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Rozwojowo-zależna indukcja biogenezy mitochondriów podczas różnicowania neuralnego ludzkich indukowanych pluripotencjalnych komórek macierzystych (hiPSC)

Rozprawa na stopień naukowy doktora nauk medycznych

w dyscyplinie biologia medyczna

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1. Augustyniak J, Zychowicz M, Podobińska M, Barta T, Bużańska L., Reprogramming of somatic cells: possible methods to derive safe, clinical-grade human induced pluripotent stem cells., *Acta Neurobiol Exp (Wars).* 2014;74(4):373-82., Review, pięcioletni IF=1,631
2. Augustyniak J, Lenart J, Zychowicz M, Lipka G, Gaj P, Kolanowska M, Stępień PP, Bużańska L.; Sensitivity of hiPSC-derived neural stem cells (NSC) to Pyrroloquinoline quinone depends on their developmental stage., Toxicol In Vitro. 2017 Dec;45(Pt 3):434-444., pięcioletni IF=3,197
3. Augustyniak J, Lenart J, Zychowicz M, Stępień PP, Bużańska L., Mitochondrial biogenesis and neural differentiation of human iPSC is modulated by idebenone in a developmental stage-dependent manner.; *Biogerontology*. 2017 Aug;18(4):665-677, pięcioletni IF=3,581

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Wykaz skrótów używanych w rozprawie doktorskiej

AAS – *(ang. Antibiotic Antimycotic Solution),* zestaw antybiotyków do hodowli komórkowej;

ACTB – (ang. β-Actin), gen kodujący β-aktynę;

ALDH1L1 – (*ang. Aldehyde Dehydrogenase 1 Family Member L1*), gen kodujący dehydrogenazę aldehydową L1;

AP – (*ang. Astrocyte Progenitors*), progenitory astrocytarne;

ATP – (*ang. Adenosine Triphosphate*), adenozyno-5′-trifosforan, adenozynotriofosforan;

B27 – suplement stosowany *in vitro* do różnicowania progenitorów neuralnych w kierunku neuronalnym;

bFGF – (*ang. basic Fibroblast Growth Factor),* zasadowy czynnik wzrostu fibroblastów;

CAPN10 – (*ang. Calpain 10*), gen kodujący kalpainę 10;

CCNG1 – (*ang. Cyclin G1*), gen kodujący cyklinę G1;

CoQ10 – (ang. Coenzyme Q10), koenzym Q10;

COX-1 – (*ang. Mitochondrially Encoded Cytochrome C Oxidase I*), oksydaza 1 cytochromu c, białko IV kompleksu łańcucha oddechowego ETC;

CSPG4 – (*ang. Chondroitin Sulfate Proteoglycan 4*), gen kodujący proteoglikan siarczanu chondroityny 4;

DCFH-DA – (*ang. Ddichloro-Dihydro-Fluorescein Diacetate*), dioctan dichloro-dihydro fluoresceiny;

DCX – (*ang. Neuronal Migration Protein Doublecortin*), białko związane z mikrotubulami;

DCX – (*ang. Neuronal Migration Protein Doublecortin*), gen kodujący białko związane z mikrotubulami;

DNA – (*ang. Deoxyribonucleic Acid*), kwas deoksyrybonukleinowy;

EEF1A1 – (*ang. Eukaryotic Translation Elongation Factor 1 Alpha 1*), gen kodujący Eukariotyczny Czynnik Elongacji Translacji;

EGF – (*ang. Epidermal Growth Factor),* naskórkowy czynnik wzrostu*;*

EID2 – (ang. EP300 Interacting Inhibitor Of Differentiation 2), gen kodujący inhibitor różnicowania 2 oddziałujący z białkiem EP300;

eNP – (*ang. early Neural Progenitors*), wczesne progenitory neuralne;

ESC – (*ang. Embryonic Stem Cells*), zarodkowe komórki macierzyste;

ETC – (*ang. Electron Transport Chain*), łańcuch transportu elektronów, łańcuch oddechowy;

GAPDH – (*ang. Glyceraldehyde 3-Phosphate Dehydrogenase),* gen kodujący dehydrogenazę aldehydu 3-fosfoglicerynowego;

GFAP – (*ang. Glial Fibrillary Acidic Protein*), glejowe kwaśne białko włókienkowe;

GFAP – (*ang. Glial Fibrillary Acidic Protein*), gen kodujący glejowe kwaśne białko włókienkowe;

hESC – (*ang. human Embryonic Stem Cells*), ludzkie zarodkowe komórki macierzyste;

hiPSC – (*ang. human induced Pluripotent Stem Cells*), ludzkie indukowane pluripotencjalne komórki macierzyste;

hiPSC-eNP – komórki eNP otrzymane przez różnicowanie neuralne komórek hiPSC;

hiPSC-NP – komórki NP otrzymane przez różnicowanie neuralne komórek hiPSC;

hiPSC-NSC – komórki NSC otrzymane przez różnicowanie neuralne komórek hiPSC;

HPRT1 – (*ang. Hypoxanthine Phosphoribosyltransferase1*), gen kodujący fosforybozylotransferazę hipoksantynową;

IDB – *(ang. Idebenone),* idebenon, agonista receptora PPARα oraz PPARγ; analog koenzymu Q10 o właściwościach przeciwutleniających, wykazujący zdolność do stymulacji biogenezy mitochondriów w różnych typach komórek;

iPSC – (*ang. induced Pluripotent Stem Cells*), indukowane pluripotencjalne komórki macierzyste;

MAP2 – (*ang. Microtubule-Associated Protein 2*), białko stabilizujące mikrotubule;

MAP2 – (*ang. Microtubule-Associated Protein 2*), gen kodujący białko stabilizujące mikrotubule;

miRNA- małe niekodujące RNA*;*

MKI67 – (*ang. Proliferation Marker Protein Ki-67*), gen kodujący antygen komórek proliferujących, białko obecne w jądrach komórek aktywnych mitotycznie;

Ki67 - (ang. Proliferation Marker Protein Ki-67), antygen komórek proliferujących, białko obecne w jądrach komórek aktywnych mitotycznie;

mRNA – (*ang. messenger RNA*), matrycowy RNA;

MT-CO1 – (ang. Mitochondrially Encoded Cytochrome C Oxidase I (*COX1*)), gen kodujący oksydazę I cytochromu c;

mtDNA – (*ang. mitochondrial DNA*), DNA mitochondrialny;

MYC – (*ang. MYC Proto-Oncogene*), gen kodujący protoonkogen c-MYC;

N2 – suplement stosowany do różnicowania *in vitro* mysich oraz ludzkich komórek PSC w kierunku neuralnym oraz neuronalnym;

NANOG – (*ang. Homeobox protein NANOG - Pluripotency Marker, Transcription Factor,*), gen kodujący czynnik transkrypcyjny NANOG;

NAT1 – (*ang. N-Acetyltransferase1*), gen kodujący N-acetylotransferazę 1;

ND1 – (*ang. Mitochondrially Encoded NADH, Ubiquinone Oxidoreductase Core Subunit 1*), gen kodujący podjednostkę 1 dehydrogenazy NADH;

ND5 – (*ang. Mitochondrial Membrane Respiratory Chain NADH - Ubiquinone Oxidoreductase Chain*), gen kodujący podjednostkę 5 dehydrogenazy NADH;

NEFH – (*ang. Neurofilament Protein, Heavy Chain*), gen kodujący ciężki łańcuch neurofilamentów pośrednich;

NEFL – (*ang. Neurofilament Protein, Light Chain*), gen kodujący lekki łańcuch neurofilamentów pośrednich;

NES – (*ang. Nestin*), gen kodujący nestynę;

NES – (ang. Nestin), nestyna;

NEUROD1 – (*ang. Neurogenic Differentiation Factor 1, Neuronal Differentiation 1*), gen kodujący czynnik różnicowania neurogennego;

NF200 – *(ang. Neurofilament 200*), białko neurofilamentu o masie cząsteczkowej 200 kDa;

NP – (*ang. Neural Progenitors*), progenitory neuralne;

NRF1 – (ang. Nuclear Respiratory Factor-1), gen kodujący jądrowy czynnik oddechowy 1;

NSC – (*ang. Neural Stem Cells*), neuralne komórki macierzyste;

OP – (*ang. Oligodendrocyte Progenitors*), progenitory oligodendrocytarne;

OXPHOS – (*ang. Oxidative phosphorylation*), fosforylacja oksydacyjna;

PCR – *(ang. Polymerase Chain Reaction),* reakcja łańcuchowa polimerazy*;*

PGC-1α – (*ang. Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-alpha*), koaktywator 1α receptora γ aktywowanego przez proliferatory peroksysomów;

PHB – (ang. Prohibitin), gen kodujący prohibitynę;

POU5F1 – (ang. POU Class 5 Homeobox 1), gen kodujący czynnik transkrypcyjny OCT3/4, OCT4;

PPAR – *(ang. Peroxisome Proliferator-Activated Receptors),* receptory aktywowane przez proliferatory peroksysomów*;*

PPARA – (*ang. Peroxisome Proliferator Activated Receptor Alph*a), gen kodujący receptor aktywowany przez proliferatory peroksysomów alfa (PPARα);

PPARD – (*ang. Peroxisome Proliferator Activated Receptor Delta),* gen kodujący receptor aktywowany przez proliferatory peroksysomów delta (PPARβ/δ)*;*

PPARG – (*ang. Peroxisome Proliferator Activated Receptor Gamma*), gen kodujący receptor aktywowany przez proliferatory peroksysomów gamma (PPARγ);

PPARGC1A – gen kodujący koaktywator (PGC-1α);

PQQ – (*ang. Pyrroloquinoline quinone*), pirolochinolinochinon (2,7,9-Tricarboxy-1H-Pyrrolo-(2,3-f)Quinoline-4,5-Dione, Methoxatin), agonista receptora PPARα; analog koenzymu Q10 o właściwościach przeciwutleniających, wykazujący zdolność do stymulacji biogenezy mitochondriów w różnych typach komórek;

PRKAA1 – (*ang. Protein Kinase AMP-Activated Catalytic Subunit Alpha 1*), gen kodujący białko kinazy białkowej, zależnej od kinazy AMPK;

PSC – (*ang. Pluripotent Stem Cells*), pluripotencjalne komórki macierzyste;

qPCR – *(ang. quantitative Polymerase Chain Reaction),* ilościowa reakcja łańcuchowej polimerazy*;*

qRT-PCR – (*ang. quantitative Reverse Transcription Polymerase Chain Reaction*), ilościowa reakcja łańcuchowa polimerazy na matrycy cDNA otrzymanej w reakcji odwrotnej transkrypcji*;*

RABEP2 – (*ang. Rabaptin, RAB GTPase Binding Effector Protein 2*), gen kodujący rabaptynę;

RFT – *(ang. Reactive Oxygen Species, ROS),* reaktywne formy tlenu*;*

RNA – (*ang. Ribonucleic Acid*), kwas rybonukleinowy;

RNA-seq – *(ang. RNA sequencing),* sekwencjonowanie RNA*;*

RPLPO – (*ang. Ribosomal Protein Lateral Stalk Subunit P0*), gen kodujący białko wchodzące w skład dużej podjednostki rybosomu;

RT – *(ang. Reverse Transcription),* reakcja odwrotnej transkrypcji*;*

RT-PCR – *(ang. Reverse Transcription Polymerase Chain Reaction),* reakcja łańcuchowa polimerazy na matrycy cDNA otrzymanej w reakcji odwrotnej transkrypcji*;*

SC – *(ang. Stem Cells*), komórki macierzyste;

Mitotracker® red CMXRos – barwnik fluorescencyjny, stosowany do barwienia mitochondriów w żywych komórkach, jego akumulacja zależy od ΔΨm;

SDHA – (*ang. Succinate Dehydrogenase Complex Flavoprotein Subunit A, Subunit of Succinate-Ubiquinone Oxidoreductase Complex of the Mitochondrial Respiratory Chain*), podjednostka A dehydrogenazy bursztynianowej, białko II kompleksu łańcucha oddechowego (ETC);

SDHA – (*ang. Succinate Dehydrogenase Complex Flavoprotein Subunit A, Subunit of Succinate-Ubiquinone Oxidoreductase Complex of the Mitochondrial Respiratory Chain*), gen kodujący podjednostkę A dehydrogenazy bursztynianowej, białko II kompleksu łańcucha oddechowego (ETC);

SERPINA1 – (ang. Alpha-1-Antitrypsin (AAT), also known as Protease Inhibitor (PI)), gen kodujący serpinę 1;

SIRT1 – (ang. Sirtuin 1), gen kodujący sirtuinę 1*;*

SLCO2B1 – (*ang. Solute Carrier Organic Anion Transporter Family, member 2B1*), gen kodujący polipeptyd transportujący aniony organiczne 2B1;

SOX2 – (*ang. Pluripotency Marker, Transcription Factor, SRY (Sex Determining Region Y)-Box 2*), gen kodujący czynnik transkrypcyjny SOX2;

TBP – (ang. TATA-Box Binding Protein), gen kodujący białko wiążące kasetę TATA;

TFAM – (*ang. Mitochondrial Transcription Factor A*), mitochondrialny czynnik transkrypcyjny A;

TUBB3 – (*ang. Tubulin Beta 3 Class III*), gen kodujący β-tubulinę III, neuronalne białko neurofilamentowe;

β-tubulina III - (*ang. Tubulin Beta 3 Class III*), neuronalne białko neurofilamentowe;

UBC – (ang. Ubiquitin C), gen kodujący ubikwitynę C;

ZNF324B – (ang. Zinc Finger Protein 324B), gen kodujący białko zawierające motyw palca cynkowego 324B;

ΔΨm – (*ang. Mitochondrial Membrane Potential*), potencjał błony mitochondrialnej;

1. Streszczenie polskojęzyczne i anglojęzyczne

Streszczenie

Odkrycie, że komórki somatyczne można reprogramować do indukowanych pluripotencjalnych komórek macierzystych (iPSC, *ang. induced Pluripotent Stem Cells*) pozwoliło na znaczny rozwój badań w dziedzinie medyczny regeneracyjnej, jak również badań toksykologicznych oraz farmakologicznych *in vitro*. Postęp w otrzymywaniu ludzkich iPSC (hiPSC, *ang. human iPSC*) tzw. „bezpiecznymi metodami" tj. bez integracji transgenu do genomu gospodarza (np. stosując mRNA, białka rekombinowane, miRNA, wektory episomalne) przyczynił się do wykorzystania tych komórek w spersonalizowanej terapii komórkowej. Dodatkowo hiPSC stanowią „niekontrowersyjną etycznie" alternatywę dla ludzkich zarodkowych komórek macierzystych (hESC, *ang. human Embryonic Stem Cells*) w modelowaniu *in vitro* wczesnych etapów rozwoju człowieka dzięki zdolności do różnicowania we wszystkie tkanki organizmu. W cyklu publikacji stanowiących rozprawę doktorską przedstawiono wyniki oceny wpływu stymulacji biogenezy mitochondriów na wczesne etapy różnicowania neuralnego komórek hiPSC. W tym celu z hiPSC otrzymano trzy populacje komórkowe: neuralne komórki macierzyste (NSC, *ang. Neural Stem Cells*), wczesne progenitory neuralne (eNP, *ang. early Neural Progenitors*) oraz progenitory neuralne (NP, *ang. Neural Progenitors*), przy czym komórki eNP scharakteryzowano po raz pierwszy. Badane populacje komórkowe różniły się istotnie pod względem ekspresji markerów typowych dla rozwoju neuralnego na poziomie mRNA i białek. Komórki NSC, eNP oraz NP zostały poddane ekspozycji na substancje indukujące biogenezę mitochondriów: pirolochinolinochinon (PQQ) lub idebenon (IDB). Pod wpływem tych związków wykazano istotne zmiany w parametrach ważnych dla procesów życiowych komórki: żywotności, poziomie wolnych rodników (RFT), potencjale błony mitochondrialnej (ΔΨm) oraz ekspresji genów związanych z regulacją biogenezy mitochondriów: *NRF1, PPARGC1A, TFAM*. Wzrostowi ekspresji tych genów towarzyszył wzrost pozostałych badanych markerów biogenezy mitochondriów: liczby kopii mitochondrialnego DNA (mtDNA) oraz ekspresji białek SDHA i COX-1, wyłącznie w stadium eNP. W komórkach NSC i eNP, po inkubacji z PQQ zanotowano równoczesny wzrost ekspresji genu *PPARGC1A* oraz markera astrocytów *GFAP*, przy jednoczesnym spadku ekspresji markera neuronalnego *MAP2*. IDB działał podobnie we wszystkich stadiach, z wyjątkiem eNP, gdzie stymulował ekspresję zarówno *GFAP* jak i *MAP2*. Powyższe wyniki świadczą o istnieniu w stadium eNP „przedziału wrażliwości rozwojowej" na PQQ oraz IDB, a także o pozytywnym wpływie stymulacji biogenezy mitochondriów na różnicowanie komórek hiPSC w kierunku astrocytarnym.

Abstract

Human induced pluripotent stem cells (hiPSC) generated from somatic cells through genetic reprogramming influenced greatly development of basic research in regenerative medicine as well as *in vitro* toxicology and pharmacology field. The progress in the "safe methods" of hiPSC generation (without the integration of the transgene into the host genome, eg., mRNA, recombinant proteins, miRNA's, episomal vectors) gave an opportunity to use this cells in personalized cell therapy. In addition, the hiPSC serve as ethically non-controversial *in vitro* model of early human development which is an alternative to the model of human embryonic stem cells (hESC). The cycle of publications chosen for the theses investigates the influence of stimulation of mitochondrial biogenesis on the early stages of hiPSC neural differentiation. In this study, neural differentiation of hiPSC resulted in obtaining three distinct cells populations: neural stem cells (NSC), early neural progenitors (eNP), and neural progenitors (NP) however, the population of eNP cells has been characterized for the first time. Analysis of the gene and protein expression have shown that NSC, eNP and NP cell populations were significantly different in the level of unique markers for early neural development. The obtained cell populations were investigated for their sensitivity to compounds stimulating the mitochondrial biogenesis: Pyrroloquinoline quinone (PQQ) or idebenone (IDB), which were added independently. The results revealed significant changes in the cells viability, free radical level (ROS) and mitochondrial membrane potential ($\Delta\Psi m$) upon the treatment with PQQ and IDB in all tested populations. The expression of genes related with mitochondrial biogenesis regulation: *NRF1*, *PPARGC1A* and *TFAM* were also significantly different. However exclusively at the eNP stage, after incubation with PQQ and IDB, all markers indicating stimulation of mitochondrial biogenesis were significantly elevated. This included upregulation of *NRF1*, *PPARGC1A*, *TFAM* gene expression, increased number of copies of mitochondrial DNA (mtDNA) and significant elevation of expression of proteins important for mitochondrial function: COX-1 and SDHA. Gene expression analysis of neural differentiation upon PQQ treatment revealed in NSC and eNP stages of development simultaneous increase in expression of *PPARGC1A* (main regulator of mitochondrial biogenesis) and astrocyte marker *GFAP* accompanied with repression of the neuronal marker *MAP2*. IDB in all stages of development yielded a similar effect with the exception of eNP, where stimulation of the expression of both *GFAP* and *MAP2* was observed, although the increase in GFAP expression was higher. The above data demonstrate the existence of developmental "window of sensitivity" for investigated factors (PQQ, IDB) inducing mitochondrial biogenesis at eNP stage of development and the possibility of influencing of the neural differentiation pathways via PQQ and IDB in favour of astrocytic fate.

2. Innowacyjność rozprawy

Po raz pierwszy:

- wyodrębniono oraz scharakteryzowano podczas różnicowania neuralnego hiPSC wczesne stadium rozwoju neuralnego: eNP, stanowiące stadium pośrednie między NSC a NP,
- zaprojektowano panel genów referencyjnych dla komórek NSC, eNP oraz NP w sposób umożliwiający ich prawidłową charakterystykę molekularną,
- udowodniono zależną od stadium zróżnicowania neuralnego wrażliwość komórek NSC, eNP, NP na PQQ i IDB, oraz wykazano:
- powiązanie między efektywnością stymulacji biogenezy mitochondriów, a stadium rozwojowym komórek hiPSC i określono „przedział wrażliwości rozwojowej" na badane czynniki indukujące biogenezę mitochondriów w stadium eNP,
- znaczenie indukcji biogenezy mitochondriów dla różnicowania neuralnego komórek hiPSC,
- wpływ PQQ oraz IDB na różnicowanie eNP w kierunku astrocytarnym (PQQ) oraz neuronalnym i astrocytarnym (IDB),
- molekularny związek stymulacji biogenezy mitochondriów z kierunkiem różnicowania astrocytarnego (wzrostowi ekspresji genu *PPARGC1A* zawsze towarzyszył wzrost ekspresji genu *GFAP*).

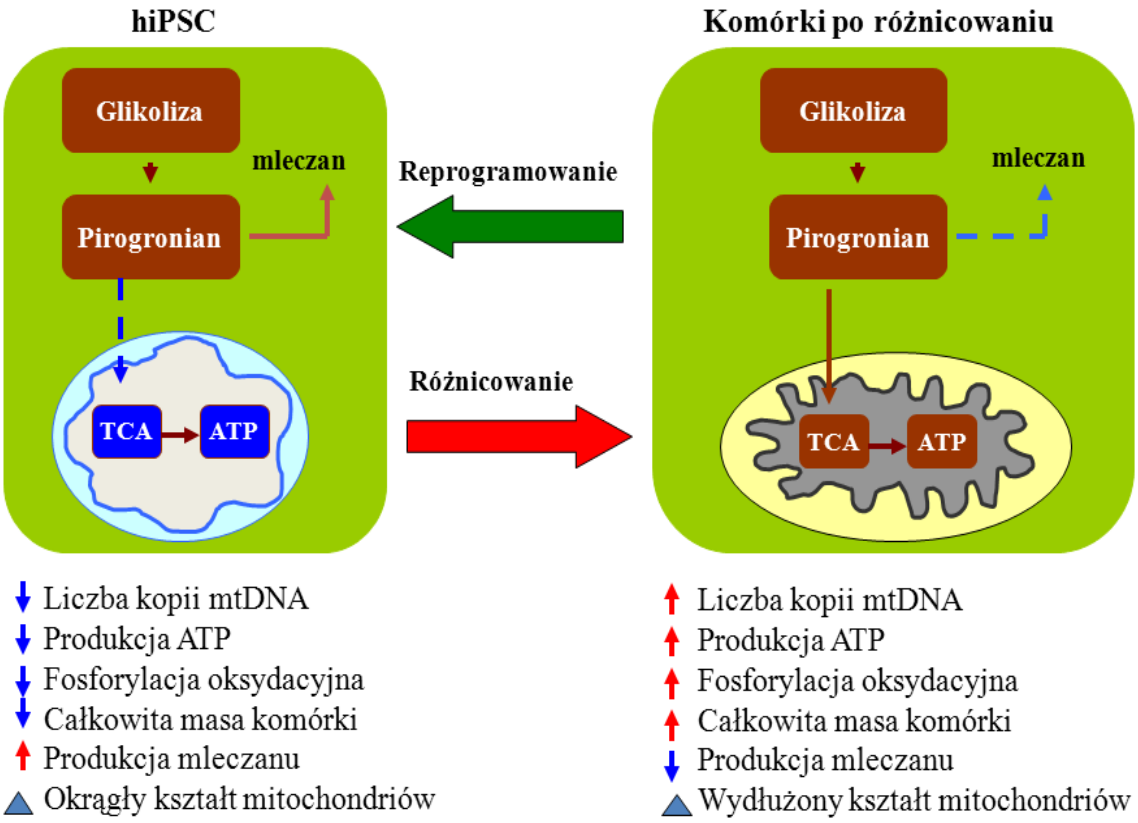
3. Wstęp

Ludzkie indukowane pluripotencjalne komórki macierzyste: hiPSC (*ang. human induced Pluripotent Stem Cells*) to komórki, uzyskane w procesie reprogramowania [1]. Opisano wiele metod reprogramowania, ale tylko nieliczne są metodami, które nie powodują włączenia transgenu do genomu gospodarza i można je zastosować do otrzymania komórek o przeznaczeniu aplikacyjnym. Należą do nich transfekcja: wektorami episomalnymi [2], mRNA, miRNA oraz transdukcja białkami rekombinowanymi [3]. Komórki otrzymane za pomocą reprogramowania cechuje wysoki potencjał samoodnowy oraz zdolność do różnicowania zarówno *in vitro,* jak i *in vivo* w komórki endo-, ekto- oraz mezodermy [1]. hiPSC mogą być stosowane *in vitro* do otrzymania: neuralnych komórek macierzystych (NSC, *ang. Neural Stem Cells);* progenitorów neuralnych: (NP, *ang. Neural Progenitors*) [4–7], ale również progenitorów astrocytarnych (AP, *ang. Astrocyte Progenitors*) [8] i oligodendrocytarnych (OP, *ang. Oligodendrocyte Progenitors*) [9]. Metody używane do różnicowania neuralnego hiPSC różnią się m.in.: stosowanymi czynnikami wzrostu oraz długością i efektywnością procesu różnicowania [6, 15].

W przedstawionym cyklu prac tej dysertacji otrzymano trzy stadia różnicowania neuralnego hiPSC: NSC, eNP (*ang. early Neural Progenitors*) i NP. Stadium pośrednie między NSC a NP: eNP charakteryzujące się specyficzną ekspresją markerów neuralnych opisano po raz pierwszy [4, 5]. Różnicowanie neuralne komórek pluripotencjalnych PSC (*ang. Pluripotent Stem Cells*) jest procesem złożonym i może być modulowane przez wiele czynników m.in. przez poziom reaktywnych form tlenu (RFT) [10], potencjał błony mitochondrialnej ($\Delta\Psi m$) [11], aktywację receptorów PPAR (*ang. Peroxisome Proliferator-Activated Receptors*) [12] oraz biogenezę mitochondriów [13, 14]. Nadal niewiele wiadomo w jaki sposób wymienione procesy wpływają na różnicowanie neuralne komórek hiPSC. Próba wyjaśnienia roli tych czynników w różnicowaniu neuralnym komórek hiPSC stała się przedmiotem badań stanowiących podstawę rozprawy doktorskiej.

