes and then transplanted into failing animal heart[2]. This approach showed superior outcome over transplantation of undifferentiated stem cells. However, transplantation of differentiated cardiomyocytes is not a major focus of clinical trials at the moment. In addition to suspension of single cells, transplantation of preformed 3D tissue patches consisting of extracellular matrix, progenitor cardiac cells and vascular elements have also tested in animal models with variable success and arrythmogenicity as a major obstacle[3].

Most commonly stem cells are classified according to their origin as embryonic stem cells (ESCs) and ASCs. ESCs are isolated from an early blastocyst. They have ability for indefinite self-renewal or differentiation into derivatives of all three germ layers, including cardiomyocytes[4]. Although human ESCs have the greatest differentiation potential, and therefore considered as gold standard in stem cell biology, their use is hampered by many issues, including ethical concerns, efficient derivation protocols, optimal culturing methods, functional competence of derived cardiomyocites, immune rejection and tumorigenicity.

Adult stem cells (ASCs) comprise a group of several types of stem cells that reside in human tissues and organs. They are distinct in their differentiation potential, e.g. bone marrow stem cells are considered to be multipotent giving rise to several different blood cell types, while resident tissue stem cells are considered to be unipotent and being able to differentiate into only one cell type. While having questionable differentiation potential ASCs are also free of ethical issues and questions of immunogenicity and graft rejection. Nevertheless, ASCs are only group of stem cells used in clinical trials for the repair of failing heart that reached phase III clinical trial[1]. The safety and feasibility of ASCs groups of stem cells in the cell therapy in myocardial infarction have been demonstrated, however the reports on the regeneration efficacy have been less consistent.

A significant advancement was made by generation of induced pluripotent stem (iPS) cells for which Yamanaka received Nobel prize in 2012[5]. iPS cells are genetically engineered cell lines generated by introducing factors of pluripotency (e.g. Oct4, NANOG, Sox2, Lin28 etc.) into already terminally differentiated cells such as fibroblasts. These cells represent a renewable source of autologous cells as can be generated from the individual patient. Thus, human iPS cell are easily available and not hampered by ethical and probably immunogenicity issues. However, differentiation potential of iPS cells is still under extensive investigation and many studies are currently addressing their genetic stability, differentiation potential, culturing methods, immunogenicity and tumorigenicity. Another problem with the use of iPSs for

genetskom stabilnošću, sposobnošću diferenciranja, metodama uzgoja te mogućim nastankom imunogenosti i i tumorogenosti. Još jedan problem u vezi korištenja iPS za regeneraciju miokarda je primjena virusnih vektora za uvođenje čimbenika matičnosti koji su ugrađeni u DNK i mijenjanju genom domaćina. Ovaj problem se moguće može zaobići izravnim uvođenjem matičnih proteina umjesto kodiranja sekvenci primjenom virusnih vektora. Naša nedavna studija pokazala je učinkovitu proizvodnju ljudskih iPS stanica reprogramiranjem fibroblasta iz prepucija uz uvođenje neepizomalnih plazmida koji samostalno kodiraju četiri čimbenika reprogramiranja, OCT4, NANOG, SOX2 and LIN28[6].

Medicinski fakultet Sveučilišta u Zagrebu prepoznao je potrebu razvoja istraživanja primjenom matičnih stanica. Trenutno postoji nekoliko skupina istraživača na Medicinskom fakultetu u Zagrebu koji istražuju mogućnosti i potencijal primjene s ciljem svakodnevnog praćenja brzog napretka ove tehnologije. Osim laboratorija za matične stanice na Hrvatskom institutu za istraživanje mozga koji se najviše bavi regenerativnim potencijalom matičnih stanica u ishemiji tkiva mozga[7], postoji još nekoliko skupina koje primjenjuju matične stanice u istraživanju regeneracije kosti i srca.