Mitochondria, to organelle odpowiedzialne za oddychanie komórkowe i produkcję energii, pełniące kluczową rolę w metabolizmie komórek zróżnicowanych. Biorą one udział w regulacji wielu procesów komórkowych, w tym procesu różnicowania komórek pluripotencjalnych (hESC, hiPSC) podczas, którego dochodzi do zmiany toru oddychania komórkowego z opartego na glikolizie anaerobowej na fosforylację oksydacyjną (OXPHOS) [16]. Wykazano, że różnicowanie komórek podczas embriogenezy prowadzi

do progresywnego wzrostu liczby kopii mtDNA, masy mitochondriów, wielkości i złożoności tych organelli [17–20]. W 2010 roku *Prigione i wsp.* [21], wprowadzili pojęcie „stanu metabolicznego komórki" łączące dojrzałość mitochondriów z różnicowaniem komórek. Mitochondria komórek hiPSC wykazują „niedojrzały fenotyp" i małą aktywność. Podczas różnicowania hiPSC zmienia się ich metabolizm energetyczny [16], co przedstawiono na Rycinie 1. Komórki te w stadium niezróżnicowanym preferują glikolizę anaerobową [16], podczas gdy komórki zróżnicowane – fosforylację oksydacyjną [22]. Związane jest to ze zwiększeniem liczby mitochondriów w odpowiedzi na zwiększone zapotrzebowanie na ATP [14, 20]. W trakcie różnicowania neuralnego, zmianom metabolicznym [16, 24–26] towarzyszą zmiany morfologii komórek oraz ekspresji głównych markerów neuralnych [4, 5].



Rycina 1. *Metabolizm hiPSC przed i po różnicowaniu (wg. Xu i wsp., 2013)*

Biogeneza mitochondriów jest kontrolowana przez geny kodujące białka mitochondrialne. Główną rolę w tym procesie odgrywa białko PGC-1α (ang. *Peroxisome proliferator-activated receptor gamma coactivator 1-alpha*) kodowane przez gen *PPARGC1A*. Białko PGC-1α aktywuje między innymi ekspresję jądrowego czynnika transkrypcyjnego NRF1 (*ang. Nuclear Respiratory Factor 1)* [27]. Białko NRF1 bierze udział w regulacji

transkrypcji genów jądrowych kodujących podjednostki kompleksów łańcucha oddechowego ETC (*ang. Electron Transport Chain*) i inne białka mitochondrialne oraz aktywuje mitochondrialny czynnik transkrypcyjny TFAM (ang. *Mitochondrial Transcription Factor A*). TFAM, odgrywa istotną rolę w inicjacji transkrypcji i replikacji mitochondrialnego DNA. Jest więc ogniwem koordynującym ekspresję genów jądrowych i mitochondrialnych, w procesie biogenezy mitochondriów [16].

Biorąc pod uwagę możliwość zastosowania komórek hiPSC w medycynie regeneracyjnej zrozumienie związku między biogenezą mitochondriów, a losem komórek hiPSC ma kluczowe znaczenie. Rola mitochondriów w procesie różnicowania komórek macierzystych SC *(ang. Stem Cells*) jest przedmiotem wielu badań [16, 28], jednak rzeczywisty wpływ mitochondriów *in vivo* na różnicowanie i plastyczność komórek SC został dotychczas słabo poznany [29]. Nie potwierdzono, czy intensywność biogenezy mitochondriów oraz zmiany metaboliczne wpływają na kierunek różnicowania komórek SC i ich plastyczność, czy też biernie towarzyszą temu procesowi oraz czy procesem tym można sterować. Ponadto w piśmiennictwie brak jest doniesień na temat wpływu stymulacji biogenezy mitochondriów na różnicowanie neuralne hiPSC. Do zbadania tego zagadnienia wykorzystano komórki NSC, eNP, NP otrzymane przez różnicowanie neuralne komórek hiPSC i poddane działaniu takich substancji jak: pirolochinolinochinon (PQQ, *ang. Pyrroloquinoline quinone)* i idebenon (IDB, *ang. Idebenone).* PQQ jak i IDB są, agonistami receptorów PPAR (PQQ - PPARα, IDB - PPARα i PPARγ) oraz analogami koenzymu Q10 (CoQ10), mogącymi pełnić rolę nośników elektronów w mitochondrialnym łańcuchu oddechowym. Związki te cechuje aktywność przeciwutleniająca oraz zdolność do indukcji biogenezy mitochondriów w różnych typach komórek [30, 31].

4. Cel pracy

Hipoteza badawcza

Wrażliwość komórek hiPSC na czynniki indukujące biogenezę mitochondriów (PQQ oraz IDB) podczas różnicowania neuralnego zależy od stadium rozwojowego komórek i ma związek z ich kierunkiem różnicowania.

Cel główny:

Określenie wrażliwości komórek hiPSC na wczesnych etapach rozwoju neuralnego *in vitro* na czynniki indukujące biogenezę mitochondriów: PQQ i IDB, oraz zbadanie molekularnego związku między biogenezą mitochondriów, a różnicowaniem neuralnyn hiPSC.

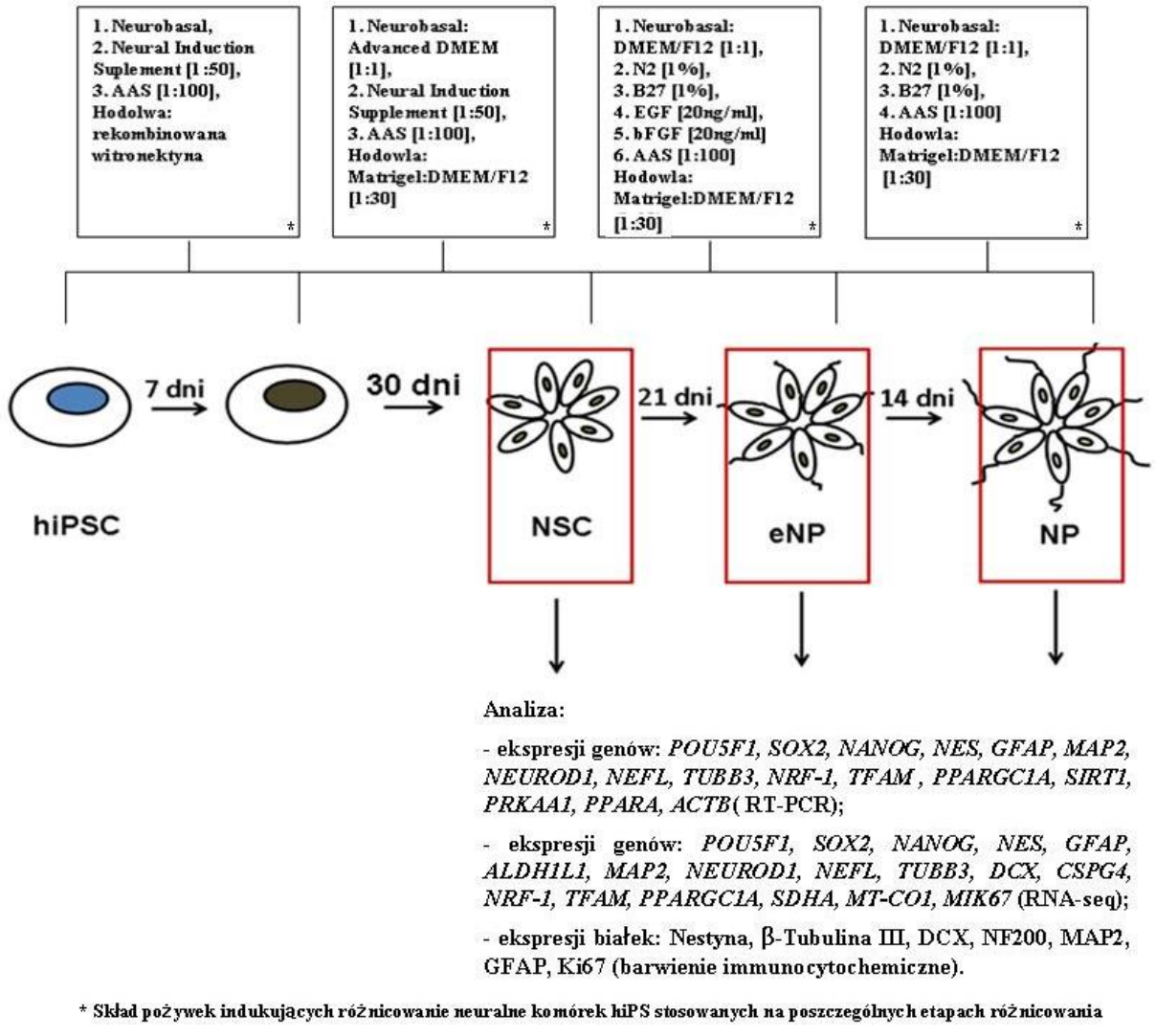
Cele szczegółowe:

1. Otrzymanie i charakterystyka populacji komórkowych NSC, eNP oraz NP.
2. Ocena wpływu PQQ i IDB w komórkach NSC, eNP, NP na:

- żywotność, poziom RFT, $\Delta\Psi m$ oraz całkowitą liczbę komórek,
- poziom wskaźników biogenezy mitochondriów: ekspresję białek COX-1 i SDHA, liczbę kopii mtDNA oraz ekspresję genów *PPARGC1A, TFAM, NRF1*,
- poziom ekspresji markerów różnicowania neuronalnego (*MAP2)* oraz astrocytarnego (*GFAP).*

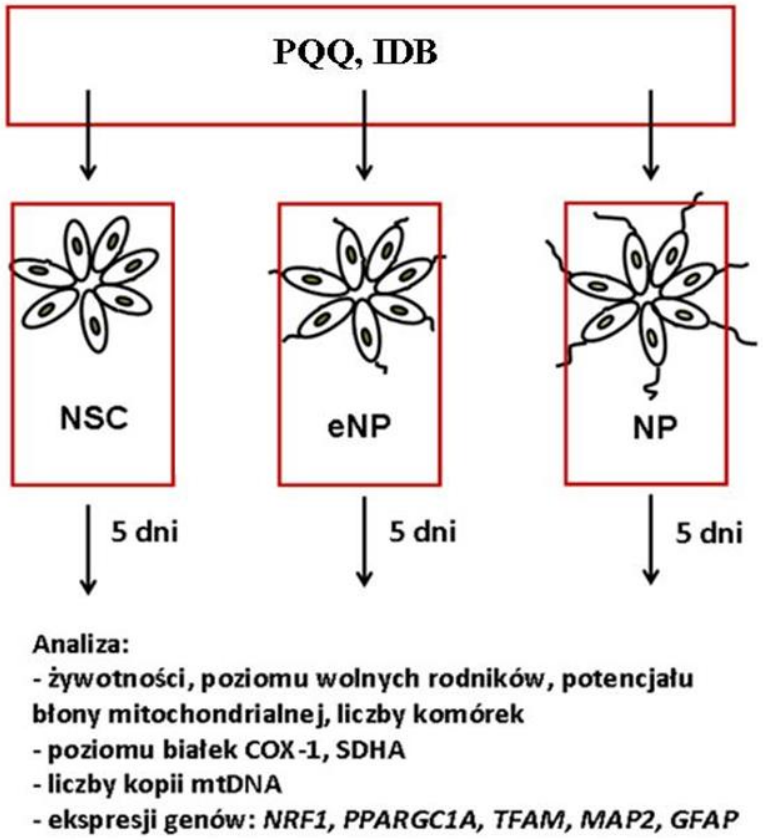
5. Zadania badawcze

1. Otrzymanie komórek: NSC, eNP, oraz NP w wyniku różnicowania hiPSC oraz ich charakterystyka za pomocą RT-PCR, RNA-seq i barwienia immunocytochemicznego [4, 5]. Schemat eksperymentu przedstawiono na Rycinie 2.



Rycina 2. *Schemat doświadczeń obejmujących* otrzymanie i *charakterystykę komórek: NSC, eNP oraz NP*

2. Inkubacja NSC, eNP, NP z PQQ [4] lub IDB [5], a następnie: a) ocena wrażliwości komórek na badane związki (oznaczenie: żywotności, poziomu RFT, ΔΨm oraz całkowitej liczby komórek); b) analiza biogenezy mitochondriów (określenie poziomu: oksydazy I cytochromu c COX-1 oraz dehydrogenazy bursztynianowej SDHA; oznaczanie liczby kopii mitochondrialnego DNA); analizę zmian profilu ekspresji genów *NRF1, TFAM, PPARGC1A, MAP2, GFAP*, po wyznaczeniu genów referencyjnych. Schemat doświadczeń przedstawiono na Rycinie 3 [4, 5].



Rycina 3. *Schemat doświadczeń oceniających wpływ substancji badanych na komórki NSC, eNP oraz NP*

6. Omówienie zalet i ograniczeń metod badawczych użytych w pracach oryginalnych

W Tabeli 1 przedstawiono zalety i ograniczenia metod badawczych użytych w pracach oryginalnych.

Tabela 1. Zalety i ograniczenia metod badawczych użytych w pracach oryginalnych

Lp.	Metoda	Zalety	Ograniczenia
1.	Analiza ekspresji genów metodą (RT-PCR)	– potwierdzenie obecności lub braku danego transkryptu;	– metoda nie pozwala na ocenę ilościową ekspresji genów;
2.	Analiza ekspresji genów metodą (RNA-seq)	– precyzyjna informacja na temat poziomów ekspresji genów; – analiza całego transkryptomu z bardzo wysoką wydajnością; – wysoka czułość i dokładność;	– niejednoznaczność w mapowaniu do genomu, spowodowana brakiem intronów w sekwencjach mRNA;
3.	Analiza ekspresji genów metodą (qRT-PCR)	– jakościowa oraz ilościowa ocena ekspresji genów; – wysoka czułość i specyficzność reakcji; – pozwala na walidację wyników otrzymanych metodą RNA-seq;	– konieczność poprawnego wyboru genów referencyjnych;
4.	Ocena liczby kopii mtDNA metodą qPCR	– umożliwia oszacowanie początkowej liczby cząsteczek DNA; – wysoka czułość i specyficzność;	– brak;
5.	Barwienie immunocyto-chemiczne	– detekcja kilku antygenów jednocześnie; – przeciwciała drugorzędowe z fluorochromami Alexa Fluor®, wytwarzają fotostabilne koniugaty;	– reakcje fałszywie pozytywne lub negatywne; – reakcje krzyżowe przeciwciał z epitopami typowymi dla różnych białek;

6.	Pomiar żywotności (alamarBlue Cell Viability Reagent)	– pomiar aktywności metabolicznej i proliferacji żywych komórek; – detekcja może być prowadzona metodą kolometryczną lub fluorescencyjną; – nie wymaga lizy komórek;	– brak;
7.	Pomiar potencjału błony mitochondrialnej ΔΨm (Mitotracker® red CMXRos)	– akumulacja barwnika zależy od potencjału błony mitochondrialnej;	– niska stabilność;
8.	Pomiar poziomu reaktywnych form tlenu RFT (DCFH-DA)	– uwzględnia biodostępność, dystrybucję oraz metabolizm przeciwutleniaczy w obrębie komórki;	– sonda DCFH-DA nie jest selektywnym wskaźnikiem tworzenia określonych RFT w komórkach; – niska stabilność;
9.	Ocena liczby komórek za pomocą barwnika Janus green	– umożliwia normalizację otrzymanych wyników z innych oznaczeń w stosunku do liczby komórek; – barwnik może być stosowany jednocześnie z metodami opartymi o fluorescencję lub luminescencję;	– brak;
10.	Test immuno-enzymatyczny MitoBiogenesis™ In-Cell ELISA Kit	– pozwala na ocenę ekspresji białek istotnych dla funkcjonowania mitochondriów: COX-1 oraz SDHA; – test nie wymaga przeprowadzenia lizy komórek;	– nie daje pełnego obrazu dotyczącego funkcjonalności mitochondriów;

7. *Podsumowanie i omówienie najważniejszych wyników*

I. Otrzymanie i charakterystyka: NSC, eNP oraz NP z komórek hiPSC

1. W wyniku różnicowania hiPSC otrzymano 3 populacje komórkowe na różnych etapach rozwoju neuralnego: NSC, eNP, NP, charakteryzujące się specyficzną: morfologią, ekspresją markerów neuralnych, neuronalnych i astrocytarnych oraz biogenezy mitochondriów na poziomie mRNA [4] (*Fig.1A, Suppl.Fig.1*) oraz ekspresją głównych markerów neuralnych na poziomie białka [4] (*Fig.1BC);* [5] (*Fig.1*).
2. W stadium NSC, w porównaniu do komórek hiPSC zanotowano spadek ekspresji genów pluripotencjalnych: *POU5F1* oraz *NANOG* [4] (*Fig.1A, Suppl.Fig.1*) oraz wzrost poziomu markerów neuralnych komórek macierzystych: Nestyny, β-Tubuliny III [4] (*Fig.1BC*); [5] (*Fig.1*).
3. Podczas różnicowania komórek NSC w kierunku eNP odnotowano wzrost poziomu ekspresji markerów neuronalnych: β-Tubuliny III oraz MAP2, pojawienie się ekspresji markera astrocytów GFAP oraz spadek poziomu ekspresji Ki67 [4] (*Fig.1BC*) [5], (*Fig.1*), co świadczy o zmniejszeniu tempa proliferacji komórek eNP.
4. Podczas różnicowania komórek eNP w kierunku NP odnotowano spadek ekspresji Nestyny oraz wzrost poziomu ekspresji GFAP [4] (*Fig.1BC*); [5] (*Fig.1*), a także dalsze zmniejszenie tempa proliferacji (spadek ekspresji Ki67) [4] (*Fig.1BC*); [5] (*Fig.1*).

II. Wpływ substancji badanych na wybrane procesy życiowe komórek NSC, eNP i NP

1. W badaniach potwierdzono, że PQQ [4] oraz IDB [5] w ustalonym, optymalnym stężeniu 0,5μM wpływa na: żywotność [4, 5] (*Fig.2A*), poziom RFT [4, 5] (*Fig.2B*), ΔΨm [4, 5] (*Fig.2C*), całkowitą liczbę komórek [4, 5] (*Fig.3C*) oraz regulację biogenezy mitochondriów [4, 5] (*Fig.3AB, Fig.4ABC, Fig.5ABC*).
2. Po ekspozycji na PQQ [4] oraz IDB [5] zanotowano obniżenie poziomu RFT [4, 5] (*Fig.2B*), któremu towarzyszył wzrost żywotności [4, 5] (*Fig.2A*) oraz wzrost całkowitej liczby komórek [4, 5] (*Fig.3C*) w populacjach komórek NSC i eNP.
3. Zestawienie najważniejszych wyników działania substancji badanych (PQQ 0,5μM oraz IDB 0,5μM) na NSC, eNP i NP przedstawiono w publikacjach [4, 5] w *Tabeli 1.*

III. Wpływ substancji badanych na biogenezę mitochondriów

1. Wykazano istotny statystycznie wzrost kopii mtDNA pod wpływem PQQ [4] (*Fig.4AB*) oraz IDB [5] (*Fig.4AB*) wyłącznie w stadium eNP.
2. Po zastosowaniu PQQ [4] oraz IDB [5] zanotowano zmiany ekspresji genów: *NRF1, PPARGC1A, TFAM* [4, 5], w zależności od stadium rozwojowego. Jednoczesny wzrost ekspresji tych genów pod wpływem PQQ [4] oraz IDB [5] odnotowano jedynie w stadium eNP [4] (*Fig.5ABC*)*;* [5] (*Fig.5ABC*).
3. Po ekspozycji na PQQ [4] oraz IDB [5] w komórkach NSC oraz eNP wykazano istotny wzrost ekspresji białek: SDHA, jak i COX-1 [4] (*Fig.3AB);* [5] (*Fig.3AB*).

IV. Wpływ substancji badanych na różnicowanie neuralne i jego związek z biogenezą mitochondriów

1. Wpływ PQQ [4] oraz IDB [5] na ekspresję genu *MAP2* i *GFAP* był różny w zależności od stadium zróżnicowania komórek. Zmianom poziomu ekspresji genu *MAP2* [4, 5] (*Fig.5D*) oraz *GFAP* [4, 5] (*Fig.5E*) towarzyszyły zmiany: ekspresji genów regulujących biogenezę mitochondriów: *NRF1, TFAM, PPARGC1A,* [4, 5] (*Fig.5ABC*), liczby kopii mtDNA [4, 5] (*Fig.4AB*) oraz ekspresji białek: SDHA, COX-1 [4, 5] (*Fig.3AB*).
2. W stadium eNP, w którym potwierdzono wzrost poziomu wszystkich markerów biogenezy mitochondriów po ekspozycji na PQQ [4] i IDB [5]. Potwierdzono wzrost ekspresji *GFAP* oraz represję *MAP2* po działaniu PQQ [4] (*Fig.5DE*) lub jednoczesny wzrost ekspresji *GFAP* oraz *MAP2* po traktowaniu komórek IDB [5] (*Fig.5DE*). Po dodaniu IDB do eNP wzrost ekspresji genu *GFAP* był wyższy niż *MAP2* [5] (*Fig.5DE*).
3. Wykazano, że wzrostowi genu *PPARGC1A* [4, 5] (*Fig.5B*) zarówno w przypadku zastosowania PQQ [4], jak i IDB [5] towarzyszy wzrost ekspresji markera astrocytów *GFAP* [4, 5] (*Fig.5E*).

V. Omówienie wyników

1. Związek PQQ i IDB z PGC-1α i receptorami PPAR jest znany z literatury [30, 32–36]. Wiadomo również, że aktywacja receptorów jądrowych PPAR wpływa na wybór kierunku różnicowania neuralnego [12]. Białko PGC-1α kodowane przez gen *PPARGC1A* odgrywa istotną rolę w biogenezie mitochondriów [30, 37]. PGC-1α ulega ekspresji na wysokim poziomie podczas rozwoju zarodkowego, ale również w progenitorach neuralnych i nowo

powstałych neuronach w postnatalnym mózgu [38]. *Xing i wsp., 2018* [37] wykazali, że nadekspresja genu *PPARGC1A* prowadzi do wzrostu ekspresji GFAP. Ponadto udowodniono, że aktywacja receptora: PPARα wiąże się z pojawieniem fenotypu astrocytarnego; PPARβ/δ neuronalnego, a PPARγ oligodendrocytarnego [12]. W populacjach NSC, eNP i NP potwierdzono ekspresję *PPARA* [4] (*Suppl.Fig.1*) oraz wykluczono ekspresję *PPARG* [7], którym towarzyszył brak markerów fenotypu oligodendrocytarnego i pojawienie się markerów astrocytów w otrzymanych populacjach [7]. Dotychczasowe wyniki sugerują, że przez aktywację szlaków zależnych od PGC-1α oraz receptora PPARα substancje badane: PQQ [4] oraz IDB [5] promują różnicowanie astrocytarne.

2. PQQ [4] oraz IDB [5] wykazują działanie wielokierunkowe, dlatego zmiany ekspresji *GFAP* [4, 5] (*Fig.5E*) oraz *MAP2* [4, 5] (*Fig.5D*) po traktowaniu komórek PQQ [4] oraz IDB [5] mogą wynikać także ze zmian poziomu RFT [4, 5] (*Fig.2B*) oraz ΔΨm [4, 5] (*Fig.2C*).
   - Wiele wskazuje na to, że poziom RFT wpływa na kierunek różnicowania komórek macierzystych [10]. Wysoki potencjał samoodnowy komórek PSC oraz ekspresja markerów pluripotencjalności OCT4, NANOG i SOX2 uwarunkowany jest przez niski poziom RFT [10]. Wraz ze wzrostem stopnia zróżnicowania maleje ekspresja enzymów antyoksydacyjnych i następuje wzrost poziomu RFT, który promuje różnicowanie neuralne [39]. Zarówno PQQ [40], jak i IDB [31] to związki o silnych właściwościach antyoksydacyjnych. PQQ chroni mitochondria przed indukowaną stresem oksydacyjnym peroksydacją lipidów i inaktywacją ETC [40]. IDB przez interakcje z ETC zwiększa wytwarzanie ATP wymaganego do funkcjonowania mitochondriów, redukuje poziom RFT, hamuje peroksydację lipidów oraz chroni błonę lipidową i mitochondria przed uszkodzeniami oksydacyjnymi [34, 35]. Ponadto IDB bierze udział w przenoszeniu elektronów bezpośrednio do III kompleksu ETC, zastępując kompleks I i przywracając produkcję ATP [36]. W prezentowanych pracach [4, 5] wykazano, że PQQ [4] oraz IDB [5] obniżają poziom RFT [4, 5] (*Fig.2B*) we wszystkich badanych populacjach komórkowych nie potwierdzono jednak wpływu RFT na kierunek różnicowania hiPSC, co wymaga dalszych badań.
   - *Schieke i wsp.* [11] udowodnili, że zmiany ΔΨm mają duże znaczenie dla kierowania różnicowaniem komórek SC, dlatego w badaniach oceniono zdolność do modyfikacji ΔΨm przez PQQ [4] (*Fig.2C*) oraz IDB [5] (*Fig.2C*). Po traktowaniu komórek związkiem PQQ [4] jedynie w populacji eNP ΔΨm [4] (*Fig.2C*) wzrósł istotnie

statystycznie. W populacji NP odnotowano jego spadek. W populacjach NSC, eNP oraz NP traktowanych IDB [5] nie wykazano znaczących zmian w ΔΨm [5] (*Fig.2C*). W badaniach zaobserwowano wzrost ekspresji markera astrocytów *GFAP* [4, 5] (*Fig.5E*), jednak występował on także w komórkach traktowanych IDB. To sugeruje, że mechanizm molekularny dotyczący zmiany kierunku różnicowania w komórkach traktowanych badanymi związkami nie był bezpośrednio związany ze zmianami ΔΨm [4, 5] (*Fig.2C*). Zbadanie tej zależności wymaga dalszych badań.

8. Wnioski

1) Przedstawione w dysertacji wyniki pozytywnie weryfikują postawioną hipotezę, wykazując, że wrażliwość hiPSC na czynniki indukujące biogenezę mitochondriów (PQQ oraz IDB) podczas różnicowania neuralnego zależy od stadium rozwojowego komórek i ma związek z ich kierunkiem różnicowania.
2) Na wczesnych etapach rozwoju neuralnego hiPSC można wyodrębnić trzy stadia rozwojowe (NSC, eNP, NP) różniące się istotnie ekspresją genów i białek (do tej pory stadium NP funkcjonowało w literaturze, jako populacja jednorodna).
3) W komórkach hiPSC różnicowanych neuralnie istnieje przedział „wrażliwości rozwojowej" na badane czynniki stymulujące biogenezę mitochondriów. Aktywacja biogenezy mitochondriów zarówno za pomocą PQQ jak i IDB w tym przedziale jest związana z różnicowaniem w kierunku astrocytarnym.
4) Nie potwierdzono znaczenia zmiany potencjału błony mitochondrialnej ($\Delta\Psi m$) oraz reaktywnych form tlenu (RFT) w regulacji różnicowania neuralnego hiPSC.
5) Zależność molekularna stymulacji biogenezy mitochondriów i zmiany drogi różnicowania w stadium eNP w kierunku astrocytarnym dotyczy aktywacji drogi przekazywania sygnału spowodowanej wzrostem ekspresji genu *PPARGC1A*. Otrzymane wyniki sugerują więc, że zarówno PQQ jak i IDB promują różnicowanie astrocytarne przez aktywację szlaków zależnych od PGC-1α oraz receptora PPARα.
6) Komórki w stadium NSC, eNP, NP otrzymane przez różnicowanie komórek hiPSC stanowią dobry model *in vitro* do badań wrażliwości wczesnych etapów rozwoju neuralnego na czynniki indukujące biogenezę mitochondriów.

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10. Kopie publikacji wchodzących w skład zbioru

10.1. Poświadczony przez Bibliotekę IMDiK PAN pięcioletni impact factor (IF) publikacji wchodzących w skład zbioru

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Reprogramming of somatic cells: possible methods to derive safe, clinical-grade human induced pluripotent stem cells

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Derivation of pluripotent stem cells from adult somatic tissues by reprogramming technology has opened new therapeutic possibilities. Current most efficient procedures for derivation of induced pluripotent stem (iPS) cells are based on the viral vectors, which represent the danger of insertional mutagenesis during incorporation of introduced genes into the host genome. To circumvent this problem, the new, safe, non-integrative and non-viral strategies of reprogramming have been developed. In this review we discuss novel DNA-free and viral-free methods of reprogramming to iPS cells including protein transduction, mRNA and microRNA delivery.

Key words: iPS cells, somatic cells reprogramming, protein transduction, mRNA and miRNA transfection

INTRODUCTION

Development of cloning technology in vertebrates clearly demonstrates that the nucleus of differentiated somatic cell may attain pluripotent stage in the cytoplasm of the oocyte (Gurdon et al. 1964). Phenotypic and molecular investigation of embryonic stem (ES) cells during the last three decades has enabled identification of genes which are responsible for the maintenance of cellular pluripotency in mammals (reviewed in Nichols and Smith 2012). However, *in vitro* conditions for successful derivation of pluripotent cells from differentiated somatic cells were not known. Yamanaka and his PhD student, Takahashi, undertook the challenge of finding the proper combination of transcription factors for genetic modification of mouse embryonic fibroblasts (MEF) into iPS cells (Takahashi and Yamanaka 2006). The procedure of reprogramming appeared to be the most efficient when a combination of 4 out of 24 tested genes encoding transcription factors Oct4, Sox2, Klf4, c-Myc (OSKM factors, also called Yamanaka factors) was applied. One year later Yamanaka's group successfully derived iPS cells also from human fibroblasts (Takahashi et al. 2007). The first method, employed for the introduction of reprogramming transcription factor genes to differentiated cells, was based on four monocistronic retroviral vectors. However, upon transduction, retroviral vectors are randomly integrated into the host genome, thus significantly increasing the risk of insertional mutagenesis and cancer (Schroder et al. 2002, Wu et al. 2003, Bushman et al. 2005, Okita et al. 2008). In order to lower the excessive amounts of random integration, polycistronic vectors containing the sequence for all reprogramming factors under a single promoter have been used (Carey et al. 2009, Zhang et al. 2011). However, this method involves integration of transgenes, and therefore it is unsuitable for the generation of clinical-grade iPS cells.

In addition to the stable integration approach, the transient transfection using episomal vector or minicircle DNA has been employed (Yu et al. 2009, Narsinh et al. 2011). This reprogramming method is non-integrating since the introduced genetic material persists in the nucleus as extrachromosomal DNA. However, potential spontaneous integration causing mutagenesis

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can occur, thus limiting the advantage of this technique for possible clinical applications (Okita et al. 2008). In that respect closer to the clinical application seems to be efficient, transgen-free induction of human pluripotent stem cells by the vectors derived from Sendai virus. However, this technique involves viral particles raising questions regarding the safety of generated iPS cells (Fusaki et al. 2009).

Safe methods for derivation of iPS cells have become the main goal of development in reprogramming technology. The most promising are DNA-free and viral-free protocols. They include introduction of reprogramming-inducing molecules into cells such as: (1) recombinant proteins (Zhou et al. 2009, Kim D. et al. 2009), (2) messenger RNA (mRNA) (Warren et al. 2010), and (3) mature microRNA (miRNA) (Miyoshi et al. 2011). The efficiency of non-integrating reprogramming methods is greatly enhanced by the use of low oxygen level conditions (Szablowska-Gadomska et al. 2011) and small molecules such as histone deacetylase inhibitors (Huangfu et al. 2008) and/or DNA methyltransferase inhibitors (Mikkelsen et al. 2008).

The strategies of somatic reprogramming such as using recombinant proteins, mRNA and miRNA are the safest and integration-free methods having the highest potential for therapeutic application of all known methods for reprogramming. These techniques are the main focus of this review and will be described below in detail.

RECOMBINANT PROTEIN TRANSDUCTION

The strategy to entirely replace gene delivery during the reprogramming process by recombinant protein transduction was reported for the first time by Kim D. and coauthors (2009) and Zhou and others (2009). Recombinant protein transduction is one of the DNA-free strategies of cellular reprogramming that rely on introduction of proteins into the target cells, bypassing the need to introduce exogenous genetic materials. However, some macromolecules including proteins have largely limited ability to cross cellular membrane, therefore, recombinant proteins have to be modified. The observation that some proteins are able to pass through the cell membrane barrier contributed to the identification of specific domains controlling this process. Cell penetrating peptides (CPP), also termed membrane translocating sequences (MTS) or protein transduction domains (PTDs), are peptides that have the ability to transport through the cell membrane large molecules in a process independent of classical endocytosis. These properties make CPP domains suitable for transfer of proteins and other molecules into living cells (Fig. 1), for spreading the protein from transfected to non-transfected cells (Beerens et al. 2003) and also for entering the nucleus (Matsui et al. 2003, Prochiantz 2000).

Naturally existing peptides having the ability to translocate through the cell membrane barrier are characterized by a high proportion of basic amino acids (e.g., arginine or lysine) (Ziegler et al. 2005, El-Sayed et al. 2009). Poly-arginine domains are the type of CPP which are frequently used in reprogramming protocols. They bind to the plasma membrane, facilitating chemical compounds or proteins to translocate through the cell membrane (Schwarze et al. 2000). Poly-arginine penetrating peptide is composed of six to twelve arginine residues and assigns recombinant proteins with high transduction capacity (Matsui et al. 2003). It has been reported that oligo-arginine residue (3R) was sufficient for delivery of functional transcription factors, but its delivery effectiveness is not as powerful as poly-arginine (11R) residue (Hitsuda et al. 2012). Thus, for cellular reprogramming pluripotency transcription factors, such as Klf4, Oct4, Sox2, Nanog and Lin28, have been produced as recombinant proteins containing nine or eleven "arginin tails" functioning as protein transduction domain (PTD).

The pioneers of the experimental procedure for reprogramming using recombinant proteins (Kim D. et al. 2009, Zhou et al. 2009) applied the method of serial transduction with proteins containing poly-arginine domain. Though the process of reprogramming somatic cells to pluripotency stage was completed, the efficiency of this process was low. However, using recombinant proteins for cellular reprogramming has some advantages, as it does not require complicated manipulation protocols and also does not incorporate any changes to the genome, thus representing a safe method for iPS cells derivation.

For the pioneering experiments, Zhou and colleagues used MEFs from OG2 transgenic mice (Oct4-GFP reporter). MEFs were cultured in media containing Oct4, Sox2, Klf4, c-Myc recombinant proteins associated with poly-arginine domain (11R) in the presence of valproic acid (VPA) – a histone deacetylase inhibitor (HDAC). After four rounds of protein supplementation and subsequent culture of $5x10^5$ MEF

cells for 23–28 days, three iPS cell clones positive for Oct4-GFP reporter gene were successfully generated. Resulting iPS cells formed compact small colonies, which were morphologically similar to mouse embryonic stem (mES) cell colonies. Global gene expression analysis revealed that derived iPS cells were similar to mES cells and subsequent genomic sequencing analyses showed that Oct4 and Nanog promoters were demethylated. Obtained iPS cells contributed to embryonic development of the three germ layers in mouse chimeras and they also possessed the ability to differentiate into neurons, cardiomyocytes, and pancreatic as well as hepatic cells (Zhou et al. 2009).

For the reprogramming of human newborn fibroblasts (HNF) Kim and colleagues (2009) used whole cell extracts from human embryonic kidney (HEK) 293 cells, which were transfected with plasmid encoding pluripotent factors, such as OCT4, SOX2, KLF4 and C-MYC. Proteins were fused to 9-arginine peptide tags to allow transport through the plasma membrane. Cell extracts were added to the fibroblast cultures six times within the first week. Efficiency of reprogramming was low (0.001%), but obtained cells possessed pluripotent stem cell properties including differentiation potency into three germ layers performed *in vitro* and *in vivo* (Kim D. et al. 2009).

Protein transduction method in reprogramming can be used routinely for developmentally immature newborn or fetal cells. However, it has been proven that it is generally more difficult for adult cells to undergo reprogramming procedures (Park et al. 2008). Szablowska-Gadomska and colleagues (2012) reported that human umbilical cord blood-derived neural stem cells (HUCB-NSC) can be reprogrammed using recombinant proteins fused with poly-arginine domains. HUCB-NSCs have been treated with HEK293 cell extracts containing KLF4-9R, OCT4-9R and SOX2-9R recombinant proteins. The induction of pluripotency in HUCB-NSC was successful when small molecules, such as histone deacetylase inhibitor Trichostatin A (TSA), DNA methyltransferase inhibitor RG-108, and 5% oxygen tension were applied in addition to the recombinant proteins. TSA and RG-108 in combination with low oxygen tension showed an important role in epigenetic stimulation and in the generation of induced pluripotent stem cells from HUCB-NSC (Szablowska-Gadomska et al. 2012).

Several studies have indicated that other peptide domains can also be functional as the potential protein transmembrane carriers (Wadia and Dowdy 2002). The cell penetrating TAT domain from HIV1 (HIV1 TAT) is one of these, and was used for reprogramming of human fibroblasts (Pan et al. 2010). However, the reprogramming to iPS cells was not fully successful, since TAT engineered proteins remained in the endosomes instead of being transported to the nucleus. To make TAT-protein-based reprogramming effective, the approach of the conjugation in complex with cationic liposomes (lipo-Tat) was applied resulting in higher transduction efficiency (by 1000 fold) (Li et al. 2012). iPS cell generation using TAT-conjugated reprogramming factors (Oct4, Sox2, c-Myc, Klf4, Nanog) was further supported by adding VPA to the culture medium (Zhang et al. 2012).

In summary, the application of recombinant proteins is considered to be a safe and non-integrative method of generation of iPS cells, although this technology has some limitations. One of them is the quality and quantity of recombinant proteins required for cellular reprogramming, since it is challenging to generate and purify sufficient quantities of desired proteins. The other limitation is linked to bacterial post-translational modification of proteins, which revealed essential disadvantages and low efficiency of iPS cell generation (Zhou et al. 2009). Since iPS cells obtained by recombinant proteins transduction have significant potential for clinical application, these technical difficulties need to be resolved (Yang et al. 2012). It is of note that the first clinical trial using iPS cells, which has already started in Japan in order to cure retinal disease age-related macular degeneration (AMD), is based on iPS cells obtained by the technology of reprogramming with recombinant proteins (Cyranoski 2013, Takahashi 2013).

TRANSFECTION WITH mRNA

Efficient reprogramming of somatic cells to pluripotency can be achieved by introducing mRNA molecules into living cells. mRNA can be obtained from purified lysed cells, synthesized from free nucleotides either chemically or enzymatically, and delivered to the cells by microinjection, electroporation or lipofection. Transfected cells translate the mRNA into the desired protein (Fig. 2), which can be transported to the nucleus for its functional outcome. The mRNA technique may offer several advantages over the classical reprogramming protocols. Technology based on

mRNA totally eliminates the risk of integration of genetic material into the genome and insertional mutagenesis inherent to all DNA-based methodologies, including those that are defined as non-integrating.

Transfection using mRNA encoding reprogramming factors was applied for the first time in 2010 (Warren et al. 2010, Yakubov et al. 2010). Warren and colleagues (2010) produced human iPS cells by repeated transfection of modified synthetic mRNAs designed to bypass innate antiviral responses. Fibroblasts and keratinocytes were transfected with four synthetic modified mRNAs encoding Oct4, Sox2, Klf4, and c-Myc in the presence of interferon inhibitor. Efficiency of synthetic mRNA transfection in cellular reprogramming was higher than using retroviral vectors (1.4% *versus* 0.04%, respectively). It was also shown that additional transfection of Lin28-encoding synthetic modified mRNAs, under hypoxic conditions, enhanced reprogramming of fibroblasts and keratinocytes to iPS cells.

iPS cells obtained using mRNA molecules show similar characteristics and morphology as derived by Yamanaka protocol using the retroviral transduction method (Takahashi et al. 2007). However, some distinctions in the molecular phenotype, differentiation capacity, and teratoma formation between viral iPS and the mRNA-iPS cells have been reported. The different capability of teratoma formation *in vivo* can be explained by the possibility of producing partially reprogrammed intermediates during mRNA-based reprogramming procedure (Chan et al. 2009).

The ability to maintain a high-level of expression of defined proteins in human cells for many days without introducing the cells to unsafe DNA-based transgenes makes the mRNA-based reprogramming procedure attractive for therapeutic applications. Today it is considered to be the optimal strategy for the fast generation of pluripotent cell lines with therapeutic potential as compared to other non-integrative methods using episomal DNA plasmids or highly-infective Sendai virus. The mRNA reprogramming method is considered to be the safest and a highly efficient method, which eliminates the need for screening the cells to confirm viral remnants (Warren et al. 2010). However, mRNA molecules delivered into the living cells usually induce a significant inflammatory response and may also cause a variety of nonspecific effects including translation block, cell cycle arrest and apoptosis (Warren et al. 2010). Frequently observed cell death after repetitive transfection of cells with even a small amount of mRNA was related to the cell immune response. The application of chemical compounds (Pepinh-TRIF, Pepinh-MYD, B18R, chloroquine, TSA) known for their ability to suppress such cellular responses did not evoke the desired effect (Drews et al. 2012). One of the possibilities to solve this problem is the use of RNA viruses to destroy or inhibit specific immune-related proteins which enable persistent infection (Bode et al. 2007). The other possibility to escape the response of the immune system during multiple transfections with mRNA is the induction of suppression of exogenous RNA-recognition receptors (PRRs). They include Toll-like receptors TLR3, TLR7, TLR8 (Alexopoulou et al. 2001, Diebold et al. 2004, Kariko et al. 2005), the RNA helicase RIG1 (RARRES3) (Yoneyama et al. 2004), protein kinase R (PKR, a.k.a. EIF2AK2) (Levin et al. 1981), and members of the oligoadenylate synthetase family of proteins (OAS1, OAS2, OAS3). These and other receptors trigger an inflammatory response upon detecting pathogen-associated molecular patterns (PAMPs) such as exogenous RNA. However, it is not yet clear how cells distinguish exogenous mRNA from the large amount of endogenous RNA (Hornung et al. 2006, Saito et al. 2008, Takahasi et al. 2008, Yoneyama and Fujita 2008, Schmidt et al. 2009).

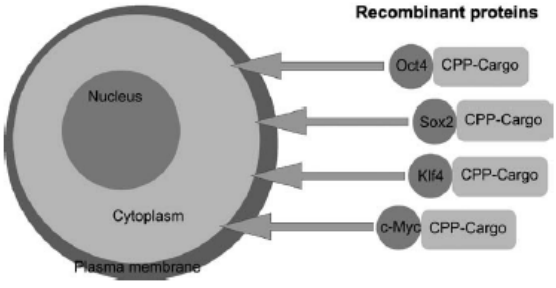

Fig. 1. Delivery of recombinant proteins to the somatic cells: (CPP) cell penetrating peptide.

The third possibility to increase the rate of recovery of cells transfected with mRNA is the knock-down of p53 (Angel and Yanik 2010). The same group has introduced the procedure of desensitizing cells to frequent transfection with mRNA. They applied small interfering RNA (siRNA) cocktail for the combined knock-down of interferon beta (IFNβ) and transcription factors Eif2ak2, Stat2 to allow sequential transfections with mRNA for the successful reprogram-

ming. Co-transfection of cells with siRNA cocktail designed to directly knock-down the expression of immune-related proteins allowed for repeated transfection with exogenous mRNA and increased viability of mRNA transfected cells (Angel and Yanik 2010). Further methodological advancement was the prolonged transfection with mRNA, which allowed for generation of iPS colonies without interferon-directed blocking (Arnold et al. 2012). Arnold and colleagues (2012) used this technology together with the combination of three transcription factors (OSK and ONT) for successful reprogramming of human Huntington's disease fibroblasts. However, the efficiency of reprogramming was low: 0.0005% of input cells as compared to 1.4% obtained by Warren and coworkers (2010) with the OSKN transcription factor combination.

Although the generation of iPS cells from adult patients is difficult, Heng and colleagues (2013) generated human iPS cells from adipose-derived mesenchymal stem cells (MSCs) from a 50-year old patient using synthetic modified mRNA encoding transcription factors in feeder-free defined conditions. Twelve karyotypically normal clonal iPS cell lines that were obtained revealed normal karyotype up to 10th passage, but after 24 passages displayed chromosomal mosaicism of normal and abnormal karyotypes. Reprogramming efficiency was at 0.005% level, and thus the procedure was considered just as the progress toward reaching clinical application (Heng et al. 2013).

Despite a few disadvantages which are recently being reduced by technological progress, the mRNA technique for iPS cell derivation is safer than viral-based classical reprogramming protocols, since it eliminates the risk of genomic integration and insertional mutagenesis. Application of the modified RNA strategy may serve in the future as the method for derivation of the clinical-grade human iPS cell lines.

TRANSFECTION WITH miRNA

miRNAs are 18–24 nucleotide-long single stranded RNA molecules usually generated from non-coding regions of gene transcripts, and function to suppress gene expression by repression of mRNA translation. miRNAs are associated with a protein complex called RNA-induced silencing complex (RISC) which inhibits the translation of targeted mRNA (Ambros 2004, Bartel 2004, Rana 2007, Kim VN et al. 2009)The reports showed that specific miRNAs can play a critical role in control of pluripotency-related genes. These conclusions were based on studies demonstrating that specific miRNAs are highly expressed in embryonic stem cells (Houbaviy et al. 2003, Suh et al. 2004, Marson et al. 2008). Several years earlier Leeand coworkers (1993) and Ruvkun (2001) confirmed the significant role of miRNAs in regulation of embryonic development and cell differentiation. Some important cellular processes that miRNAs have been implicated in include: expression of self-renewal genes in human embryonic stem (hES) cells (Xu et al. 2009), cell cycle control of ES cells (Wang et al. 2008), alternative splicing (Makeyev et al. 2007) and heart development (Latronico and Condorelli 2009).

Several miRNAs could mediate reprogramming of somatic cells to iPS cells, or they enhance iPS cell reprogramming when expressed with combinations of the OSKM factors (Judson et al. 2009). Specific miRNAs, such as miR290-295 in mouse or miR-302/367 in human, facilitate iPS cells to maintain the ES cell phe-

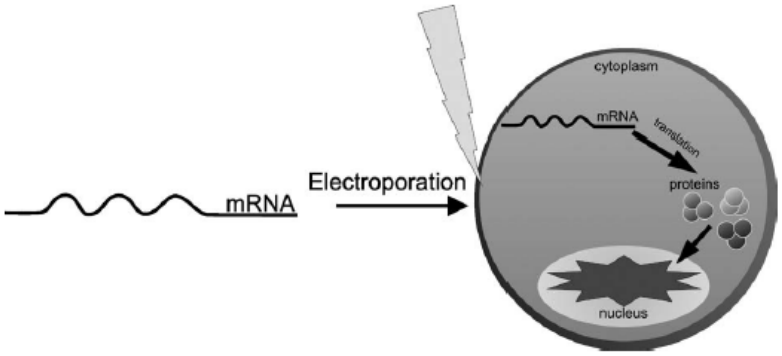

Fig. 2. mRNA delivery to the living cell and activation of the gene response.

notype by stimulation of expression of pluripotent genes (Wang et al. 2007, 2008, Babiarz et al. 2008, Wang and Blelloch 2009). These miRNAs have the ability to regulate the cell cycle which probably is connected with their capacity to enhance iPS cell reprogramming (Judson et al. 2009). Furthermore, cell-specific miRNAs can replace the function of c-Myc during reprogramming (Judson et al. 2009). Lin and others (2011) reported co-suppression of four epigenetic regulators: Lysine-specific histone demethylase 1A (also known as AOF2, KDM1 or LSD1), histone H3K4 demethylase (AOF1), histone deacetylase complex-repressor component (MECP1-p66) and Methyl-CpG-binding domain protein 2 (MECP2) by miR-302. The consequence of AOF2 silencing connected with DNA-methyltransferase-1 (DNMT1) deficiency resulted in global genomic DNA demethylation and H3K4 modification during somatic cell reprogramming (SCR), while supplementation of AOF2 changed pluripotent stage of iPS cells propagating their differentiation (Lin et al. 2011). Involvement of different miRNA clusters in the activation and inhibition of the specific cellular processes during reprogramming is presented on Figure 3.

The mir-302 cluster is located in the 4q25 locus of human chromosome 4 (Puca et al. 2001) and is predominantly expressed in hES and iPS cells (Suh et al. 2004, Wilson et al. 2009), while during early embryonic development and *in vitro* differentiation the expression of miR-302 is lost (Suh et al. 2004, Ren et al. 2009). The majority of miR-302-targeted genes are transcripts of developmental signals and oncogenes (Lin et al. 2008). The MiR-291/294/295 family presents a similar expression profile in mice (Judson et al. 2009).

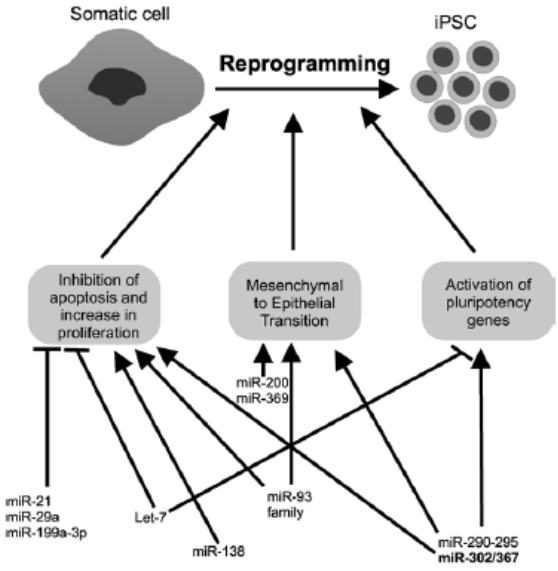

Fig. 3. The role of miRNA in the activation and inhibition of the specific cellular processes during reprogramming.

As demonstrated by Lin and coworkers (2008), miR-302 cluster not only improves the efficiency of SCR but also enhances the stemness and pluripotency of the reprogrammed cells. In addition, miR-302 may silence cyclin-dependent kinase inhibitor 1 (CDKI1, p21Cip1) thus promoting cell proliferation (Dolezalova et al. 2012). In human cells miR-302 cluster was also shown to be implicated in the inhibition of G1-S cell cycle transition by simultaneous suppression of cyclin E-CDK2 and cyclin D-CDK4/6 pathways (Lin et al. 2010).