U suradnji i uz podršku dr. Željka J. Bošnjaka sa Medical College of Wisconsin, u čijem je laboratoriju je izvršena većina pokusa, naši istraživački napori usmjereni na razvoj eksperimentalnih ljudskih staničnih modela generiranih iz matičnih stanica koji, za razliku od eksperimentalnih životinjskih modela, pouzdano rekapituliraju ljudski, na genotipu, temeljen fenotip kardiomiocita. Otkrili smo da prethodna obrada s inhalacijskim anestetikom izofluranom pruža usporedive odgovore i citoprotekciju u ljudskim kardiomiocitima dobivenih iz matičnih stanica i odraslim ljudskim stanicama te stanicama srca štakora[8]. Štoviše, pomoću IPS-kardiomiocita dobivenih od dijabetičara tipa 2, otkrili smo neučinkovitost prethodne obrade u dijabetičara zbog djelomičnog blokiranja dijabetičkim fenotipom i visokim vrijednostima glukoze koji pojačavaju negativne učinke[9]. Također smo proveli opsežnu karakterizaciju kardiomiogeneze kod ljudskih iPS stanica kao dijela globalnih napora za uvođenje ljudskih iPS stanica u translacijska istraživanja i regenerativnu medicinu[10]. Poboljšali smo metodologiju "usmjeravanja diferencijacije" matičnih stanica u kardiomiocite koja se temelji na endodermski induciranom kardiomiogenskom signalizacijom s koštanim morfogenetskim proteinom-4, faktorom rasta fibroblasta i aktivinom-A. Ova vrlo učinkovita metodologija daje 60 i 80% kardiomiocita u razbijanju nakupina stanica bilo kada se koriste ljudske iPS stanice kao i ESC-i. Iako je kardiomiogeneza manje učinkovita u ljudskim iPS stanicama nego u ESC, opsežno profiliranje genske ekspresije ukazalo je na usporedivu kardiomiogenezu u tim linijama dodatno potvrđujući sposobnost ljudskih iPS stanica za učinkovitu kardiomiogenezu i in vitro modeliranje. Naš trenutni interes u istraživanju matičnih stanica je usmjeren na detaljno ispitivanje mehanizama koji uzrokuju neučinkovitost prethodne obrade kod dijabetičara koristeći kardiomiocite diferencirane od iPS stanica koje se dobivaju od dijabetičara.

Iako se suočavamo sa ograničenjima u financiranju istraživanja, znanstvenici uključeni u istraživanje matičnih stanica nastavljaju razvijati ovu metodologiju na Medicinskom fakultetu u Zagrebu. Nedavno nagrađeni europski projekti i uska suradnja s brojnim skupinama iz EU i SAD pomažu održati korak s istraživanjima matičnih stanica u većini naprednih zemalja.

myocardial regeneration is the use of viral vectors for introduction of stemness factors that incorporate into DNA and alter host genome. This issue can be potentially circumvented by direct introduction of stemness proteins instead of their coding sequence with viral vectors. Our recent study demonstrated efficient generation of human iPS cells by reprogramming foreskin fibroblasts with introduction of non-episomal plasmids which independently encode four reprogramming factors, OCT4, NANOG, SOX2 and LIN28[6].

The School of Medicine University of Zagreb recognized a need for development of research aimed toward stem cells applications. Currently there are several groups at Zagreb School of Medicine which are investing their potential and manpower with a goal to follow a fast progress of this technology on an everyday basis. Apart from Laboratory for Stem Cells at Croatian Institute for Brain Research which is mostly focused on regenerative potential of stem cells in the ischemia of brain tissue[7], there are several more groups which use stem cells in research focused on regeneration of bone and heart.

In collaboration and support from Dr. Zeljko J. Bosnjak, Medical College of Wisconsin, in whose laboratory majority of experiments have been performed, our research efforts focused on development of experimental human cell models generated from stem cells that, unlike experimental animal models, reliably recapitulate human genotype- driven cardiomyocyte phenotype. We found that preconditioning with inhalational anesthetic isoflurane elicits comparable responses and cytoprotection in human stem cell-derived cardiomyocytes and adult human and rat cardiac cells[8]. Moreover, using iPS-cardiomyocytes derived from type 2 diabetic patients, we found that inefficient preconditioning in diabetics is in part blocked by diabetic phenotype and that high ambient glucose exacerbates the negative effects[9]. We also conducted extensive characterization of cardiomyogenesis in human iPS cells as a part of global efforts in introducing human iPS cells to translational research and regenerative medicine10. We have improved methodology of "directing differentiation" of stem cells into cardiomyocytes, which is based on endoderm-induced cardiomyogenic signaling with bone morphogenetic protein-4, fibroblast growth factor and activin-A. This highly efficient methodology yields 60 and 80% of cardiomyocytes in beating cell clusters when human iPS cells and ESCs are used, respectively. Although cardiomyogenesis is less efficient in human iPS cells than in ESCs, the extensive gene expression profiling demonstrated comparable cardiomyogenesis in these lines further corroborating competence of human iPS cells for efficient cardiomyogenesis and in vitro modeling. Our current interest in stem cell research is aimed at dissecting mechanisms underlying inefficient preconditioning in diabetics using cardiomyocytes differentiated from iPS cells that are generated from diabetic patients.

Although faced with restrictions in research funding, scientists involved in stem cell research will continue to develop this methodology at Zagreb School of Medicine. Recently awarded European projects and strong collaborations with numerous groups from EU and USA are helping to keep a pace with stem cell research in the most advanced countries.

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