Next to the miR-302s, the miR-367 expression is essential for iPS cell reprogramming by the miR-302/367 cluster (Betel et al. 2008, Zhang and Wu 2013). The miR-302/367 cluster has been shown to be a direct target of Oct4 and Sox2 transcription factors, since levels of miR-302/367 cluster correlate with Oct4 transcripts in ES cells during early embryonic development, indicating an important role of these miRNAs in ES cell homeostasis and maintenance of pluripotency (Card et al. 2008). Moreover, expression of the miR-302/367 cluster can directly reprogram mouse and human somatic cells to a pluripotent cell state without the presence of reprogramming transcription factors (Anokye-Danso et al. 2011). The efficiency of reprogramming obtained by Anokye-Danso and colleagues (2011) was higher when integrating viral vectors encoding miRNAs were used, compared to the method based on direct transfection of mouse and human cells with mature miRNAs (miR-200c, miR-302, miR369) (Miyoshi et al. 2011). The same combination of miRNA was investigated and found to successfully generate induced pluripotent stem cells from human somatic cells. The reprogramming was effective when transfections with mature miRNA were repeated 4 times in 48 h intervals. After 30 days post-transfection miRNA-derived iPS cells expressed genes typical for undifferentiated ES cells, including Nanog, Oct4, Sox2, Cripto, Dppa5 and Fbx15, Ssea-1, as well as E-cadherin which is the epithelial cell marker highly

expressed in ES cells (Miyoshi et al. 2011). However, the reprogramming efficiency using this method based on incorporation of mature miRNA molecules was about 0.01%. These experiments showed that miRNAs can reprogram somatic cells to pluripotency and miR-367 is required for miR-302/367 reprogramming. Moreover, the supplementation with histone deacetylase inhibitors, such as valproic acid or sodium butyrate in miR-302/367 reprogramming further enhanced this process (Anokye-Danso et al. 2011, Zhang and Wu 2013). The miR-302/367 expression along with HDAC2 suppression allows for highly efficient iPS cell reprogramming (10%) without the expression of the commonly used reprogramming factors. Moreover, the miRNA-based method was more efficient than previously described strategies, including transfection of synthetic mRNAs or OSKM factors (Warren et al. 2010).

Reprogramming methods using mature miRNAs do not require vector-based gene transfer, therefore they can be considered to be a potential solution for the personalized medical applications.

CONCLUSIONS

Reprogramming methods are constantly developing due to the amazing technological progress in the stem cell field. The performance of the applied methods is expected to be improved, while maintaining a high degree of safety. The present state of the art in the advancement of reprogramming procedures suggests that utilizing integration-free and virus-free methods under feeder-free conditions is the most promising step toward safe translation of iPS cells to future possible personalized regenerative medicine.

Improving both the efficiency and biological safety of reprogramming using recombinant proteins, mRNA and miRNAs is an opportunity for more rapid introduction of iPS cells to therapeutic application.

However, despite the overall methods of reprogramming, it is very crucial to extensively investigate the iPS cell-derived cell lines considered to be used in the clinic. The issues that must be evaluated in addition to the reprogramming technology raised in this review are the appropriate somatic origin of iPS cells and a proper differentiation of iPS in order to exclude the immunogenic potential of undifferentiated cells and elimination of the risk of tumorigenesis in the host tissue.

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10.3. Kopie prac oryginalnych

10.3.1. Augustyniak J, Lenart J, Zychowicz M, Lipka G, Gaj P, Kolanowska M, Stępień PP, Bużańska L.; Sensitivity of hiPSC-derived neural stem cells (NSC) to Pyrroloquinoline quinone depends on their developmental stage., Toxicol In Vitro. 2017 Dec;45(Pt 3):434-444.

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Sensitivity of hiPSC-derived neural stem cells (NSC) to Pyrroloquinoline quinone depends on their developmental stage

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ABSTRACT

Pyrroloquinoline quinone (PQQ) is a factor influencing on the mitochondrial biogenesis. In this study the PQQ effect on viability, total cell number, antioxidant capacity, mitochondrial biogenesis and differentiation potential was investigated in human induced Pluripotent Stem Cells (iPSC) - derived: neural stem cells (NSC), early neural progenitors (eNP) and neural progenitors (NP). Here we demonstrated that sensitivity to PQQ is dependent upon its dose and neural stage of development. Induction of the mitochondrial biogenesis by PQQ at three stages of neural differentiation was evaluated at mtDNA, mRNA and protein level. Changes in *NRF1*, *TFAM* and *PPARGC1A* gene expression were observed at all developmental stages, but only at eNP were correlated with the statistically significant increase in the mtDNA copy numbers and enhancement of SDHA, COX-1 protein level. Thus, the "developmental window" of eNP for PQQ-evoked mitochondrial biogenesis is proposed. This effect was independent of high antioxidant capacity of PQQ, which was confirmed in all tested cell populations, regardless of the stage of hiPSC neural differentiation. Furthermore, a strong induction of *GFAP*, with down regulation of *MAP2* gene expression upon PQQ treatment was observed. This indicates a possibility of shifting the balance of cell differentiation in the favor of astroglia, but more research is needed at this point.

1. Introduction

Differentiation ability and the therapeutic potential for personalized diagnosis and treatment are the main qualities of human induced pluripotent stem cells (hiPSC) (Takahashi et al., 2007; Szablowska-Gadomska et al., 2012). hiPSC can generate neural stem cells, as well as neural and glial progenitors (Choi et al., 2014). Neural stem cells (NSC) and neural progenitors (NP) can subsequence be differentiated into neurons, astrocytes and oligodendrocytes. Neural stem cells can be obtained from hiPSC by direct differentiation under adherent conditions (Chambers et al., 2010), or indirectly, through the stage of three-dimensional aggregates formed by hiPSC (Embryoid Bodies) in a suspension culture (Karumbayaram et al., 2009; Szablowska-Gadomska et al., 2012). The latter method is considered currently as the closest model of *in vitro* embryo neurogenesis.

Mitochondria play a central role in cell metabolism by controlling the cellular respiration and energy production. Somatic cells reprogramming into iPSC is associated with decrease in mitochondria content and activity, and with metabolic shift toward glycolysis. The opposite occurs during differentiation (Wanet et al., 2015). In 2010 Prigione with colleagues, introduced a "metabolic state hypothesis" linking mitochondrial state and cellular metabolism to the stage of differentiation. The differentiation process is an inverse process to the reprogramming and is associated with increase the mtDNA copy number, ATP levels and oxidative phosphorylation intensity, while the decreasing production of lactic acid. Mitochondria take a part in the stem cells differentiation decisions and development of stem cells by the level of free radicals (ROS) modulation and intensity of oxidative phosphorylation (Xu et al., 2013), but also by the increase of mitochondrial mass, which is proportional to the neuronal mass growth (Zheng et al., 2016). However, the mechanisms linking mitochondrial biogenesis to the neural stem cell differentiation are still unknown.

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Substances which induce mitochondrial biogenesis may play a role in neural stem cells (NSC) development. One of them is Pyrroloquinoline quinone, a well-studied compound modulating mitochondria DNA content and positively influencing the biogenesis of mitochondria (Bauerly et al., 2006; Chowanadisai et al., 2010). PQQ is responsible for the increase of the amount of mitochondria by modulation in *CREB* (cAMP-response-element-binding protein) phosphorylation and subsequent *PPARGC1A* (Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α)) directed up-regulation of *NRF-1* (nuclear respiratory factor-1), *NRF2* (nuclear factor, erythroid 2-like 2) and *TFAM* (transcription factor A, mitochondrial) expression (Chowanadisai et al., 2010).

Despite the plethora of studies on PQQ, its activity on different stages of neural differentiation has not been evaluated. We present here our studies on the response of neural stem cells generated from hiPSC to Pyrroloquinoline quinone treatment. We were investigating whether sensitivity to different concentrations of PQQ and induction of mitochondrial biogenesis depend on the stage of differentiation of NSC. For that purpose we generated from hiPSC three cell populations at different developmental stages: neural stem cells (NSC), early neural progenitors (eNP) and neural progenitors (NP). NSC, eNP and NP phenotype has been confirmed with qualitative and quantitative analysis of protein expression (immunocytochemistry) and gene expression (RT-PCR and RNA-seq). Subsequently, we tested the influence of PQQ on cell viability, total cell number, ROS level, mitochondrial membrane potential and differentiation capacity. The biogenesis of mitochondria has been checked by analysis: succinate dehydrogenase (SDHA) and cyclooxygenase isoenzymes (COX-1) protein level, mtDNA copy number (qPCR) and *NRF-1*, *TFAM* and *PPARGC1A* genes expression (RT-qPCR). The influence of PQQ on neural differentiation capacity of hiPSC was evaluated by the analysis of the expression of genes related to neuronal (*MAP2*) and astrocytic (*GFAP*) differentiation.

2. Materials and methods

2.1. Cell culture and neural differentiation

The induced pluripotent stem cells (iPS) were feeder free cell line derived from CD34 + fraction of human cord blood cells by transfection with EBNA1-based episomal system comprising of seven factors: *SOX2*, *OCT4*, *KLF4*, *MYC*, *NANOG*, *LIN28*, *SV40L T* antigen (The Gibco® Human Episomal iPSC Line, Life Technologies, Thermo Fisher Scientific). The cells were grown on recombinant human Vitronectin (Thermo Fisher Scientific), maintained in culture in Essential 8 Medium (Thermo Fisher Scientific). The neural commitment to the stage of NSC was performed according to Yan et al., 2013 with some modifications, while further differentiation was proceeded with our protocol implementing neural differentiation medium type I and type II. Briefly, at the 80% of confluence Essential E8 Medium was changed to the neural induction medium (Gibco® PSC Neural Induction Medium, Thermo Fisher Scientific) and cells were cultured for 7 days with medium changed every second day. On the 7th day of culture cells were plated in 96 or 6 well plates (Nunc) coated by 1:30 Matrigel:DMEM/F12 ratio (BD Matrigel™ Basement Membrane Matrix, BD Biosciences) in Neural Expansion Medium (Neural Induction Supplement 1:50, Neurobasal, DMEM/F12, 1:1) to obtain neural stem cell (NSC) population. NSCs at passage 6 were used for derivation of early neural progenitors (eNP). To obtain eNP stage of development, cells were grown for 14 days in neural differentiation medium type I: Neurobasal, DMEM/F12 [1:1], N2 supplement 1%, B27 supplement 1%, EGF [20 ng/ml], bFGF [20 ng/ml]. The third tested cell population – neural progenitors (NP), was obtained by withdrawing of EGF and bFGF from the above mentioned medium (neural differentiation medium type II) and culturing for the following 14 days. NSC, eNP and NP phenotype has been confirmed with RT-PCR, RNA-seq (mRNA copy number) and immunocytochemistry staining (protein expression), as described below.

2.2. Immunofluorescence staining

After fixation with 4% of PFA (15 min) cells were washed with PBS, permeabilized with 0,1% Triton X-100 and blocked with 10% goat serum. The following primary antibodies were applied overnight: NESTIN (1:500 Millipore), β-TUBULIN III (1:1000, Sigma-Aldrich), DCX (1:500, Cell Signaling Technology), MAP-2 (1:500, Sigma-Aldrich), Ki67 (1:500, Novocastra), NF200 (1:200, Sigma-Aldrich), GFAP (1:500, Dako). After washing with PBS, appropriate secondary antibodies (Alexa Fluor 488 and 546, 1:1000, Thermo Fisher Scientific) were applied for 1 h, and cell nuclei were contra stained with Hoechst 33,258 (Sigma-Aldrich). The measurement of fluorescence was performed by calculating the % of the area of selected positive marker staining per image. Images and calculations were obtained using Confocal Laser Microscope LSM 510 (Zeiss) and ZEN 2012 blue edition software in the Laboratory of Advanced Microscopy Techniques, Mossakowski Medical Research Centre, Polish Academy of Sciences.

2.3. PQQ treatment

Cells were seeded at a 96-well or 6-well plate covered with the solution of Matrigel:DMEM/F12, 1:30 ratio (BD Matrigel™ Basement Membrane Matrix, BD Biosciences) at density 5×10^5 cells/cm^2 in Neural Expansion Medium for Neural Stem Cells (NSC) population; 2) in neural differentiation medium (type I differentiation medium) for Early Neural Progenitors (eNP) population; 3) in neural differentiation medium (type II differentiation medium) for Neural Progenitors (NP) population. After 24 h the medium was replaced with fresh medium supplemented with PQQ (Sigma) at concentrations 0,5 μM; 0,25 μM; 0,125 μM; 0 μM.

2.4. Alamar blue cell viability assay

Cell viability was measured by Alamar blue viability assay (Sigma-Aldrich) after 5 days of incubation with PQQ at various concentrations (0–0,5 μM). Untreated NSC, eNP and NP cell populations were used as the controls. Alamar Blue (0,1 mg/ml Sigma) was added to the culture medium (1:10) for 3 h in 37 °C. The fluorescence intensity of resorufin was measured by Fluoroscan Ascent (FL, Labsystems) plate reader at 544 nm and 590 nm wavelengths for excitation and emission, respectively. The results were normalized to the whole cell number with Janus green normalization staining (Abcam) according to the manufacturer's protocol. Final data are presented as % of the untreated control.

2.5. Reactive oxygen species detection

Dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay (Sigma-Aldrich) was used to measure the ROS level at the experimental and control cell populations. The DCFHDA reagent at a concentration of 1 μM was added to the cell cultures then incubated for 3 h. After the fat-soluble DCFH-DA enters the cell, it is hydrolyzed by lipase into DCFH, then the non-fluorescent DCFH is oxidized by intracellular ROS to become fluorescent DCF, detected by quantitative fluorescent measurements. The fluorescence of DCF was measured at a wavelength: 485–538 nm on plate reader (Fluoroscan Ascent FL (Labsystems). The normalization of the fluorescent measurements to the whole cell number for each sample was performed with Janus green normalization staining (Abcam) according to the manufacturer's protocol. Final data are presented as % of untreated control.

2.6. Mitochondrial membrane potential assay

Changes in the mitochondrial membrane potential were evaluated at three different stages of hiPSC neural differentiation (NSC, eNP and NP) in control cells and after 5 day of cell exposition to PQQ (0–0,5 μM), using the mitochondrial membrane potential (Δψm)-

sensitive fluorochrome Mitotracker® Red CMXRos (Thermo Fisher Scientific). The level of mitochondrial membrane potential, revealed as the red dye fluorescence intensity, was measured 4 h after MitoTracker Red CMXRos (50 nM) was added to the cells and detected at a wavelength: 544–590 nm on plate reader (Fluoroscan Ascent FL (Labsystems). Janus green normalization staining (Abcam) was used for normalization of the fluorescence readouts to the whole cell number for each sample and final data are presented as % of untreated with PQQ control.

2.7. Succinate dehydrogenase (SDH-A) and cyclooxygenase isoenzymes (COX-1) protein expression assay

The protein level of succinate dehydrogenase A (SDH-A) was evaluated by enzyme-linked immunosorbent assay (ELISA) (MitoBiogenesis In-Cell ELISA Colorimetric kit, Abcam). After 5 days of incubation with PQQ, NSC, eNP and NP cells were fixed with 4% PFA on 96-well Matrigel coated plate (RT), then the cells were incubated for 5 min with 0.5% acetic acid in order to block the endogenous alkaline phosphatase activity. After washing with PBS, permeabilization of the cell membrane by 1 × Permabilization Buffer for 30 min was performed. Unspecific antibody binding was blocked with 2 × Blocking Buffer for 2 h and the incubation with primary antibodies anti-SDH-A was performed for 24 h. The incubation for 60 min with the secondary antibody conjugated with alkaline phosphatase was followed by the reaction with the substrate for alkaline phosphatase. The absorbance was measured colorimetrically immediately after reaction took place, at OD 405 nm wavelength (Fluostar plate reader OMEGA, BMG LABTECH). To determine the protein level of cyclooxygenase isoenzymes (COX-1), the enzyme-linked immunosorbent assay (MitoBiogenesis In-Cell ELISA Colorimetric kit, Abcam) was performed. After the fixation of NSC, eNP and NP control and experimental cell populations, permeabilization was performed with 1 × Permabilization Buffer. After incubation with 2 × Blocking Buffer, primary antibody anti-COX-1 for 24 h was added. Secondary antibody conjugated with horseradish peroxidase was applied for 60 min. After antibody removal, the substrates for horseradish peroxidase were added and absorbance was immediately measured at a wavelength of OD 600 nm (Fluostar plate reader OMEGA, BMG LABTECH). SDH and COX-1 protein expression level (readouts as the absorbance level) were normalized to cell number with Janus green normalization staining (Abcam) according to the manufacturer's protocol. After these steps SDHA and COX-1 protein expression were presented as % of untreated with PQQ control.

2.8. Total cell number measurements

After 5 days of incubation with PQQ the total cell number was evaluated by Janus Green reagent (Abcam) in tested cell populations. Cells were fixed with 4% PFA (RT). Incubation with 1 × Janus green reagent (5 min) was followed by the reaction with 50 µl of 0,5 M HCl (10 min in RT). Before the HCL was added, the cells were washed with PBS until Janus green dye was completely removed. The absorbance was measured using a Fluostar plate reader OMEGA (BMG LABTECH) at a wavelength: OD 595 nm. Total cell number was obtained from standard curve. The results are presented as % of untreated control.

2.9. Gene expression analysis

2.9.1. Primer design

All primers were design using Primer3 software (*http://bioinfo.ut.ee/primer3-0.4.0/*) based on the mRNA and DNA nucleotide sequences from GenBank (*http://www.ncbi.nlm.nih.gov/genbank/*). Primers specificity was checked in Primer-BLAST (*http://www.ncbi.nlm.nih.gov/tools/primer-blast/*). The properties of each RT-PCR, qPCR and RT-qPCR primer, including melting temperature, GC content, and PCR suitability were evaluated using free online software (*http://www.bioinformatics.org/sms2/pcr_primer_stats.html*). UNAFOLD tool (*http://eu.idtdna.com/UNAFold*) was used to simulate folding of amplicons in melting temperature of primers. Genes amplification with designed primers were tested *in silco* (http://www.bioinformatics.org/sms2/pcr_products.html). uMELT (Melting Curve Predictions Software) were used to melting-curve prediction (*https://www.dna.utah.edu/umelt/umelt.html*). All primers used in this study were synthesized in the Laboratory of DNA Sequencing and Oligonucleotide Synthesis, Institute of Biochemistry and Biophysics Polish Academy of Sciences, (*http://oligo.pl/*). After *in silico* validation all the primer pairs were tested in a qPCR set-up step. Standard curves was generated to determine the efficiency of amplification, by pooling undiluted cDNA from the samples across all conditions, and diluting the pooled cDNA by 1:5, 1:25, 1:125, 1:625 and 1:3125. PCR efficiencies (Suppl. Tables 2 and 3) (E) were calculated as $E = (10^{-1/\text{slope}} - 1) \times 100$. A melting-curve analysis was performed at the end of the amplification, to confirm the presence of a single PCR product.

2.9.2. DNA and RNA isolation

DNA and total RNA was isolated from cells using ZR-*Duet*™ DNA/RNA Mini Prep Kit (Zymo Rsearch) according to the manufacturers' protocols. Genomic DNA was eliminated from RNA samples by a DNase treatment using a Clean-Up RNA Concentrator kit (A & A Biotechnology, Gdynia, Poland). The concentration of RNA and DNA was determined by measuring the absorbance at 260 nm using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher scientific, Waltham, USA) and its purity was assessed by calculating the 260/280 absorbance ratio. RNA integrity was assessed by electrophoresis on 2% agarose gels.

2.9.3. RT

cDNA to RT-PCR was transformed by reverse transcription (High-Capacity RNA-to-cDNA™ Kit, Thermo Fisher Scientific).

2.9.4. RT-PCR

Expression of genes *POU5F1* (POU Class 5 Homeobox 1, encoded OCT4), *SOX2* (SRY (sex determining region Y)-box 2), *NANOG* (Nanog Homeobox), *NES* (Nestin), *MAP2* (Microtubule Associated Protein 2), *NEUROD1* (Neuronal Differentiation 1), *NEFL* (Neurofilament Light), *TUBB3* (Tubulin Beta 3 Class III), *GFAP* (Glial Fibrillary Acidic Protein), *NRF1* (Nuclear Respiratory Factor 1), *TFAM* (Transcription Factor A, Mitochondrial), *PPARGC1A* (PPARG Coactivator 1 Alpha), *ACTB* (Actin Beta) were analyzed at mRNA level by RT-PCR method-using 2 × PCR Master Mix Plus (A & A Biotechnology) in the presence of specific primers (Suppl. Table 1), (LightCycler 96, Roche). PCR was performed using a Q5® High-Fidelity DNA Polymerase (Promega). The PCR primer sequences are showed in the Suppl. Table 1. The PCR was performed in a CivivCycler Thermocycler (Biotech INC) using the following cycle parameters: initial denaturation 98 °C, 35 cycles of 98 °C for 10 s, 66 °C, 67 °C, 68 °C or 69 °C (depending on target) for 30 s, 72 °C for 44 s and final extension - 72 °C for 2 min. Amplification products were separated by electrophoresis on 1.5% agarose gel (Suppl. Fig. 1). All primers were verified *in silico* (point 2.9.1.) and *in vitro*.

2.9.5. RT-qPCR

The cDNA *NRF1*, *TFAM*, *PPARGC1A*, *MAP2* and *GFAP amplicons* were analyzed by real-time PCR using the iTaq™ universal SYBR® Green supermix (Bio-Rad) in LightCycler® 96 (Roche Diagnostics GmbH, Mannheim, Germany). The amplification was performed with 1 µl (10 ng) of cDNA template in 25 µl of reaction mixture containing 12,5 µl of iTaq™ universal SYBR® Green supermix (Bio-Rad) and 0,25 µl each primer. The amplification program included an initial denaturation step at 95 °C for 3 min, 45 cycles of denaturation at 95 °C for 10 s, and annealing/extension at 58 °C for 1 min. All samples were tested in triplicate. The sequences of the specific primers used in experiments are presented in Suppl. Table 2. For all genes PCR efficiency was tested

(Suppl. Table 2).

2.9.5.1. Validation of reference genes for RT-qPCR analysis

In order to compare gene expression between tested cell populations we decided to select and assay the expression levels of a panel of 16 reference genes (Suppl. Fig. 2, Suppl. Table 3). The candidates were initially selected from relevant literature: (Synnergren et al., 2007; Coulson et al., 2008; She et al., 2009; Eisenberg and Levanon, 2013; Vossaert et al., 2013). Reverse transcription and real-time PCR were performed as described in point 2.9.4. Sequences of primers (Suppl. Table 3) used as housekeeping genes were designed and verified *in silico* (point 2.9.1) and *in vitro*, as described previously. Validations of housekeeping genes for the three tested cell populations were prepared in NormFinder software (Suppl. Fig. 2).

2.9.6. qPCR

Detection of the expression of *mt-ND1* (Mitochondrially Encoded NADH:Ubiquinone Oxidoreductase Core Subunit 1), *SLCO2B1* (Solute Carrier Organic Anion Transporter Family Member 2B1), *mt-ND5* (Mitochondrially Encoded NADH:Ubiquinone Oxidoreductase Core Subunit 5), *SERPINA1* (Serpin Family A Member 1) DNA was carried out using real-time RT-qPCR with a iTaq™ universal SYBR® Green supermix (Bio-rad) and LightCycler 96 (Roche). The quantitative real-time PCR (Q-PCR) was performed with 1 µl (10 ng) of DNA template in 25 µl of reaction mixture containing 12,5 µl of iTaq™ universal SYBR® Green supermix (Bio-rad) and 0,25 µM/l each primer. PCR reactions were subjected to hot start at 95 °C for 3 min followed by 45 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. The ratio of mtDNA to nuclear DNA was calculated for two pairs of genes: *mt-ND5* with *SERPINA1* and *mt-ND1*with *SLCO2B1*. For the analysis of mt-DNA copy number specific primers were design and verified *in silico* and *in vitro*, as described previously (point 2.10.1). In order to improve and enhance the specificity of amplification of the *mt-ND5*, *SERPINA1*, *mt-ND1*, *SLCO2B1* DNA, with the design primers pseudogenes amplification was excluded *in silico* by using: BLAT Search Genome software (https://genome.ucsc.edu/cgi-bin/hgBlat?command=start). Sequence of the primers used for qPCR are shown in the Suppl. Table 4.

2.9.7. qPCR, RT-qPCR data analysis

The quantification cycle (Cq) values and the baseline settings of each amplification plate were automatically calculated by the qPCR instrument software (LightCycler® 96 Software, Roche Diagnostics GmbH, Mannheim, Germany). The data were analyzed using GeneEx 6.1 software (Multid Analyses AB, Göteborg, Sweden). Relative gene expression was performed in relation to a comparator (references genes). The reference genes chosen for gene expression analysis were showed in the Supplementary data. As a comparator NormFinder validated 13 genes for gene expression analysis for NSC, and 3 for eNP, and NP. The software calculates gene expression based on 13 genes for gene expression analysis for NSC, and 3 for eNP, and NP. NormFinder algorithm has been used to determine the most stable reference gene(s). This analysis provides not only a ranking of the assessed genes based on their stability value, but also calculates the optimal number of genes necessary for normalization.

2.9.8. RNA-Seq

The quality and quantity of isolated RNA was verified on 2200 TapeStation (Agilent) and Quantus fluorometer (Promega) respectively. Only RNA with RNA Integrity Number (RIN) value ≥ 7.5 was subjected to further library preparation. The library of RNA was prepared by Truseq Stranded mRNA kit (cat.no. RS-122-2101, Illumina, USA) according to the protocol. The quality and quantity of the library was measured on 2200 TapeStation and on Quantus. Sequencing was performed with the use of NextSeq 500/550 High Output Kit-75 cycles (cat.no. FC-404-2005, Illumina, USA) on NextSeq 500 sequencer (Illumina, USA). The control populations were performed for genome-wide RNAseq analysis. The libraries were subjected to sequencing using the Illumina NextSeq 500 76 bp single-end mode. Before using in statistical analysis the raw RNAseq data (FASTQ) was trimmed for adapter sequences and filtered using Trimmomatic (Bolger and Lohse, 2014). Then the reads were mapped with STAR (Dobin et al., 2013) aligner using the reference genome (GRCh38) coupled with Ensembl 88 transcriptome annotations. Transcription levels were calculated using HTseq-count (Anders et al., 2015) (Tange, 2011) and were further used for differential gene expression (DGE) analysis using edgeR (Robinson et al., 2010). Data are presented as normalized counts per million ($\log_2$), and differential gene expression results.

2.10. Statistical analysis

The results from 3 independent experiments, each in 4 replicates were analyzed using the following statistical tests after Kolmogorov–Smirnov normality test: 1) One-way ANOVA, Tukey's post-test or Bonferroni Multiple Comparison Test, 2) Two-way ANOVA, Bonferroni Multiple Comparison Test, 3) t-student test. The statistical significance of the results was determined for *p*-value; 1) $p < 0,05$ (*); $p < 0,001$ (**); $p < 0,0001$ (***); $p < 0,00001$ (****) for comparison inside the group; 2) $p < 0,05$ (#); $p < 0,001$ (##); $p < 0,0001$ (###); $p < 0,00001$ (####) for comparison between group. Statistical analysis was performed using GraphPad Prism 5.0. RNA-Seq results from 3 independent experiments are presented as normalized counts per million ($\log_2$), and differential gene expression results have been obtained using generalized linear model (GLM) linear regression in R (*edgeR*) (Robinson et al., 2010), *p*-value < 0.05 (*); $p < 0,001$ (**); $p < 0,0001$ (***); $p < 0,00001$ (****). Results on the graphs are given as the mean with standard error of measurement (SEM). Data in the text are shown as the mean with standard deviation (SD).

3. Results

3.1. hiPSC neural differentiation

Human induced pluripotent stem cells were neurally committed and differentiated into three cell populations: NSC, eNP, and NP at different stages of development as described in Materials and methods section. The characteristics of various cell phenotypes in NSC, eNP, and NP populations were demonstrate with RNA-Seq (NGS) analysis of genes involved in maintaining of pluripotency (*POU5F1*, *NANOG*, *SOX2*) regulation and proliferation (*MKI67*) as well as genes engaged in neural differentiation process, like early neural marker *NES*, neuronal markers *TUBB3*, *DCX*, *NEFH*, *NEFL*, *NEUROD1*, *MAP2*, glial markers: for astrocytes: *GFAP*, *ALDH1L1* (Aldehyde Dehydrogenase 1 Family Member L1), and oligodendrocytes: *CSPG4* (Chondroitin Sulfate Proteoglycan 4) (Fig. 1A). Differentiation protocol applied in this study resulted in loss of pluripotency markers (*POU5F1* and *NANOG*) in all three populations. *SOX2*, besides being marker of pluripotency is also indicator of neural lineage commitment and, thus, is still present in analyzed cells. Similar mRNA copy number level for neural/neuronal markers, such as *NES*, *TUBB3*, *NEFH* as well as proliferation marker *MKI67* were observed in all tested populations. However, during acquiring more mature phenotype cells start to express more mRNA copy number for *DCX*, *NEFL*, *MAP2*.Transition from NSC to eNP resulted in statistically significant elevation of expression of astrocytic markers, such as *GFAP* and *ALDH1L1*, while the level of mRNA for the early oligodendroglial marker *CSPG4* was constantly present in cells regardless of time of differentiation (Fig. 1A).

Additional genes analyzed by RNA-Seq in the three populations include contributors of the complex of the mitochondrial respiratory chain *SDHA* (Succinate Dehydrogenase Complex Flavoprotein Subunit A) and *MT-CO1* (Mitochondrially Encoded Cytochrome C Oxidase I). The mRNA copy number for these genes is on the same level for NSC,

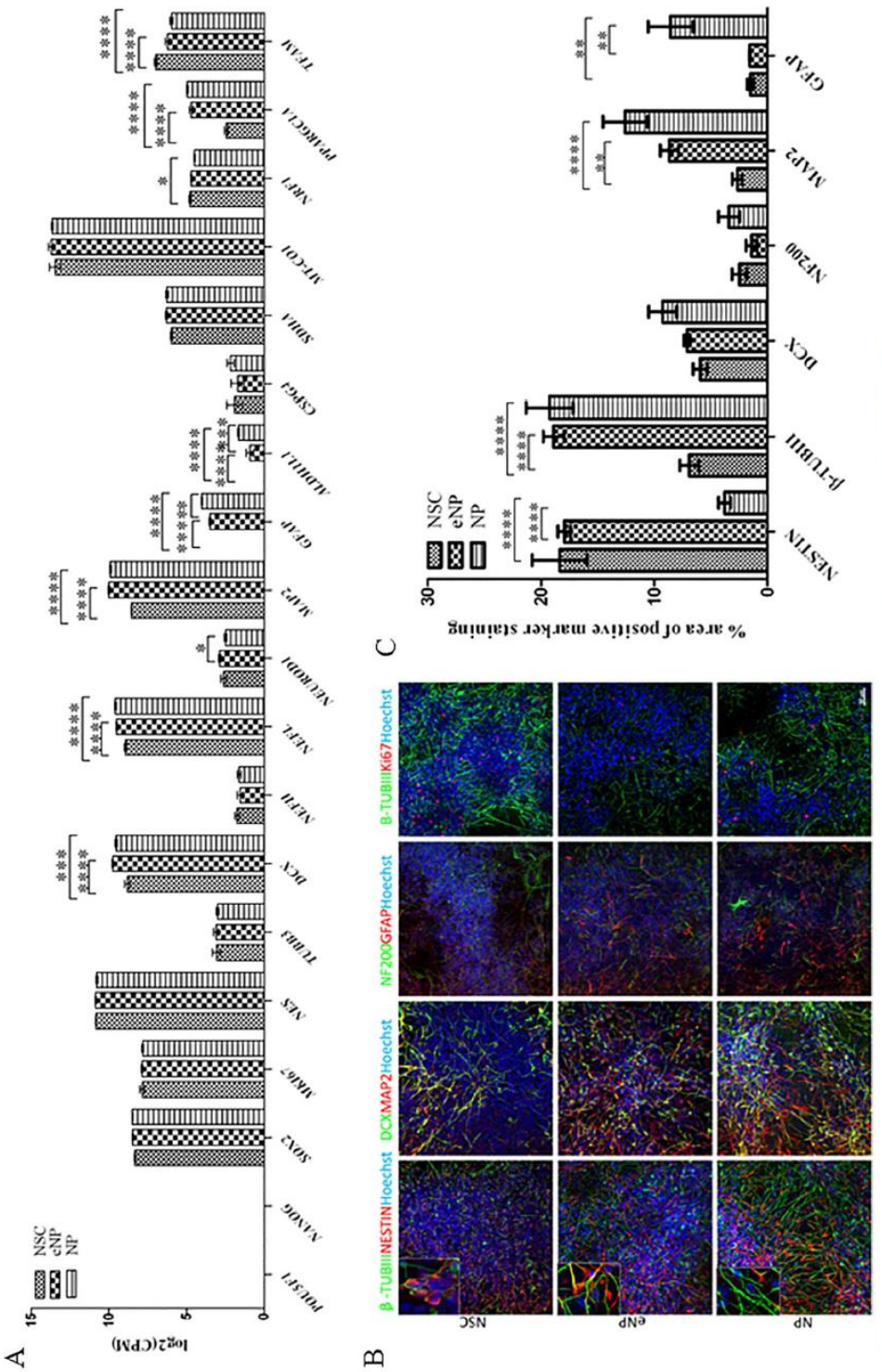

Fig. 1. Three cell populations resulted from neural differentiation of human iPSC: neural stem cells (NSC), early neural progenitors (eNP), neural progenitors (NP). (A) RNA-Seq (NGS) analysis of genes involved in: maintaining of pluripotency (*POU5F1, NANOG, SOX2*), proliferation (*MKI67*), early neural (*NES, SOX2*) and neuronal differentiation (*NES, TUBB3, DCX, NEFH, NEFL, NEUROD1, MAP2*), glial differentiation (*GFAP, ALDH1L1, CSPG4*), complex of the mitochondrial respiratory chain (*SDHA, MT-CO1*) and biogenesis of mitochondria (*NRF1, PPARGC1A, TFAM*). Results are presented as normalized counts per million (log_2), and differential gene expression results have been obtained using generalized linear model (GLM) linear regression in R (*edgeR*), *p* value < 0.05 (*); $p < 0,001$ (**); $p < 0,0001$ (***); $p < 0,00001$ (****). (B) The expresion of selected proteins in NSC, eNP and NP cell populations immunostained for neural (NESTIN), neuronal (β-TUBIII, DOUBLECORTIN-DCX, MAP2, NF200), astrocytic (GFAP) and proliferation (Ki67) markers. Scale bar 50 µm. (C) Quantitative results of immunofluorescence data are presented as area [%] of positive marker staining. Brackets shows statistical significance between populations (one way ANOVA, Bonferroni multiple comparison post-test, (*) $p < 0,05$; (**); (****) $p < 0,0001$.

eNP and NP cell populations.

On the Fig. 1A we present RNA level of representative genes important for mitochondrial biogenesis. The expression of *NRF1* and *TFAM* decreased during differentiation, while *PPARGC1A* significantly increased in neural progenitors (eNP and NP).

To confirm on the protein level the expression of genes important for neural differentiation, immunocytochemical staining was performed. The cellular immunostaining and the quantitative evaluation of the expression of neural markers in the investigated cell populations is presented on Fig. 1B and C, respectively. The change in cell morphology along differentiation, from more rounded and flattened in NSC to elongated, with small cell body and long protrusions forming neuronal network in NP populations are clearly visible (Fig. 1B). Proliferation of NP cells (Ki67 staining), as compared to eNP and NSC population decreased (Fig. 1B), however there is no significant difference in the expression of *MKI67* on the mRNA level (Fig. 1A). The expression of NESTIN on protein level significantly decreased during transition from eNP to NP stage, while the difference between NSC and eNP is not significant (Fig. 1C). The difference in the expression of *NES* between all three populations on mRNA level was also not significant (Fig. 1A). The expression of neuronal markers, such as β-TUBIII and MAP2 significantly increased during transition from NSC population to eNP, while the difference in the expression of this markers between eNP and NP is not significant (Fig. 1C), The amount of astrocytic (GFAP) staining significantly increased in the NP population as compared to NSC and eNP populations (Fig. 1B and C).

3.2. Viability assay

The PQQ treatment resulted in statistically significant increase of the viability of NSC (***, $p < 0{,}0001$) at all tested concentrations and eNP population for 0,125 μM–0,25 μM (*, $p < 0{,}05$ for dose: 0,125 μM, 0,25 μM and ***, $p < 0{,}0001$, for 0,5 μM). However, the viability of NP cell population was not changed by PQQ treatment with the exception for the highest tested dose 0,5 μM, where the significant decrease of viability was observed (*, $p < 0{,}05$). When different tested groups were compared, significant difference was observed: viability of NSC was enhanced as compared to NP at all tested dose (###, $p < 0{,}0001$), and for NSC/eNP comparison at 0,125 μM PQQ concentration (##, $p < 0{,}001$). Thus at the highest dose (0,5 μM), PQQ revealed a positive impact on cell viability at: NSC (138,46% ± 12,58) and eNP (130,08% ± 5.65) stages and marginally decreased cell viability (4,02% ± 6.13) in neural progenitors (NP). The results are shown in Fig. 2.

3.3. Reactive Oxygen Species (ROS) detection

After 5 days of PQQ exposition the intracellular ROS levels significantly decreased (when compared to the untreated control) in all tested groups: NSC, eNP and NP, but only at the highest 0,5 μM. In NP population ROS level was significantly decreased upon PQQ treatment at all tested concentrations (0,125 μM and 0,5 μM, ***, $p < 0{,}0001$ and 0,25 μM,**, $p < 0{,}001$). For NSC and eNP cell populations only highest dose of PQQ (0,5 μM) induced significant decrease (***, $p < 0{,}0001$ for NSC) and (*, $p < 0{,}05$ for eNP). No significant differences between tested groups were detected. Thus, in our study we have observed decrease in the ROS level in all stages of tested neural development. At the highest PQQ dose (0,5 μM) ROS ratio (*vs* control) was noted as: 74,03%(± 6.73) for NSC; 83,37%(± 14,38) for eNP and 75,41%(± 2.55) for NP. Differences in ROS level between tested populations were not significant. The previous reports also showed that significant changes for antioxidant-related genes during differentiation were not detected (Prigione and Adjaye, 2010), therefore the antioxidant potential of cells could be at stable level, and not dependent from stage of neural differentiation. The results are shown in Fig. 2B.

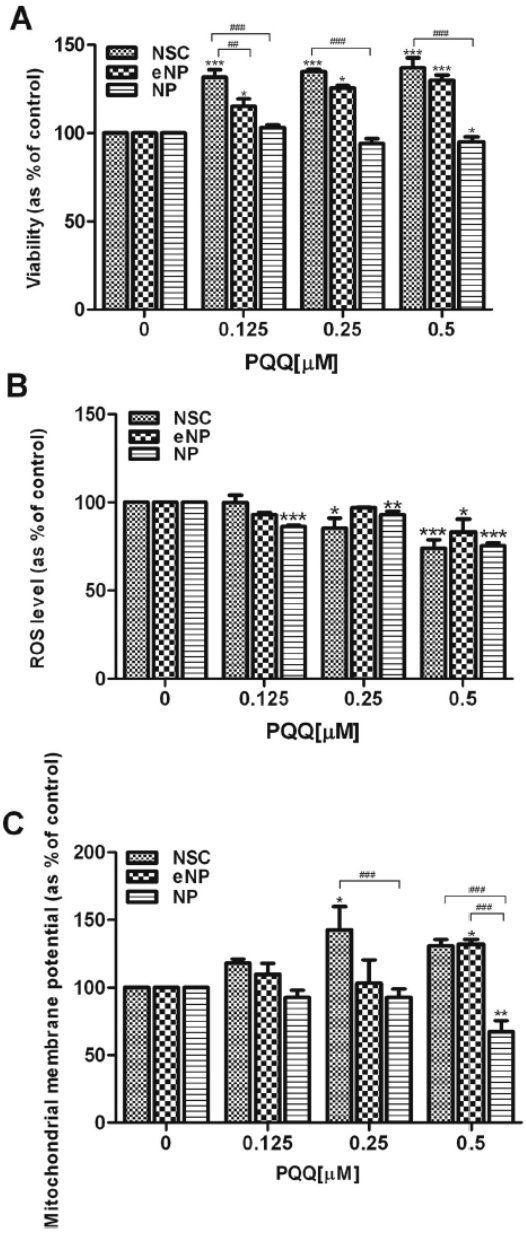

Fig. 2. A The viability of hiPSC at three different stages of neural differentiation: neural stem cells (NSC), early neural progenitors (eNP) and neural progenitors (NP) after 5 days exposure to the different concentrations of PQQ. B) ROS level measured in tested cell populations after 5 days exposure to the different concentrations of PQQ evaluated by DCFH-DA assay. C) The mitochondrial membrane potential measured in tested cell population after 5 days exposure to the different concentrations of PQQ evaluated by MitoTracker Red CMXRos staining. After normalization of data to cell number (obtained by Janus Green staining), results has been shown as % of control untreated with PQQ. Brackets shows statistical significance between samples *vs.* control (one way ANOVA, Tukey's post-test): (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0{,}001$; (****) $p < 0{,}0001$; and comparison between groups (two way ANOVA, Bonferroni post-test): (#) $p < 0{,}05$; (##) $p < 0{,}01$; (###) $p < 0{,}001$; (####) $p < 0{,}0001$.

3.4. Mitochondrial membrane potential

PQQ at dose 0,25 μM significantly increase mitochondrial membrane potential in NSC population (*, $p < 0,05$). The mitochondrial membrane potential was also significantly increased (*, $p < 0,05$) in eNP population at the highest dose of PQQ (0,5 μM). However, statistically significant decrease of mitochondrial membrane potential was detected in NP (**, $p < 0,01$). The evaluation of differences between tested cell populations revealed significant difference between NSC and NP at PQQ dose 0,25 μM and 0,5 μM (###, $p < 0,001$), then between eNP and NP only at the highest PQQ dose 0,5 μM (###, $p < 0,001$). Functional mitochondria were labeled by MitoTracker Red CMXRos (Molecular Probes, Thermo Fisher Scientific), as described previously. PQQ enhance mitochondrial membrane potential at all tested concentrations at NSC and eNP, but not at NP stage of differentiation. The data for 0,5 μM o PQQ were shown as: (130,82 ± 6,49) at stage NSC: and eNP (131,99; ± 3.60) at eNP and (0,5 μM:68,26 ± 15,96) at NP stage of differentiation. The results are shown in Fig. 2C.

3.5. SDH-A and COX-1 protein expression and total cell number

Succinate dehydrogenase complex, subunit A, flavoprotein variant is one of a major protein in the complex of the mitochondrial respiratory chain. SDHA protein expression level was evaluated by enzyme-linked immunosorbent assay (ELISA) after 5 days of NSC, eNP and NP PQQ different concentration treatment. Statistically significant increase in SDHA protein expression was shown for NSC (**, $p < 0,01$), at the highest concentration of PQQ and for eNP (*, $p < 0,05$) at the dose 0,125 μM, 0,5 μM. SDHA at the highest PQQ 0,5 μM tested dose amounted 122,75%(± 2.12) (NSC); 104,56%(± 2.54) (eNP); 95,96%(± 11,22) (NP). Comparison between different cell populations revealed significant difference in the SDHA protein expression between NSC and NP (###, $p < 0,001$). The results are shown in Fig. 3A.

Cytochrome c oxidase I (COX1) also known as mitochondrially encoded cytochrome *c* oxidase I (MT-CO1), is a main subunit of the cytochrome *c* oxidase complex protein. At the highest dose (0,5 μM) PQQ significant increase of COX-1 protein expression level in NSC (*, $p < 0,01$ and eNP (***, $p < 0,001$) population is observed. On the other hand 0,5 μM PQQ significantly decreased COX-1 protein expression in NP population (***, $p < 0,001$). COX-1 at 0,5 μM PQQ amounted 107,37%(± 3.88) for NSC; 135,23%(± 5.25) for eNP and 62,88%(± 11,18) for NP cell populations. Statistical evaluation of differences between NSC, eNP and NP revealed significant difference in 0,25 μM PQQ between NSC *vs.* eNP (##, $p < 0,01$) and eNP *vs.* NP (##, $p < 0,01$). At the highest dose significant difference was indicated between NSC *vs.* eNP (###, $p < 0,001$); eNP *vs.* NP (####, $p < 0,0001$) and NSC *vs.* NP (####, $p < 0,0001$). The results are shown in Fig. 3B.

Thus, the exposure to PQQ increased level of markers indicative for induction of mitochondrial biogenesis (SDHA, COX-1) at NSC and eNP stage of differentiation. Reduction of SDHA and COX-1 protein expression were observed in a NP population.

Total cell number in control and experimental populations were estimated by Janus Green staining. At the highest dose PQQ significantly enhance total cell number of NSC and eNP (*, $p < 0,05$) and significantly decrease cell number in NP population (*, $p < 0,05$). Total cell number was significantly higher in NSC *vs* eNP population at 0,125 μM, 0,5 μM doses: (####, $p < 0,0001$) and 0,25 μM (##, $p < 0,01$) respectively. Positive influence of 0,5 μM PQQ on the total cells number of NSC (120,11%(± 13,59)) and eNP (110,00%(± 6.37)) but not on NP cells culture (0,5 μM; 75,38%(± 13,48)) was observed. Significant difference between the eNP and NP was revealed at all tested doses: 0,125 μM (###, $p < 0,001$); 0,25 μM and 0,5 μM (####, $p < 0,0001$). The results are shown in Fig. 3C.

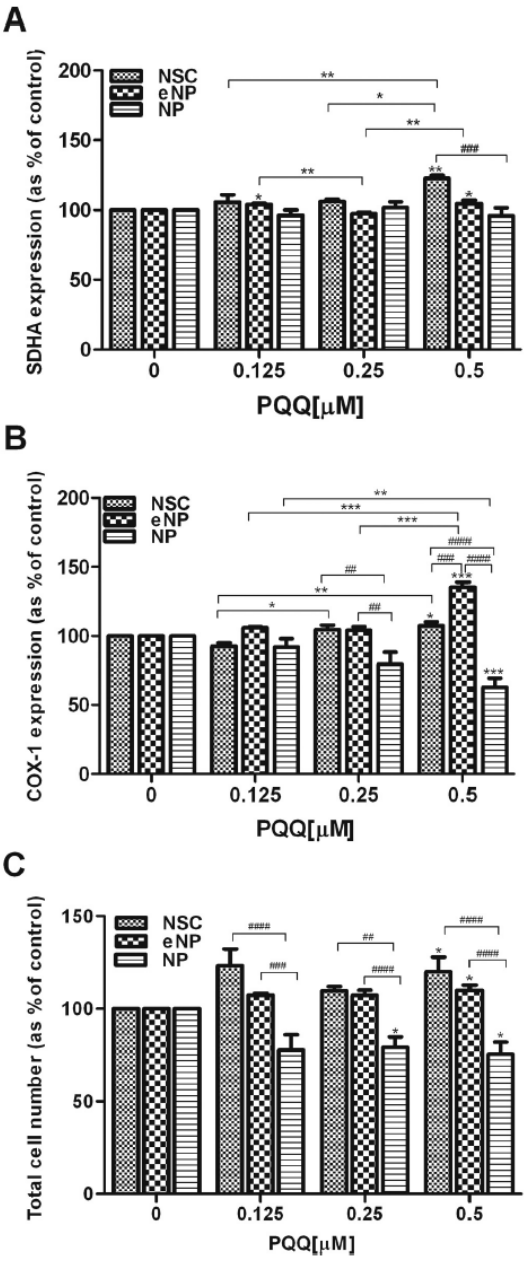

Fig. 3. Mitochondrial biogenesis indicators measured in tested cell populations: neural stem cells (NSC), early neural progenitors (eNP) and neural progenitors (NP) after 5 days of exposure to the different concentrations of PQQ. (A) The Succinate dehydrogenase (SDH) level; (B) The Cyclooxygenase isoenzymes (COX-I) level. SDHA and COX-1 level was measured by MitoBiogenesis™ In-Cell ELISA Kit. After normalization of data (A,B) to cell number (obtained by Janus Green staining), results have been shown as % of control untreated with PQQ. (C) Total cells number obtained from standard curve measured with Janus Green staining. Brackets shows statistical significance between samples *vs.* control (one way ANOVA, Tukey's post-test): (*) $p < 0,05$; (**) $p < 0,01$; (***) $p < 0,001$; (****) $p < 0,0001$; and comparison between groups (two way ANOVA, Bonferroni post-test): (#) $p < 0.05$; (##) $p < 0,01$; (###) $p < 0,001$; (####) $p < 0,0001$.

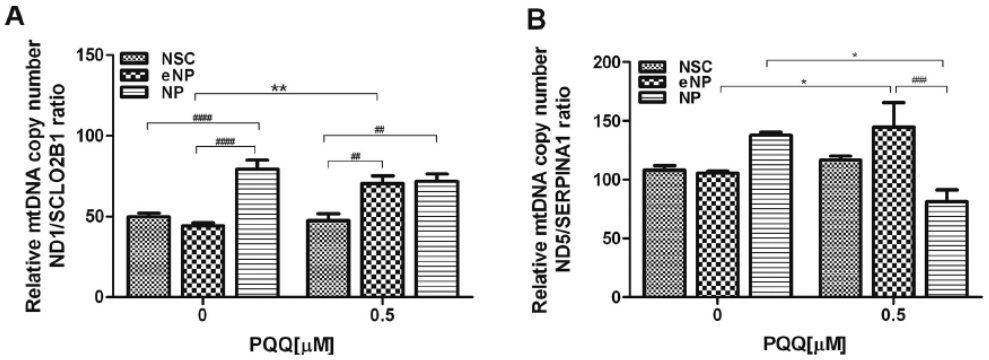

Fig. 4. The relative mtDNA copy number evaluated by q-PCR measurement of *ND1*, *ND5*, *SLCO2B1 and SERPINA1* level in tested cell populations: neural stem cells (NSC), early neural progenitors (eNP) and neural progenitors (NP) after 5 days exposure to the 0,5 μM of PQQ. (A) The ratio of NADH dehydrogenase subunit 1 (ND1) to SLCO2B1 and (B) the ratio of (*ND5*) to *SERPINA1* are presented as mean (SEM). Brackets shows statistical significance between samples *vs*. control, (*t*-Student): (*) $p < 0,05$; (**) $p < 0,01$; (***) $p < 0,001$; (****) $p < 0,0001$; and comparison between groups (two way ANOVA, Bonferroni post-test): (#) $p < 0,05$; (##) $p < 0,01$; (###) $p < 0,001$; (####) $p < 0,0001$.

3.6. mtDNA copy number

Representative mitochondrial (*ND1 and ND5*) and nuclear (*SCLO2B1* and *SERPINA1*) genes have been chosen to evaluate quantitatively changes in mtDNA copy number during neural differentiation. After treatment of NSC, eNP and NP with 0,5 μM PQQ for 5 days total cellular DNA was isolated and the number of mtDNA copy was evaluated by qPCR.

The relative mtDNA copy number was calculated as a ratio of: 1) mitochondrial *ND1* gene and nuclear *SCLO2B1* gene or 2) mitochondrial *ND5* and nuclear *SERPINA1* gene. Fig. 4A shows a relative copy number *ND1/SCLO2B1* ratio treated group *vs*. untreated control. The incubation with PQQ significantly increases mtDNA copy number *ND1/SCLO2B1* ratio at the eNP (**, $p < 0,01$) stage, but not at the NSC and NP stages of hiPSC neural differentiation. Analysis of differences between PQQ treated and control cell populations revealed significant difference between NSC and NP in both control (####, $p < 0,0001$) and experimental (##, $p < 0,01$) groups. Significant difference between eNP and NP was shown for the control group (####, $p < 0,0001$), while difference between NSC and eNP was significant only in treated group (##, $p < 0,01$).

Fig. 4B shows a relative copy number *ND5/SERPINA1* ratio treated group *vs*. untreated control. PQQ significantly increases relative mtDNA copy number *ND5/SERPINA1* ratio in treated group *vs*. untreated control at the eNP (*, $p < 0,05$) stage of differentiation; but significant decrease at NP (*, $p < 0,5$) is observed. Impact of PQQ on the NSC cell population was not statistically significant. Significant difference between eNP and NP was shown for the experimental group (###, $p < 0,001$).

In summary PQQ induce increase in mtDNA copy number for both tested mitochondrial genes only at eNP stage. This is confirmed by the increase in control *vs*. experimental (0,5 μM PQQ) 44,17(± 4.30)/70,40(± 9.35); 105,60(± 2.94)/144,67(± 29,63) for *ND1/SCLO2B1* ratio and *ND5/SERPINA1* ratio respectively.

3.7. Relative gene expression analysis (RT-qPCR) of representative genes involved in mitochondrial biogenesis and neural differentiation

The *NRF1*, *TFAM*, *PPARGC1A*, *MAP2*, *GFAP* relative gene expression was evaluated using RT-qPCR method. Total RNA was isolated from treated (0,5 μM PQQ) and untreated (control) cells on the fifth day of the experiment from hiPSC at the NSC, eNP and NP cell populations (Fig. 5).

The relative *NRF1* gene expression in treated (0,5 μM PQQ) *vs*. untreated control group was the highest at the stage of early progenitors (eNP). Significant increase in relative *NRF1* gene expression value after treatment by PQQ in comparison to control was also detected at the stage of neural stem cells (NSC), but not at the neural progenitors (NP) stage of development.

In our experiments PQQ induced changes in *NRF1* relative gene expression level at all tested stages of differentiation. At the stage of NSC and eNP PQQ increase relative expression of *NRF1* by fold change 1,01(± 0.09) and 2,08(± 0.27) for NSC and eNP respectively. Different response was observed at the stage of NP, where *NRF1* expression fold change was at the level − 0,39,(± 1.26). Statistically significant difference at the relative *NRF1* gene expression level was observed between NSC *vs*. NP cells populations (*, $p < 0,05$) and eNP *vs*. NP, (***, $p < 0,001$). (Fig. 5A).

In the case of *PPARGC1A* gene expression, the strongest response for the PQQ treatment was found for the stage NSC. The relative *PPARGC1A* expression was increased upon PQQ treatment at NSC and eNP populations, but not at the late neural progenitors NP population. The differences between all tested populations were statistically significant (***, $p < 0,001$) (Fig. 5B). The relative expression of peroxisome proliferator-activated receptor (PPAR) gamma coactivator 1-α *PPARGC1A*(*PGC1 alpha*) - the master regulator of mitochondrial biogenesis was enhanced in NSC 5,51(± 0.33) and eNP 2,88(± 0.37) populations, while repressed by PQQ at stage NP–0,21(± 0.96).

In the case of *TFAM*, PQQ increase relative *TFAM* expression level in the early neural progenitors (eNP) while at NSC and NP stages such effect was not observed. Statistically significant difference at *TFAM* relative gene expression was demonstrated between all tested cell populations at different stages of neural development (***, p < 0,001) (Fig. 5C). PQQ change *TFAM* relative gene expression level at all tested stages of differentiation. Difference between all tested populations was recorded. At NSC stage − 1,10(± 0,30) and NP − 2,47(± 0,25) was observed. At eNP stage small increase in relative *TFAM* genes fold expression were detected 0,17 (± 0,22).

The *MAP2* gene expression level was negatively influenced by PQQ treatment at all three investigated stages of differentiation. The strongest inhibition of the relative *MAP2* gene expression was detected at neural progenitor (NP) stage. Statistically significant difference between NSC *vs*. NP and eNP *vs*. NP was indicated (***, $p < 0,001$) (Fig. 5D).

The expression level of *GFAP* gene was strongly influenced by PQQ treatment. The increase of the relative *GFAP* gene expression was observed at all exposed cell populations but mostly at NSC and eNP stages. The increase in the relative expression of GFAP was observed in NSC: 4,95(± 0.27), eNP: 4,63(± 0.60) and NP: 1,01(± 0.51). Statistically significant differences were detected at both: neural stem cells (NSC)

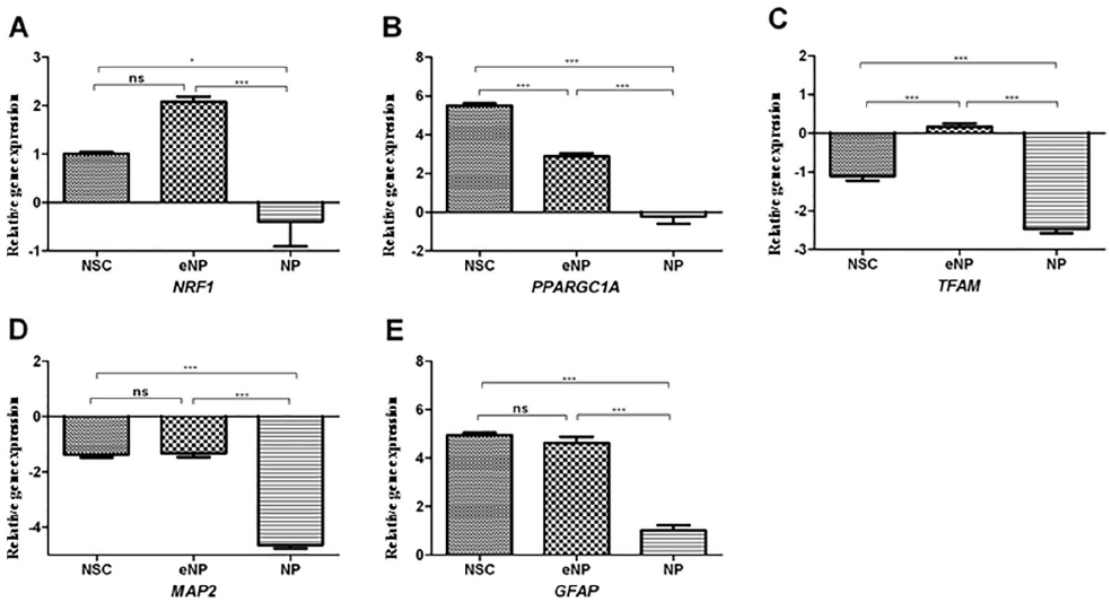

Fig. 5. Real-time (RT-qPCR) evaluation of expression of genes involved in mitochondrial biogenesis and neural differentiation in investigated cell stages: NSC, eNP, NP. A relative expression of (A) *NRF1*; (B) *TFAM*, (C) *PPARGC1A*, (D) *GFAP*, (E) *MAP2* was measured after 5 days treatment of cell cultures with 0,5 μM PQQ. Data were represented as the mean (SEM) from three independent experiments at each in 4 replicates. Brackets shows statistical significance between samples *vs.* control (*t*-Student) (*) $p < 0,05$; (**) $p < 0,01$; (***) $p < 0,001$; (****) $p < 0,0001$; and comparison between groups (two way ANOVA, Bonferroni post-test): (#) $p < 0,05$; (##) $p < 0,01$; (###) $p < 0,001$; (####) $p < 0,0001$.

and early neural progenitors (eNP) revealing significantly higher relative expression of GFAP than neural progenitors (NP) population (***, $p < 0,001$) (Fig. 5E). The increase in relative GFAP expression was accompanied by a relative MAP2 repression in the cells of NSC;1,38(± 0.27); eNP:1,33(± 0.34) and NP:4,64(± 0.31).

4. Discussion

Transition from the pluripotent to differentiated neuronal cells is linked to the mitochondrial biogenesis and the metabolic switch, where the balance between glycolysis and oxidative phosphorylation (OXPHOS) is shifted to the latter (Zheng et al., 2016). While the association of the fate control and energy metabolism was widely investigated in adult neural stem cells (Rafalski and Brunet, 2011) and in the restricted neural progenitors differentiating into post-mitotic neurons (Zheng et al., 2016), the earlier phases of neural commitment still remain a "black box". Thus we aimed to compare the three different stages of neural development from human iPSC, namely: "neural stem cells" (NSC), "early neural progenitors" (eNP) and "neural progenitors" (NP) upon the exposure to PQQ, a well known agent stimulating mitochondrial biogenesis (Chowanadisai et al., 2010). The neural commitment from hiPSC was performed according to Yan et al., 2013, but further differentiation was carried out with our protocol as described in Materials & methods section.

In our experiments we measured the acquisition of specific stages of hiPSC neural differentiation. The main pluripotency factors *POU5F1* (OCT4) and *NANOG* were not detected by quantitative RNAseq analysis in all three tested populations, (Fig. 1A). However data from RT-PCR indicated the presence of *NANOG* in control populations, but at the low level of expression (Suppl. Fig. 1). This is consistent with the finding, that the balance of *OCT4/NANOG* expression is changing in favor of *NANOG* when cells leave the pluripotency stage in order to acquire lineage specification (Radzisheuskaya et al., 2013). Together with the strong expression of *SOX2* pluripotency marker which is also typical for neural commitment, we conclude, that our "NSC" population acquired neural fate. Obtained by us cell populations were further characterized by the expression of early neural (NES, β-TUBIII), advanced neuronal (NF-200, MAP2, NEUROD1), and astrocytic (GFAP) markers on the protein level (immunofluorescence) (Fig. 1B and C) and mRNA level (RNAseq, Fig. 1A and RT-PCR, Suppl. Fig. 1). From the above markers only astrocytic markers *GFAP* and *ALDH1L1* (not analyzed on protein level) were hardly detected in NSC stage, while the rest were present in all three stages of development, however with different intensity. The quantitative evaluation of GFAP on the protein level revealed significant difference not only between NSC and NP populations, but also between eNP and NP. Thus the mRNA (Fig. 1A) and protein (Fig. 1B and C) expression panels revealed, that the phenotype of the tested populations during the differentiation from NSC through eNP, to NP change from early to advanced neuronal markers acquiring morphology, that show branched, neuronal network (inserts in Fig. 1B) with the representative astrocytic population at NP stage.

We compared three different stages of cell development upon PQQ treatment at concentrations in the range of 0–0,5 μM. The most efficient concentration (0,5 μM) in our biochemical assays was subsequently employed for molecular biology studies. Table 1 shows the summary of the influence of PQQ (0,5 μM) on hiPSC at different stages of neural differentiation.

We have found that the sensitivity to PQQ is dependent on the stage of hiPSC neural differentiation. However high antioxidant capacity of PQQ was confirmed in all tested cell populations, regardless of the stage of hiPSC neural differentiation. In our experiments the ROS production was significantly inhibited at all tested cell populations (Fig. 2B) and mitochondrial membrane potential was significantly raised in NSC and eNP (Fig. 2C). The only exception from the above was that mitochondrial membrane potential at NP stage, which was significantly lowered, but that may be explained by the possible different dose response as compared to earlier stages of development.

Furthermore the most effective PQQ effect was at the eNP and NSC

Table 1
Summary of the influence of PQQ (0,5 μM) on NSC, eNP, NP.

		NSC	eNP	NP
Viability		↑ (***)	↑ (***)	↓ (*)
ROS level		↓ (***)	↓ (*)	↓ (***)
Mitochondrial membrane potential		↑ (ns)	↑ (*)	↓ (**)
Total cell number		↑ (*)	↑ (*)	↓ (*)
Protein expression	SDHA	↑ (**)	↑ (*)	↓ (ns)
	COX-1	↑ (*)	↑ (***)	↓ (***)
mtDNA copy number	*ND1/SCLO2B1*	↓ (ns)	↑ (**)	↓ (ns)
	ND5/SERPINA1	↑ (ns)	↑ (*)	↓ (ns)
Relative gene expression	*NRF1*	↑ (1,01)	↑ (2,08)	↓(− 0.39)
	TFAM	↓ (− 1.10)	↑ (0,17	↓ (− 2.47)
	PPARGC1A	↑ (5,51)	↑ (2,88)	↓ (− 0.21)
	MAP2	↓ (− 1.38)	↓ (− 1.33)	↓ (− 4.64)
	GFAP	↑ (4,95)	↑ (4,63)	↑ (1,01)

* $p < 0,05$; ** $p < 0,01$; *** $p < 0,001$, ns- non significant.

stage regarding other cellular processes tested as viability and increase of total cells number. The opposite effect was observed in the NP stage of development (Figs. 2, 3C), where dramatic switch in cellular response to PQQ appeared (Table 1). That suggests different than ROS level related mechanisms underlying the PQQ induced change in developmental fate decisions. The previous reports also showed that significant changes for antioxidant-related genes during differentiation were not detected (Prigione and Adjaye, 2010), therefore the antioxidant potential of cells could be at stable level, and not dependent from stage of neural differentiation.

We have found the specific "developmental window" of sensitivity to PQQ in hiPSC neural differentiation, which is eNP stage of development. As indicated in Table 1, this is the only stage, where PQQ positive effect for the cell is accompanied by elevated mitochondrial biogenesis.

In order to assess mitochondrial biogenesis at the protein level we determined expression of: Flavoprotein (FP) subunit A of succinate dehydrogenase (SDH) and Cytochrome C Oxidase I (COX-1; mt-CO1). Exposure to PQQ has increased SDHA and COX-1 protein expression in NSC and eNP stage of differentiation (Fig. 3A, B).

To further estimate mitochondrial biogenesis we also measured mtDNA copy number as the ratio between 1) *mt-ND1* to *SCLO2B1* gene copy number and 2) *mt-ND5* to *SERPINA1* gene copy number. The significant increase mtDNA copy number in our studies was recorded only at eNP cell population (Fig. 4A, B). Mitochondrial DNA copy number increase upon PQQ addition was reported earlier (Scacco et al., 2003; Alvarez-Fischer et al., 2011) however, to the best of our knowledge our data are the first to show that PQQ enhances mtDNA copy number during neural differentiation of human iPSC and only at stage of early neural progenitors (eNP).

We have also measured the expression of genes important for the enhancement of mitochondrial number, such as *NRF1*, *PPARGC1A* and *TFAM* (Fig. 5A, B, C), which were previously shown to be regulated by PQQ treatment (Chowanadisai et al., 2010). It is well documented, that the key player of mitochondrial to nuclear crosstalk linking mitochondrial biogenesis to respiration is peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC1 alpha) protein (Wu et al., 1999; Scarpulla, 2011). Pathways of mitochondrial biogenesis through PGC-1α involve NRF1 and NRF2 which subsequently regulate the transcription of respiratory chain cytochrome *c* oxidase subunit 4I1 (*COX4*) and responsible for replication of mtDNA mitochondrial transcription factor A (*TFAM*) (Dominy and Puigserver, 2013).

The increase in relative gene expression of *NRF1* and *PPARGC1A* was detected only at the early stages of neural development (NSC) and (eNP) (Fig. 5A, B). *TFAM* relative gene expression appeared to be down-regulated by PQQ at neural stem cells (NSC) and neural progenitors (NP), while at stage of eNP was up-regulated (Fig. 5C). Thus elevated mitochondrial biogenesis (shown as elevated *TFAM* expression which correlates mtDNA copy number increase) is proved only for eNP.

The PQQ induced significant increase of mitochondrial biogenesis, measured by elevated mtDNA content and high expression of mitochondrial proteins SDHA and COX-1, which appeared only in eNP as well as significant up regulation of *TFAM* expression coexist with the "developmental switch" from neuronal to astrocytic differentiation, as shown quantitatively by down-regulation of neuronal (*MAP2*) and up-regulation of glial (*GFAP*) genes expression (Fig. 5D, E).

In conclusion PQQ was found to act in a different way depending on stage of neural differentiation of human iPS cells. In our study mitochondrial biogenesis was detected only at eNP stage of differentiation. Positive effect of PQQ exposition on cell parameters such as viability, total cells number was observed for neural stem cells (NSC) and early neural progenitors (eNP) but not for late neural progenitor (NP).

The presented here change in the cellular fate induced by PQQ treatment at the selective stages of development which are correlated with mitochondrial biogenesis, may be of interest for the wide range of toxicological studies and the future possible therapeutic applications, especially when the patient specific human iPSC are considered.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.tiv.2017.05.017.

Transparency document

The Transparency document associated with this article can be found, in online version.

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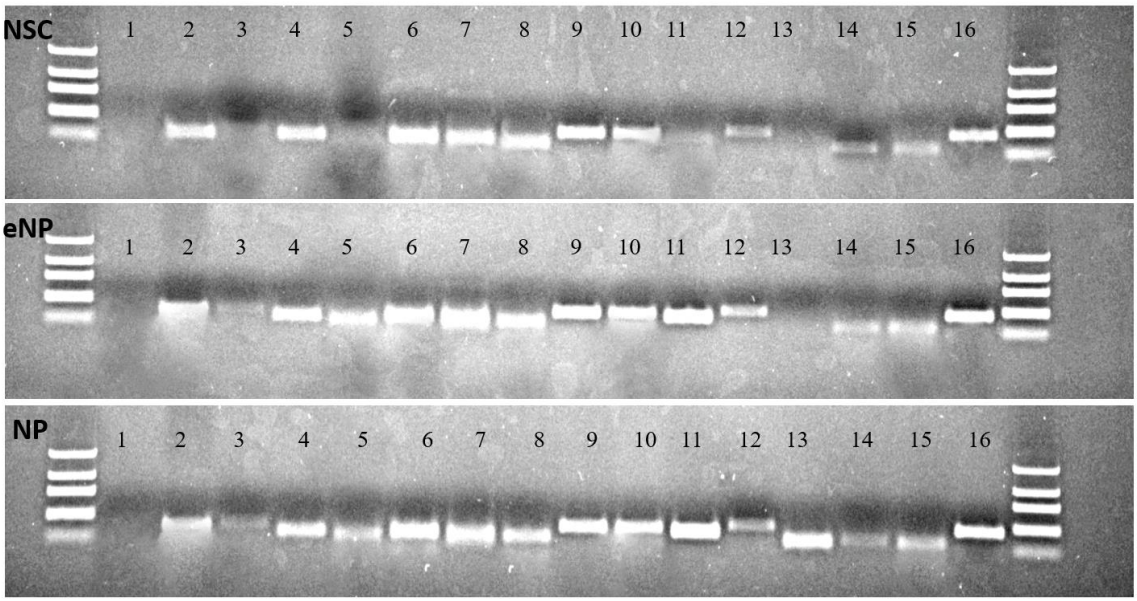
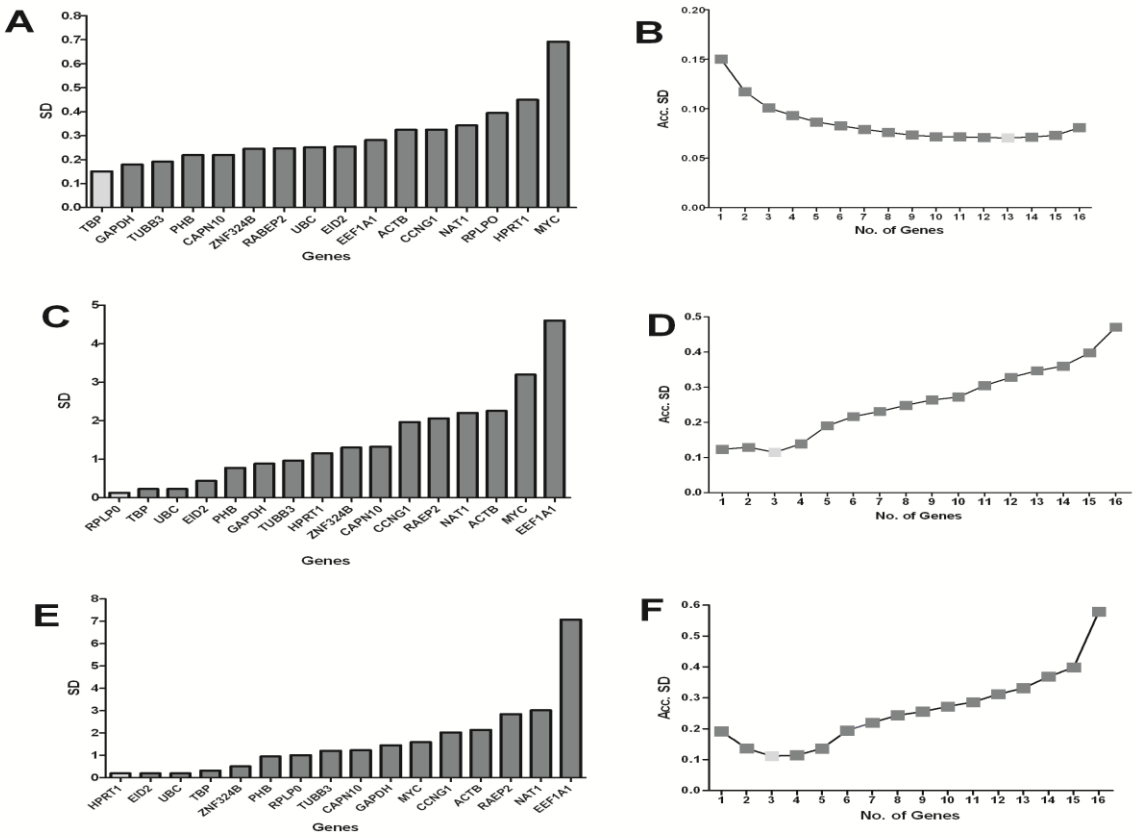


1. *POU5F1*; **2**. *SOX2*, **3**. *NANOG*, **4**. *NES*, **5**. *GFAP*, **6**. *MAP2*, **7**. *NEUROD1*,**8**. *NEFL*, **9**. *TUBB3*, **10**. *NRF1*, **11**. *TFAM*, **12**. *PPARGC1A*, **13**. *SIRT1*, **14**. *PRKAA1*, **15**. *PPARA*, **16**. *ACTB*

Suppl.Fig.1. *Neural differentiation of hiPSC was confirmed, at mRNA level, for the presence of specific pluripotency markers (1-3), neural marker (4), neuronal and glial markers (5-10), as well as important genes for mitochondrial biogenesis (NRF1, TFAM, PPARGC1A, SIRT1, PRKAA1, PPARA). RT-PCR experiments has been performed at: neural stem cells (NSC), early neural progenitors (eNP) and neural progenitor (NP). ACTB expression was used as reference genes.*



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Supp.Fig.2. *Gene expression was measured by RT-qPCR for a panel of selected reference genes. The stability of gene expression was calculated for NSC (A), eNP (C) and NP (E) cells using NormFinder algorithm; the lowest values correspond to the most stable genes. Determination of the optimal number of reference genes for normalization for NSC (B), eNP (D) and NP (F) cells based on the calculation of the Acc. S.D., NormFinder suggests using 13 reference genes for normalization in eNP cells, 3 reference genes in eNP and 3 genes for NP cells.*

Suppl.Tab.1. **Primers used for RT-PCR**

Primers	*Genbank number*	*Primers sequence*	*Amplicon length*
POU5F1 F	NM_002701.5	GAGAGGGGTTGAGTAGTCCCTT	100
POU5F1 R		CGAAATCCGAAGCCAGGTGTC	
SOX2 F	NM_003106.3	CGGAAAACCAAGACGCTCAT	140
SOX2 R		TAACTGTCCATGCGCTGGTT	
NANOG F	NM_024865.3	AATAACCTTGGCTGCCGTCT	150
NANOG R		AGCCTCCCAATCCCAAACAAT	
MAP2 F	NM_002374.3	TGCCTCAGAACAGACTGTCAC	101
MAP2 R		AAGGCTCAGCTGTAGAGGGA	
GFAP F	NM_002055.4	GTGAAGACCGTGGAGATGCG	76
GFAP R		TGCCTCACATCACATCCTTGT	
NES F	NM_006617.1	CCCCGTCGGTCTCTTTTCTC	96
NES R		TCGTCTGACCCACTGAGGAT	
NEUROD1 F	NM_002500.4	ATCTTGCACAGGGAGTCACC	90
NEUROD1 R		TACTGCCGTCCAGTCCCATA	
NEFL F	NM_006158.4	AGCGTGGGAAGCATAACCAG	80
NEFL R		CTGGTCTGTAAACCGCCGTA	
TUBB3 F	NM_006086.3	CAACCAGATCGGGGCCAAGTT	146
TUBB3 R		GAGGCACGTACTTGTGAGAAGA	
SIRT1 F	NM_001314049.1	GTTTCAGAAGACTCAAGTTCACCAG	77
SIRT1 R		GCTTGGTCTAAAAGTGTGACAATCA	
PRKAA1 F	NM_001314049.1	GCCATGCGCAGACTCAGTTC	92
PRKAA1 R		CCCAGAATGTAGTGGCCGAT	
PRARA F	NM_006251	GCTTCGCAAACTTGGACCTG	85
PRARA R		ACAGAAGACAGCATGGCGAA	
NRF1 F	NM_001001928.2	CAGCCGCTCTGAGAACTTCAT	148
NRF1 R		GTCTTCATCAGCACTCAGCATACTA	
TFAM F	NM_005011.4	TGAAAGATTCCAAGAAGCTAAGGGT	132
TFAM R		TAACGAGTTTCGTCCTCTTTAGCAT	
PPARGC1A F	NM_003201.2	TAGTAAGACAGGTGCCTTCAGTTC	174
PPARGC1A R		CTCGATGTCACTCCATACAGACTC	

F- forward; R-reverse

Suppl.Tab.2. **Primers used for RT-qPCR**

Primers	*Genbank number*	*Primers sequence*	*PCR efficiency E(%)*	*Amplicon length (bp)*
NRF1 F	NM_005011.4	CAGCCGCTCTGAGAACTTCAT	106	148
NRF1 R		GTCTTCATCAGCACTCAGCATACTA		
TFAM F	NM_003201.2	TGAAAGATTCCAAGAAGCTAAGGGT	96	132
TFAM R		TAACGAGTTTCGTCCTCTTTAGCAT		
PPARGC1A F	NM_013261.3	TAGTAAGACAGGTGCCTTCAGTTC	88	174
PPARGC1A R		CTCGATGTCACTCCATACAGACTC		
MAP2 F	NM_002374.3	TGCCTCAGAACAGACTGTCAC	110	101
MAP2 R		AAGGCTCAGCTGTAGAGGGA		
GFAP F	NM_002055.4	GTGAAGACCGTGGAGATGCG	78	76
GFAP R		TGCCTCACATCACATCCTTGT		

F- forward; R-reverse

Suppl.Tab.3. Primers used for reference gene validation RT-qPCR

Primers	*Genbank number*	*Primers sequence*	*PCR efficiencyE(%)*	*Ampliconlength (bp)*
ACTB F	NM_001101.3	GCTCACCATGGATGATGATATCGC	1,95	169
ACTB R		CACATAGGAATCCTTCTGACCCAT		
GAPDH F	NM_002046.5	GTTCGACAGTCAGCCGCATC	2,12	90
GAPDH R		TCCGTTGACTCCGACCTTCA		
HPRT1 F	NM_000194.2	AGGCGAACCTCTCGGCTTTC	1,92	166
HPRT1 R		CTGGTTCATCATCACTAATCACGAC		
TUBB3 F	NM_006086.3	CAACCAGATCGGGGCCAAGTT	2,03	146
TUBB3 R		GAGGCACGTACTTGTGAGAAGA		
EID2 F	NM_153232.3	GGCATCGCTCTGTCCAGTTA	2,14	74
EID2 R		GCTTGGACATCTCAGACCGT		
CAPN10 F	NM_023083.3	TCTCACCGGGCTACTACCTG	2,05	86
CAPN10 R		CCCGGTAGAGAAGACTCGGA		
RABEP2 F	NM_024816.2	AGGAAGGGGCAAATGGTGAG	2,08	96
RABEP2 R		CAGCCTTCATGGTTTCCATTTCTG		
ZNF324B F	NM_207395.2	CATTGGAAGGACAAACCTAGGATGATG	1,93	164
ZNF324B R		CTTATCTGCTCCAAAGCTATCACTGTC		
NAT1 F	NM_001160170.3	TGGTTGCCGGCTGAAATAAC	2,09	93
NAT1 R		TCTGTCTAGGCCAGTCTCCT		
TBP F	NM_003194.4	GCAAGGGTTTCTGGTTTGCC	2,14	80
TBP R		CAAGCCCTGAGCGTAAGGTG		
PHB F	NM_001281496.1	TGGAAGCAGGTGAGAATGGAG	2,05	76
PHB R		ATCATGGAGCAGAGGAGGACT		
UBC F	NM_021009.6	ACGGGACTTGGGTGACTCTA	2,15	82
UBC R		ATCGCCGAGAAGGGACTACT		
CCNG1 F	NM_004060.3	GCCTCTCGGATCTGATATCGT	2,04	138
CCNG1 R		CATTCAGCTGGTGTAGCAGT		
MYC F	NM_002467.4	CCCTCCACTCGGAAGGACTA	2,07	96
MYC R		GCTGGTGCATTTTCGGTTGT		
EEF1A1 F	NM_001402.5	TGTTCCTTTGGTCAACACCGA	2,07	122
EEF1A1 R		ACAACCCTATTCTCCACCCA		
RPLP0 F	NM_001002.3	CCTCGTGGAAGTGACATCGT	2,10	76
RPLP0 R		CTGTCTTCCCTGGGCATCAC		

F- forward; R-reverse

Suppl.Tab.4. Primers used for qPCR

Gene symbol	*Genbank number*	*Primers sequence*	*Amplicon length (bp)*
SERPINA1 F	NM_000295.4	CAGTGAATAAATGAGGCGTACATCC	89
SERPINA1 R		GACTGTTTCTCATGCCTCTGGAAAG	
SLCO2B1 F	NM_007256.4	CCTGATGCCTAGGTTTCTTTTCTTG	85
SLCO2B1 R		GGTCATCTGCCTACCCTAGAAC	
mt-ND1 F	NC_012920.1	TACGGGCTACTACAACCCTTC	77
mt-ND1 R		ATGGTAGATGTGGCGGGTTT	
mt-ND5 F	NC_011137.1	CATTACTAACAACATTTCCCCCGC	70
mt-ND5 R		GGCTGTGAGTTTTAGGTAGAGGG	

F- forward; R-reverse

10.3.2. Augustyniak J, Lenart J, Zychowicz M, Stępień PP, Bużańska L., Mitochondrial biogenesis and neural differentiation of human iPSC is modulated by idebenone in a developmental stage-dependent manner.; Biogerontology. 2017 Aug;18(4):665-677.

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RESEARCH ARTICLE

Mitochondrial biogenesis and neural differentiation of human iPSC is modulated by idebenone in a developmental stage-dependent manner

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Abstract Idebenone, the synthetic analog of coenzyme Q10 can improve electron transport in mitochondria. Therefore, it is used in the treatment of Alzheimer's disease and other cognitive impairments. However, the mechanism of its action on neurodevelopment is still to be elucidated. Here we demonstrate that the cellular response of human induced pluripotent stem cells (hiPSC) to idebenone depends on the stage of neural differentiation. When: neural stem cells (NSC), early neural progenitors (eNP) and advanced neural progenitors (NP) have been studied a significant stimulation of mitochondrial biogenesis was observed only at the eNP stage of development. This coexists with the enhancement of cell viability and increase in total cell number. In addition, we report novel idebenone properties in a possible regulation of neural stem cells fate decision: only eNP stage responded with up-regulation of both neuronal (*MAP2*), astrocytic (*GFAP*) markers, while at NSC and NP stages significant down-regulation of *MAP2* expression was observed, promoting astrocyte differentiation. Thus, idebenone targets specific stages of hiPSC differentiation and may influence the neural stem cell fate decision.

Keywords Idebenone · hiPSC · Neural progenitors · Developmental neurotoxicity · Mitochondrial biogenesis · mtDNA copy number · Neuronal · Astrocytic differentiation

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Introduction

Human induced pluripotent stem cells (hiPSC) can generate neural stem cells, as well as neural and glial progenitors (Choi et al. 2014). Differentiation of hiPSC into neural progenitors is associated with the metabolic switch from glycolysis to oxidative phosphorylation (OXPHOS) and is correlated with an increase in the number of mitochondria (Zheng et al. 2016).

Mitochondria play a central role in energy production, apoptosis, and redox homeostasis. Loss of mitochondria functionality is observed in aging and age related neurodegenerative disorders. Manipulation of the activity and number of mitochondria is an interesting therapeutic option for age-related neurodegenerative diseases (Reddy 2009, Luo et al. 2015). In the process of reprogramming (Takahashi et al. 2007) somatic cells are converted into induced pluripotent stem cells. Among many physiological changes, de-differentiation involves the restructuring of mitochondria (mitochondria number, morphology, activity, and mtDNA amount). During differentiation, which is a process opposed to reprogramming, mitochondria plays a leading role as well (Wanet et al. 2015). Thus, a participation of mitochondria in reprogramming and differentiation is not only important in therapy and regenerative medicine but is also crucial for understanding stem cell biology.

Idebenone, an analogue of CoQ10, was synthesized in Japan in the 1980′s (Kanabus et al. 2014). This drug was intended for use for the treatment of neurodegenerative disorders and diseases that exhibit mitochondrial etiology. Idebenone was tested with varying degrees of success in Friedreich's ataxia (Parkinson et al. 2013), Leber hereditary optic neuropathy (LHON) (Klopstock et al. 2011), mitochondrial encephalomyopathy (MELAS) (Napolitano et al. 2000), Duchenne muscular dystrophy (DELOS) (Buyse et al. 2015), multiple sclerosis (Villoslada 2016), dementia (Bergamasco et al. 1994), and Alzheimer's disease (Weyer et al. 1997). Idebenone activates electron transport chain in mitochondria and exerts strong neuroprotective effect both in vitro and in vivo (Murphy et al. 1990; Ratan et al. 1994, Miyamoto and Coyle 1990). One of the most important features of idebenone is its antioxidant capacity (Jaber and Polster 2015). Mechanisms implicated in neuroprotection and antioxidative properties involve stabilization of the BAX/Bcl-2 ratio (Kernt et al. 2013). Reduced by complex II form of idebenone protects mitochondria against lipid peroxidation (Suno and Nagaoka 1989).

Until now, to our knowledge, the impact of idebenone on neural development in the stem cells models, including hiPSC has not been studied. Idebenone was tested in iPSC-derived Friedreich ataxia cardiomyopathy model on the drug screening platform and was shown not to influence tested cells in contrary to deferiprone (the iron chelator), which attenuated disease phenotype (Lee et al. 2016).

In this study, we present results showing the response of neural stem/progenitor cells generated from hiPSC to idebenone exposition at three different stages of neural differentiation: neural stem cells (NSC); early neural progenitors (eNP) and neural progenitors (NP). We have investigated cell viability, ROS level, mitochondrial membrane potential and mitochondrial biogenesis (SDHA and COX-1 protein level, mtDNA copy number), changes in total cell number as well as the expression of *NRF1, TFAM, PPARGC1A, MAP2, GFAP* genes. We have shown that idebenone can positively influence viability and total cell number, significantly increase mitochondrial biogenesis and can change lineage specification during neural differentiation of hiPSC.

Materials and methods

Cell culture and idebenone exposition

Before the exposition to idebenone (Sigma-Aldrich) at concentrations of 0.5; 0.25; 0.125 μM; control NSC, eNP and NP were generated from human induced pluripotent stem cells (hiPSC) (The Gibco® Human Episomal iPSC Line, Thermo Fisher Scientific), as described in Augustyniak et al. 2017. Briefly, for neural differentiation, we used protocol adapted from Yan et al. 2013, with some modifications. Culture media and reagents were purchased from Thermo Fisher Scientific. At the undifferentiated stage, hiPSC were grown on a 6-well plate on rh-Vitronectin in Essential 8 Medium. The medium was replaced every other day. At 80% hiPSC confluency, Essential E8 Medium was changed to the PSC neural induction medium. hiPSC were grown in PSC Neural Induction Medium for the next days. The neural stem cells, was obtained after six passages maintained on Matrigel (BD Matrigel™ Basement Membrane Matrix, Corning) in Neural Expansion Medium (neural induction

supplement 1:50, Neurobasal, Advanced DMEM, 1:1). The second stage of differentiation (eNP) was obtained from NSC by transferring cells to neural differentiation medium type I: Neurobasal, DMEM/F12 [1:1], N2 supplement 1%, B27 supplement 1%, EGF (20 ng/ml), bFGF (20 ng/ml), and culturing them for next 14 days. The third stage of differentiation (NP) was obtained from eNP by culturing in differentiation medium type I without EGF and bFGF (neural differentiation medium type II). Before exposition to idebenone, all three cells populations were seeded at a density of 5×10^5 cells/cm^2 on 6-well, 24-well or 96-well (Nunc) plates covered with the solution of Matrigel:DMEM/F12 (1:30) in medium dedicated to NSC (neural expansion medium), eNP (differentiation medium type I), NP (differentiation medium type II). The next day the media were replaced by the fresh ones supplemented with idebenone at concentrations of control; 0.125; 0.25; 0.5 µM. The cells were incubated with idebenone for 5 days.

Immunocytochemistry

NSC, eNP, and NP were characterized by immunofluorescence staining. Images were prepared in Laboratory of Advanced Microscopy Techniques, Mossakowski Medical Research Centre Polish Academy of Sciences using Confocal Laser Microscope LSM 510 (Zeiss). hiPSC- derived neural stem cells, early neural progenitors and late neural progenitors were seeded on coverslips covered with solution of Matrigel: DMEM/F12, (1:30 ratio) in a 24-well plate (5×10^5 cells/cm^2) in the medium dedicated to the stage of development. At 80% confluency cells were fixed with 4% of PFA (15 min). At the next steps, cells were washed with PBS and 0.1% Triton X-100 was used for cells permeabilization. Before primary antibodies (Supplementary Table 1) were added, blocking solution of 10% goat serum was applied for 1 h and cells were incubated with primary antibodies for 24 h. After this time secondary antibodies (Supplementary Table 1) were added and incubated for 1 h in the dark. Nuclei were contrast gained with Hoechst 33258 (Sigma-Aldrich).

Alamar Blue viability assay

After 5 days of exposure to idebenone at doses of 0–0.5 µM, the Alamar blue viability assay (Sigma-Aldrich) was performed. Fluorescence of resorufin was read at wavelengths: 544 nm (excitation) and 590 nm (emission) 3 h after adding reagent to the culture medium (1:10). The results are shown as the ratio (%) of the fluorescence intensity of test samples to the control (untreated) samples measured by Fluoroscan Ascent (FL, Labsystems) plate reader. Data presented on the graphs are normalized to cell number which was obtained by Janus Green (Abcam) staining performed according to the manufacturer's protocol.

ROS level detection

After 5 days of exposition of NSC, eNP, and NP to idebenone, ROS level was measured by DCFH-DA (dichloro-dihydro-fluorescein diacetate, Sigma-Aldrich) assay. Cells were incubated with DCFH-DA reagent (1 µM) for 3 h. After this time fluorescent DCF(2′,7′-dichlorofluorescin) was detected by a plate reader Fluoroscan Ascent (FL, Labsystems) at wavelengths: 485 nm (excitation)—538 nm (emission). The results are shown as the ratio (%) of the fluorescence intensity of test samples to the untreated control. Normalization of ROS level results to cell number was obtained using Janus Green (Abcam) staining.

Mitochondrial membrane potential determination

After 5 days of exposition to idebenone, NSC, eNP and NP mitochondrial membrane potential was measured using fluorochrome Mitotracker® Red CMXRos (Thermo Fisher Scientific) detected at the wavelength: 544 nm (excitation) 590 nm (emission) on a plate reader. The measurements were performed 4 h after the 50 nM solution of MitoTracker Red CMXRos was added to the culture medium. The results are shown as the ratio (%) of the fluorescence intensity of the test sample to the untreated control, after normalization of data to the total cell number with Janus Green staining according to manufacturer's protocol.

SDHA and COX-1 protein level determination

Cells at the three stages of neural differentiation were seeded separately on a 96-well plate covered with Matrigel solution (1:30). Idebenone was added to cells for 5 days. After this time the levels of two

mitochondrial proteins was measured with MitoBiogenesis In-Cell ELISA Colorimetric kit (Abcam), according to the manufacturer's instructions, on cells fixed with 4% PFA. SDHA, mt-COX-1 proteins level was obtained on a Fluostar plate reader OMEGA (BMG Labtech). After washing with PBS, cells were incubated for 30 min in 1X Permeabilization Buffer. Prior to the addition of primary antibodies (anti-SDHA and anti-COX-1), the cells were incubated in 2X Blocking Buffer for 2 h followed by a mix of secondary antibodies conjugated with enzymes: (1) alkaline phosphatase and (2) horseradish peroxidase for 60 min. After this time substrate for alkaline phosphatase was added and absorbance was measured at OD 405 nm wavelength, then the substrate for horseradish peroxidase was added, and absorbance was measured at OD 600 nm wavelength. Changes in SDHA and COX-1 levels were normalized to total cell number measured according to manufacturer's protocol using Janus Green (Abcam) staining method. SDHA and COX-1proteins levels were shown independently as the absorbance ratio (%) of cell samples treated by idebenone to the untreated control.

Total cell number determination

After 5 days of incubation with idebenone, cells were fixed with 4% PFA (RT). 1X Janus green reagent (Abcam) was added for the 5 min. After this time cells were washed five times with PBS, then 0.5 M HCl (10 min in RT) was added. Absorbance used to determine total cell number was measured on Fluostar plate reader OMEGA (BMG Labtech) at a wavelength: OD 595 nm. Total cell number was calculated from the standard curve and presented as the % of control cells untreated with idebenone.

Gene expression analysis

DNA and RNA isolation

Total RNA and total DNA was extracted from NSC, eNP, NP samples with ZR-*Duet*™ DNA/RNA MiniPrep Kit (Zymo Research) according to the manufacturers' protocols. Before reverse transcription reaction, total RNA was purified with Clean-Up RNA Concentrator kit (A&A Biotechnology, Gdynia, Poland), and RNA integrity on 2% agarose gels was validated. RNA and DNA concentration was measured using NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, USA). The purity of nucleic acids was assessed by calculating the 260/280 absorbance ratio.

RT-qPCR

cDNAs to RT-qPCR reaction were obtained by High-Capacity RNA-to-cDNA™ Kit, (Thermo Fisher Scientific) on CivivCycler Thermocycler (Biotech INC). Gene expression analysis was evaluated with 1 µl (10 ng) cDNA template in 25 µl reaction mixture containing 12.5 µl iTaq™ Universal SYBR® Green supermix (Bio-rad) and 0.25 µM/µl each primer (Supplementary Table 2). RT-qPCR was performed in the following steps: initial denaturation step at 95 °C for 3 min, 45 cycles of denaturation at 95 °C for 10 s, and annealing/extension at 60 °C for 1 min. Samples were tested in four replicates. For each stage of development (NSC, eNP, NP) reference gene was selected in NormFinder software as shown in Fig. 1. As a reference genes for a NSC stage were used: TBP, UBC, GAPDH, EID2, RABEP2, ZNF224B, PHB, CCNG1, CAPN10, EEF1A1, TUBB2; for eNP: RPLP0; for NP: EID2, RPLP0, UBC. Potential reference genes were described by Synnergren et al. 2007; Coulson et al. 2008; She et al. 2009; Eisenberg and Levanon 2013; Vossaert et al. 2013. GeneEx 6.1 software (MultiD Analyses AB, Göteborg, Sweden) was used to analyze the data by Pffafl method (Pfaffl, 2001) (the quantification cycle (Cq) values and the baseline settings automatically calculated by the qPCR instrument software) from LightCycler® 96 Software (Roche Diagnostics GmbH, Mannheim, Germany).

qPCR

The mtDNA copy numbers were calculated as (1) *mt-ND1/SCLO2B1* ratio and (2) *mt-ND5/SERPINA1* ratio (Yu et al., 2012) on the quantification cycle (Cq) values and the baseline settings automatically calculated by the qPCR instrument software (LightCycler® 96 Software, Roche Diagnostics GmbH, Mannheim, Germany). The qPCR was performed with 1 µl (10 ng) DNA template in 25 µl reaction mixture containing 12.5 µl iTaq™ Universal SYBR® Green Supermix (Bio-rad) and 0.25 µM/µl each primer (Supplementary Table 3). PCR conditions were as folows: hot start at 95 °C for 3 min followed by 45

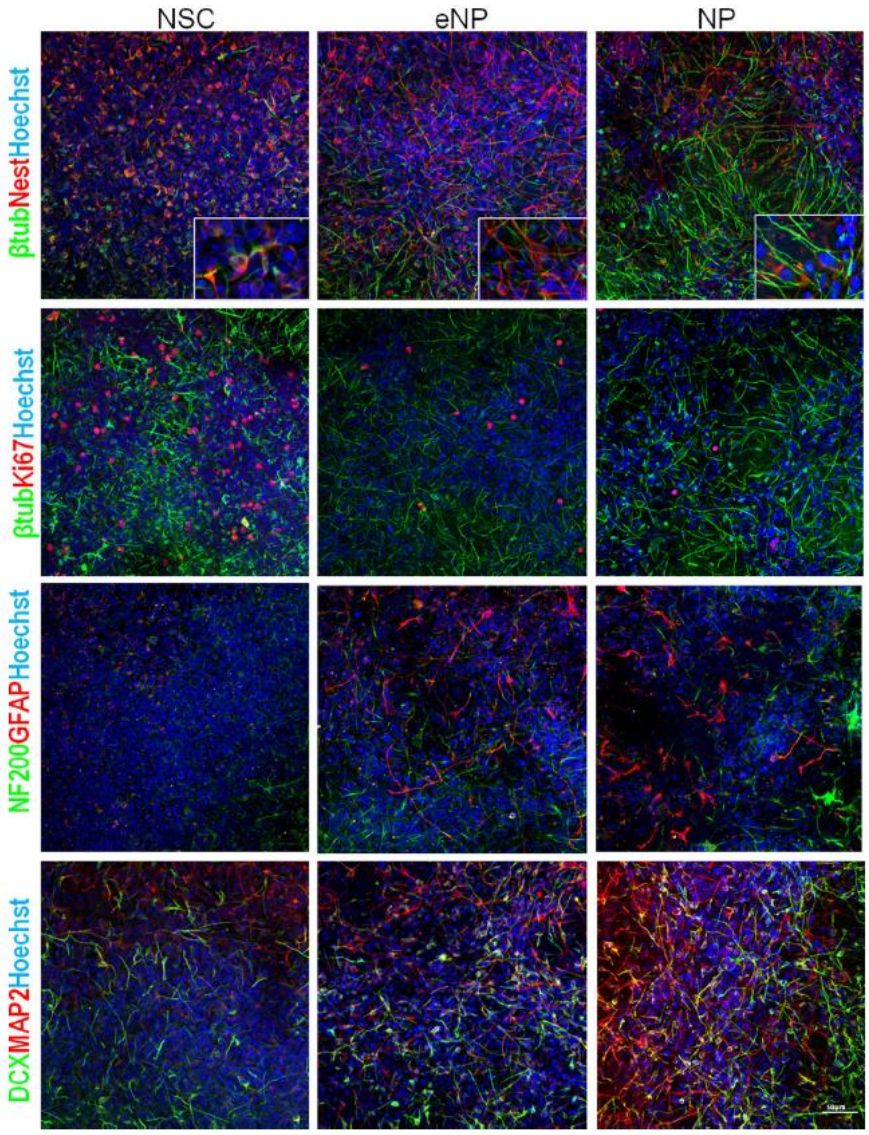

Fig. 1 Immunocytochemical confirmation of: neural stem cells (NSC), early neural progenitors (eNP) and neural progenitors (NP). NSC culture exhibit dense and packed morphology, expressing the high level of early neural marker Nestin, proliferation marker Ki67 and early neuronal marker Doublecortin (DCX). During differentiation process, the morphology of cells changed to more elongated, branched, with decreasing Ki67 expression and increasing more advanced neuronal (β-TUBULIN3, MAP2, NF200) and astrocytic (GFAP) markers. *Scale bar* 50 µm

cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s.

Statistical analysis

GraphPad Prism5.0. was used to perform statistical analysis. Kolmogorov-Smirnov was used as a normality test. After checking whether the distribution is normal test groups were compared with (1) the t-student test, (2) One-way ANOVA followed by Tukey's Multiple Comparison Test (3) two-way ANOVA followed by Bonferroni Multiple Comparison Test; (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$; (****) $p < 0.0001$; for comparison inside the group; 2)

(#) $p < 0.05$; (##) $p < 0.01$; (###) $p < 0.001$; (####) $p < 0.0001$ for comparison between group. Results represent three independent experiments, each in at least four replicates. Results presented at the graphs were shown as mean with standard error of measurement (SEM). Data in the text were present as mean with standard deviation (SD).

Results

Neural differentiation of hiPSC

NSC, eNP and NP were obtained from hiPSC as described before (Augustyniak et al. 2017). The panel of immunocytochemical images (Fig. 1) shows the staining of specific neural markers in cell the populations tested in this report. They included: Nestin (NEST)—the marker of the early stage of neural commitment; β-tubulin III (βTUB III), Doublecortin (DCX), Neurofilament 200 (NF200) and Microtubule Associated Protein 2 (MAP2)—the markers of neuronal differentiation; Glial Fibrillary Acidic Protein (GFAP)—the astrocytic marker and Ki67—indicating proliferating cells. From NSC through eNP to NP stage, lineage-related cells acquire more neuronal phenotype, as revealed by the enhanced expression of βTUB III, NF200, and MAP2, while decreasing level of NEST. DCX level was detected on similar level at all tested stages of development. Astroglial marker—GFAP is gradually increasing during differentiation, while the proliferation marker Ki67 gradually disappears, being the most abundant in NSC stage of development. The advancement in neuronal differentiation, as revealed by the expression of typical markers on the protein level, is shown to be correlated with the change in morphology from rounded to elongated cells with long protrusions (insets in Fig. 1). Detailed phenotype characterization of NSC, eNP and NP on mRNA level by RNA-seq method was presented previously by our group (Augustyniak et al. 2017).

Influence of idebenone on cells viability

We observed an increase in viability at NSC at all tested doses. Only in NP stage, at dose 0.25 µM cell viability was lowered significantly [87.75% (±3.93)], but at 0.5 µM small, no significant increase was observed. The largest increase of viability was detected at eNP stage [138.19% (±10.78)] at the highest dose 0.5 µM of idebenone. The increase in cells viability was also high and significant for NSC for doses 0.25 µM [128% (±6.31) and 0.5 µM (130% (±4.40)].

When the tested populations were compared at the highest dose of idebenone (0.5 µM), the differences between NSC [130% (±4.40)] versus NP [103.81% (±2.45)] and eNP [138.19% (±10.78)] versus NP [103.81% (±2.35)], were highly significant (####; $p < 0.0001$). The results are shown in Fig. 2a.

Antioxidant properties of idebenone

Antioxidant properties of idebenone, revealed by reactive oxygen species (ROS) detection as the decrease in ROS level, have been observed at eNP and NP stages of neural differentiation. The most effective antioxidant capacity was recorded at the highest tested dose 0.25 µM for NSC [79.77%(±14.47)] and 0.5 µM for eNP [85.45% (±4.81)]; NP [88.44% (±7.09)]. A significant difference between compared populations (NSC vs. eNP vs. NP) at the dose 0.5 µM was not notified, as presented in Fig. 2b.

Impact of idebenone on mitochondrial membrane potential

There were no significant changes in the accumulation of fluorescent dye between stages of differentiation at any tested dose of idebenone (Fig. 2c). Mitochondrial membrane potential was constant and for the cells treated with 0.5 µM idebenone revealed the level: NSC 106.66% (±18.54), eNP 98.76% (±13.38) and NP 103.16% (±3.64) of the control (100%) (Fig. 2c).

Effect of idebenone on SDHA and COX-1 expression

The SDHA (Flavoprotein (FP) subunit A of succinate dehydrogenase) protein level shown a significant effect for the highest dose of idebenone 0.5 µM: [123.12% (±20.94)] in the eNP and [117.04% (±10.79)] in the NSC stage. We did not observe any statistically significant difference of SDHA protein level between: NSC versus eNP contrary to eNP versus NP (#, $p < 0.05$) (Fig. 3a).

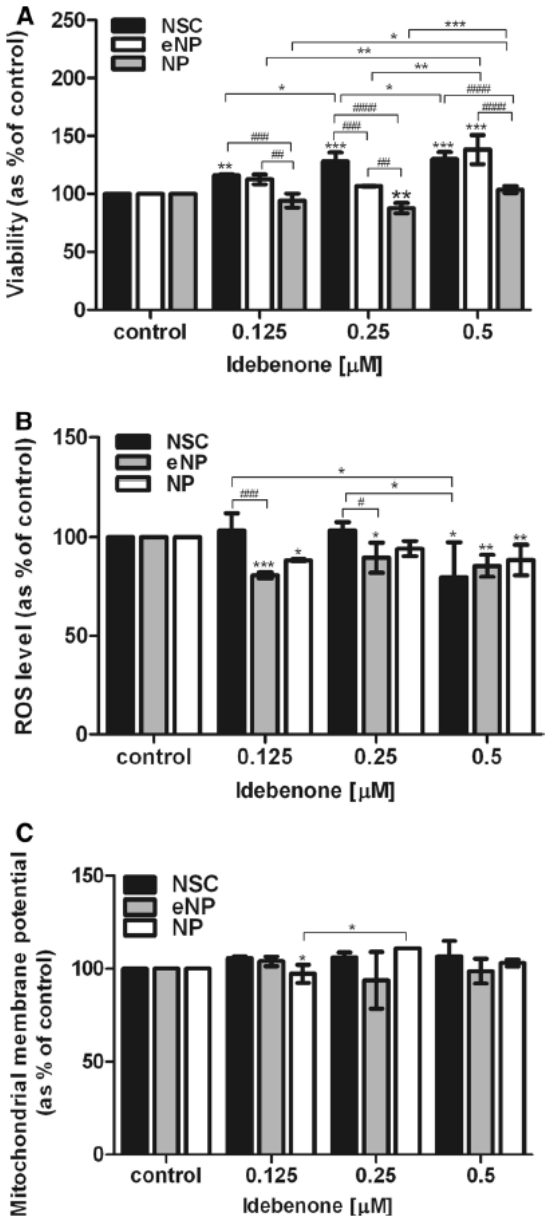

Fig. 2 The cells at three different stages of neural differentiation: NSC, eNP and NP after 5 days of exposure to the different concentrations of idebenone were tested for (a) viability measured by Alamar Blue assay; (b) ROS level measured by DCFH-DA assay; (c) the mitochondrial membrane potential measured by MitoTracker Red CMXRos staining. After normalization to total cell number results are shown the mean (SEM) of fluorescencee intensity (%) of treated group versus control. *Brackets* show statistical significance between samples versus control (one way ANOVA, Tukey's post-test): *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ and comparison between groups (two way ANOVA, Bonferroni post-test): $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$; $^{\#\#\#\#}p < 0.0001$

Idebenone significantly increased COX-1 (cytochrome C oxidase I) protein level in the early neural progenitors at dose 0.25 μM [144.13% (± 16.26)]; (**, $p < 0.01$) and 0.5 μM [137.24% (±11.83)]; (**, $p < 0.01$). At the highest dose significant enhancement of COX-1 protein level was detected in NSC stage of development [113.90% (±3.01)]; (* $p < 0.05$). No significant changes between treated and untreated control were detected at stage of NP at the dose 0.25 μM and 0.5 μM after 5 days of exposition to idebenone. We noticed significant difference in response to idebenone between: NSC versus eNP (#, $p < 0.05$); eNP versus NP (####, $p < 0.0001$), and NSC versus NP (#, $p < 0.05$) in the highest dose (Fig. 3b).

Influence of idebenone on the total cell number

Idebenone increased total cell number of NSC in all tested doses: 0.125 μM [113.64% (±8.17)]; 0.25 μM [119.75% (±18.59)]; 0.5 μM [138.57% (±12.69)], but only for the highest dose it was statistic significant. The statistically significant differences between treated cells and untreated control were shown at highest dose of 0.5 μM for NSC [138.57% (±12.69)] and eNP [118.29% (±0.11)]. At stage of late neural progenitor (NP) idebenone inhibited total cell number, about ~7–12% versus control samples at all tested doses, however this effect was found to be statistically significant only for 0.25 μM dose of idebenone [88.10% (±3.87)] (Fig. 3c).

Effect of idebenone treatment on mitochondrial DNA content

Idebenone significantly increased ratio of *ND1/SCLO2B1* (*,$p < 0.5$) from 42.60 (±2.73) to 55.33 (±4.03) only at early neural progenitors stage (Fig. 4a). *ND1/SCLO2B1* ratio remains unchanged not significant in the two other stages (NSC and NP). Significant difference between tested groups was observed at untreated cells populations: NSC versus NP (##, $p < 0.01$) and eNP versus NP (##, $p < 0.01$). In cells treated by idebenone significant difference in response to stimulations was noted between NSC and NP (##, $p < 0.01$).

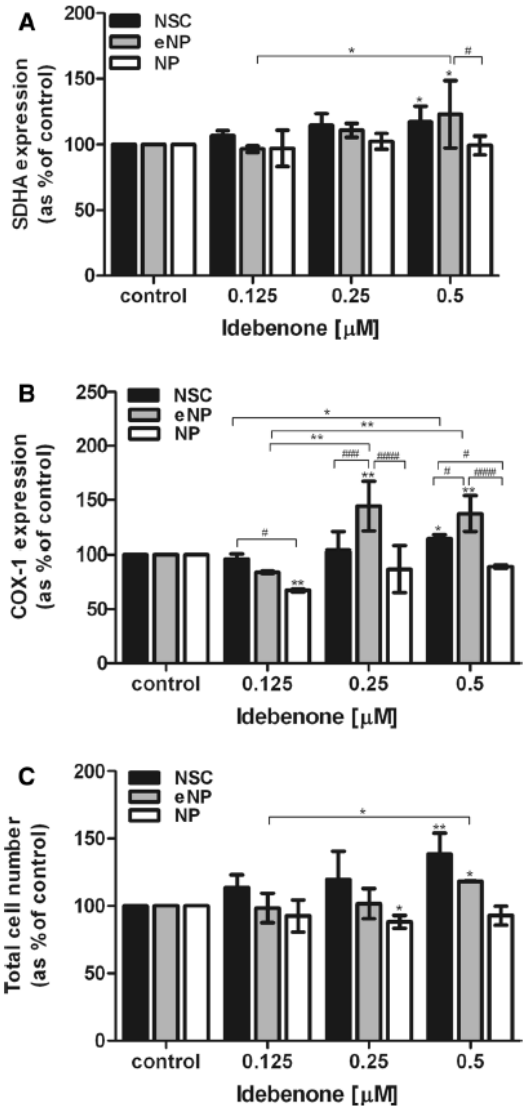

Fig. 3 In the developmental stages: NSC, eNP and NP after 5 days exposure to the different concentrations of idebenone. **a** The Succinate Dehydrogenase Complex Flavoprotein Subunit A (SDHA) level. **b** The Cyclooxygenase (COX-1) level; **c**) Total cell number obtained from standard curve (Janus green staining) was measured. Results are shown as mean (SEM) relative percent (%) of absorbance versus control. The SDHA and COX-1 protein level were normalized to cell number with Janus green staining kit. Brackets show statistical significance between samples versus control (one way ANOVA, Tukey's post-test): *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; and comparison between groups (two way ANOVA, Bonferroni post-test): $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$; $^{\#\#\#\#}p < 0.0001$

ND5/SERPINA1 ratio analysis has shown significant increase at eNP stage (****, $p < 0.0001$) [128.00(±7.00)] as compared to untreated control [99.50(±2.50)]. At NSC and NP stages *ND5/SERPINA1* ratio was not changed at significant level (Fig. 4b). The significant difference between populations treated by idebenone was obtained for NSC [62.67(±22.87)] versus NP [111.50 (±9.50)] (#, $p < 0.05$) and NSC versus eNP [128.00 (±7.00)]. In untreated samples significant difference appeared only between NSC [76.25 (±11.03)] and NP [137.67 (±3.77)] (##, $p < 0.01$), as presented in Fig. 4b.

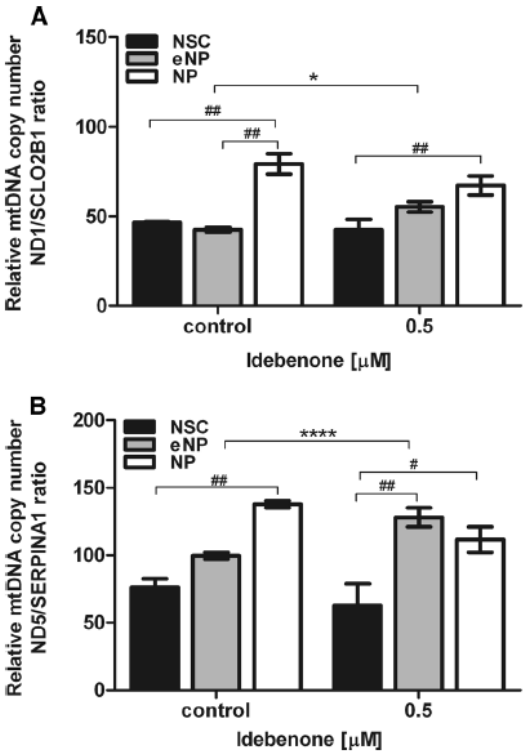

Fig. 4 The relative mtDNA copy number estimated with qPCR measurement of *ND1, ND5, SLCO2B1* and *SERPINA1* level in NSC, eNP and NP after 5 days of exposure to the 0.5 μM idebenone: **a** *ND1/SLCO2B1* ratio and **b** *ND5/SERPINA1* ratio. Data are presented as mean (SEM). *Brackets* show statistical significance between samples versus control, (t-Student): *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ and comparison between groups (two way ANOVA, Bonferroni post-test): $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$; $^{\#\#\#\#}p < 0.0001$

Effect of idebenone on the relative gene expression involved in mitochondrial biogenesis and neural differentiation

RT-qPCR analysis showed that *NRF1* mRNA expression level after exposure to idebenone at the stage of the early neural progenitors (eNP) increased about ~sevenfold [6.86 (±0.416)]. The increase in expression has also been observed in NSC and NP stages but it was much lower and ranged [0.311 (±0.156)] and [1.092 (±0.113)], respectively. We have also observed statistically significant differences in *NRF1* mRNA expression level between every tested developmental stage (***, $p < 0.001$) (Fig. 5a).

Idebenone enhanced *PPARGC1A* gene expression in all tested cell populations. The best efficiency in enhancing of *PPARGC1A* level is observed at the eNP stage, where relative gene expression was elevated about ~11-fold [10.824 (±0.998)]. Idebenone stimulated about ~5 times increase of *PPARGC1A* expression in NSC [5.304 (±0.164)] and NP [4.903 (±1.098)] stage of development. Relative gene expression in NSC versus eNP and eNP versus NP differed significantly (***, $p < 0.001$) while NSC versus NP population was not significantly different (Fig. 5b).

Idebenone raises the expression of *TFAM* gene about ~fivefold [4.865 (±0.233)], in early neural progenitors. At the developmental stages NSC and NP expression of *TFAM* was reduced about three times [−2.578 (±0.252)] and one time [−0.612(±0.131)], respectively (Fig. 5c). Difference in the *TFAM* gene expression was statistic significantly between all compared cells populations (***, $p < 0.001$).

Idebenone enhanced the *MAP2* relative gene expression only at the stage of early neural progenitors ~threefold increase [3.313 (±0.537) of expression]. At the stage of neural stem cells and neural progenitors (NP) the drug has the opposite effect: in NSC expression of *MAP2* decreased about ~onefold [−1.088 (±0.168)] and for NP about ~twofold [−2.391 (±0.347)]. The observed changes were significantly different between all stage of differentiation (***, $p < 0.001$) (Fig. 5d).

Idebenone was found to up-regulate about ~ninefold [9.040 (±0.925)] the *GFAP* gene expression level in early neural progenitors (eNP). Up regulation has been observed also at NSC ~fivefold [4.765 (±0.124)] and NP ~threefold [2.805 (±0.591)] stages of differentiation. Significant differences were recorded in all tested populations: NSC versus eNP versus NP (***, $p < 0.001$) (Fig. 5e).

Discussion

In this paper, we demonstrate the cellular and molecular response of hiPSC-derived cell populations to the synthetic analog of coenzyme Q10, idebenone during early stages of neural development. To the best of our knowledge, the effect of idebenone has not been studies in neural stem cells so far. We assumed that due to the positive effect of idebenone on electron transport in mitochondria (Haefeli et al. 2011; Erb et al. 2012) and metabolic changes characteristic of stem cell development, such treatment may influence the mitochondrial biogenesis and neural differentiation pathway of hiPSC-derived neural stem cells in a stage-dependent manner: neural stem cells, early neural progenitors and neural progenitors.

Neural stem cells are multipotent cells characterized by self-renewal, the ability to proliferate without a limit and the capacity to produce neural progenitors which finally can be differentiated into neurons, astrocytes and oligodendrocytes (Clarke et al. 2000). Neural progenitors can be multipotent, bipotent or unipotent. They differ from NSC by limited capacity to self-renew, the restricted ability of neuronal and glial differentiation and in morphology what is revealed by elongated cell shape and the presence of protrusions extending from the cell body (Seaberg and van der Kooy 2003). During hiPSC differentiation cells gradually turn off naive pluripotency (Radzisheuskaya and Silva 2014) and acquire lineage markers shown as elevated expression of SOX2 and NESTIN (Verpelli et al. 2013) We have confirmed in our previous study (Augustyniak et al. 2017) the loss of pluripotency markers: *OCT4* in all tested populations and gradual silencing of *NANOG* with elevated expression of *SOX2* and *NESTIN* during neural commitment from hiPSC and gradual upregulation of the expression of *MAP2* and *GFAP* during further differentiation, which is also consistent with observations of other groups (Denham and Dottori 2011, Kwon et al. 2012, Verpelli et al. 2013). In the neural progenitors, which are more advanced in development, neurites are extended and the increase of the expression of MAP2 is observed (Denham and Dottori 2011). Nestin is a marker of neural differentiation,

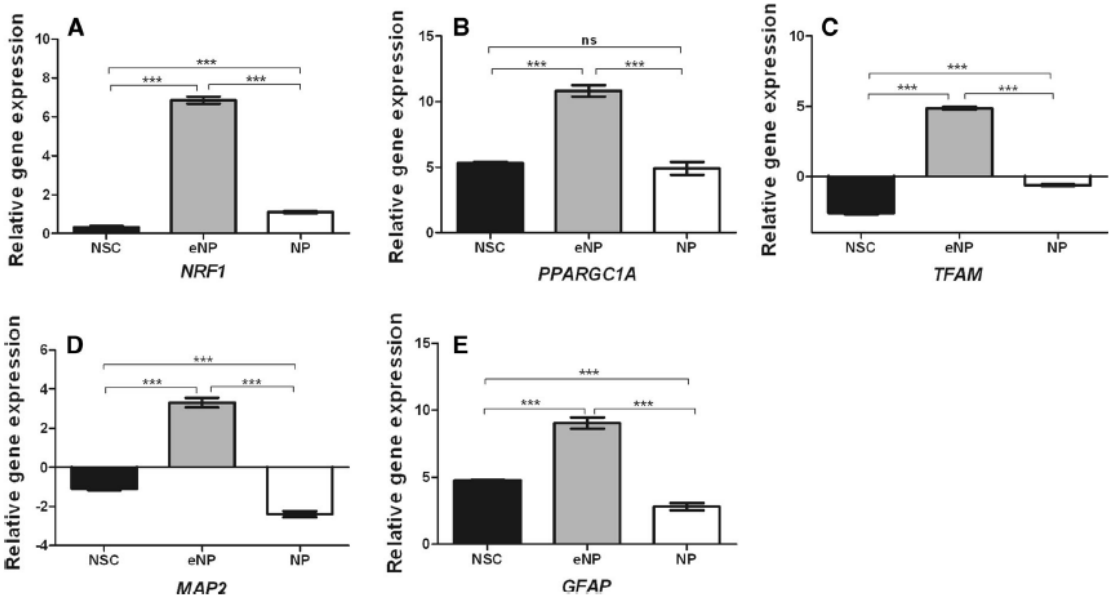

Fig. 5 Real-time (RT-qPCR) evaluation of expression of genes involved in mitochondrial biogenesis and neural differentiation at the NSC, eNP, NP developmental stages. Relative expression of **a** *NRF1*, **b** *PPARGC1A*, **c** *TFAM*, **d** *GFAP*, **e** *MAP2* was measured after 5 days of cell treatment with 0.5 μM idebenone. Data are presented as the mean (SEM) from three independent experiments, each in four replicates. Brackets show statistical significance comparison between groups (one way ANOVA, Tukey's post-test): *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

which can be detected at the earliest. Thus, the expression of early neural marker such as Nestin was shown after the induction of neural differentiation in the NSC at protein level (immunocytochemistry) and mRNA level (RT-PCR), with the tendency to diminish expression at the last NP stage. The expression of key proteins typical for neural development stages NSC, eNP and NP untreated with idebenone was tested at the all populations is presented in Fig. 1. It revealed gradual increase of the expression of βTUB III, DCX, NF200, MAP2 and GFAP along differentiation. The results obtained for tested markers confirmed data, which were previously obtained by our group on both protein (quantification of immunocytochemistry) and mRNA (quantitative analysis with RNA-seq) level (Augustyniak et al. 2017). The latter additionally proved, that presented cell populations significantly differ between each other and can be used as the independent stages of neural differentiation.

To elucidate whether idebenone can influence the neural differentiation process and content of mitochondria, and gene expression of key regulators of mitochondrial biogenesis have been investigated in the three above-mentioned cell populations. Thus, viability, total cell number, ROS level and mitochondrial membrane potential, expression of proteins (SDHA, COX-1) and genes (*NRF1, TFAM, PPARGC1A*), mtDNA copy number (revealed as *ND1/SCLO2B1* and *ND5/SERPINA1* ratio), important marker of mitochondrial biogenesis and the level of neural differentiation related markers (*MAP2* and *GFAP*) were evaluated upon the treatment with idebenone. We have observed that most effective was the highest concentration of idebenone tested by us (0.5 μM) therefore, the further comparison between NSC, eNP and NP cells was performed at the highest tested concentration of idebenone. The summary of the influence of idebenone (0.5 μM) on three different stages of neural differentiation is presented in Table 1.

The population of NP did not respond significantly to the drug treatment for the most of the tested parameters (Table 1). In contrast to NSC and eNP populations, no significant changes were detected in cell viability (Fig. 2a), SDHA (Fig 3a), COX-1 protein level (Fig. 3b) and total cell number (Fig. 3c). The only exception was the significant reduction of the

ROS level which is in agreement with previously reported function of idebenone as an antioxidant (Jaber and Polster 2015) and similar to the response of other tested populations (Fig. 2b).

The expression of proteins and genes implicated in the mitochondrial biogenesis was also tested. In our study we decided to measure the levels of two proteins: a major catalytic subunit of succinate-ubiquinone oxidoreductase (SDHA) a complex of the mitochondrial respiratory chain (Hirawake et al. 1994) (Fig. 3a) and mitochondrial complex IV: cytochrome c oxidase subunits (COX-1) (Anderson et al. 1981). The elevated level of SDHA (nuclear encoded) and COX-1 (mitochondrial encoded) is used as a marker of the one of the mitochondrial biogenesis marker since both of these proteins are localized and function in mitochondria. Thus, SDHA and COX-1 level were significantly elevated in NSC and eNP stages, while NP population was not affected.

The developmental stage dependent elevation of mitochondrial biogenesis after idebenone treatment was also confirmed by the analysis of mitochondrial DNA content. The relative DNA copy number was calculated from representative mitochondrial to nuclear genes ratio (*ND1/SCLO2B1* and *ND5/SERPINA1*). Idebenone induced significant increase of mtDNA copy number only in the stage of eNP, while NSC and NP populations did not respond significantly (Fig. 4a, b).

The genes that have been evaluated in our study: *NRF1*, *PPARGC1A* and TFAM are the key regulators of signaling pathways implicated in mitochondrial biogenesis (Kanabus et al. 2014). PGC1-alpha encoded by *PPARGC1A* is the major factor which by the cascade of nuclear-encoded hormone receptors, transcription factors, and transcriptional co-activators, including PPARs, estrogen-related receptors, thyroid hormone receptors, nuclear respiratory factors NRF1 and NRF2 and the transcription factors CREB and YY1 (Andreux et al. 2013; Dominy and Puigserver 2013; Kanabus et al. 2014) is able to increase the number of mitochondria. Several neurodegenerative diseases (Huntingtońs Disease, Alzheimeŕs Disease, and Parkinsońs Disease) are related to the impaired expression and function of PGC1 alpha. Signaling cascade regulated by PGC1 alpha is an attractive therapeutic target for mitochondrial-based diseases (Valero 2014; Kanabus et al. 2014). TFAM is a mitochondrial transcription factor 1 regulating the mitochondrial DNA replication and repair (Tiranti et al. 1995). Therefore in our research, we examined gene expression of *NRF1, PPAPGC1A, TFAM* and demonstrated by RT-qPCR analysis, that all these factors are significantly elevated only in eNP stage of development (Fig. 5a, b, c; Table 1). Up-regulation of *NRF1* and *PPARGC1A* and down-regulation of *TFAM* were shown in cells of NSC and NP stage of development.

Table 1 Summary of the influence of Idebenone (0.5 μM) at three different stages of neural differentiation (*, $p<0.05$; **, $p<0.01$, ***, $p<0.001$, ****, $p<0.0001$, ns-non-significant)

	NSC	ENP	NP
Viability	↑ (**)	↑ (**)	↑ (ns)
ROS level	↓ (*)	↓ (**)	↓ (**)
Mitochondrial membran potential	↑ (ns)	↓(ns)	↑ (ns)
Total cell number	↑ (**)	↑ (*)	↓ (ns)
Protein expresion			
SDHA	↑ (*)	↑ (*)	↓ (ns)
COX-1	↑ (*)	↑ (**)	↓ (ns)
mtDNA copy number			
ND1/SCLO2B1 ratio	↓ (ns)	↑ (*)	↓ (ns)
ND5/SERPINA1ratio	↑ (ns)	↑ (****)	↓ (ns)
Gene expression (fold change)			
NRF1	↑ (0.31)	↑ (6.86)	↓(1.09)
TFAM	↓ (−2.58)	↑ (4.87)	↓ (−0.62)
PPARGC1A	↑ (5.30)	↑ (10.83)	↓ (4.90)
MAP2	↓ (−1.09)	↑ (3.31)	↓ (−2.39)
GFAP	↑ (4.77)	↑ (9.04)	↑ (2.805)

The last but not least was the coexistence of the abovementioned changes with the neural fate commitment. For that purpose, the gene expression of *MAP2* and *GFAP*, a neuronal and astroglial marker, respectively, was investigated in the idebenone treated populations. While the *GFAP* was significantly upregulated in all tested populations, *MAP2* was shown to be repressed in NSC and NP populations and upregulated only at an eNP stage. What's more, the upregulation of *GFAP* at eNP stage was twice as strong as in other tested stages (Fig. 5d, e; Table 1). Thus surprisingly, we observed that only at the stage of early progenitors idebenone could up-regulate both *MAP2* and *GFAP* gene expression, which suggests that a bidirectional induction of differentiation is possible only at this specific stage of differentiation. Furthermore, the specific for eNP upregulation of all tested genes involved in the mitochondrial biogenesis as well as significant upregulation of viability may suggest that eNP stage is the "developmental window of sensitivity" for the neuroprotective function of the idebenone. More research is needed to elucidate the effect of idebenone on mitochondrial biogenesis and stem cell fate decision during neural differentiation, however, based on the presented results we can strongly support the hypothesis that idebenone protective effect is developmental stage dependent and that future targeted treatment of the selected stage of neural development may exert better therapeutic effect.

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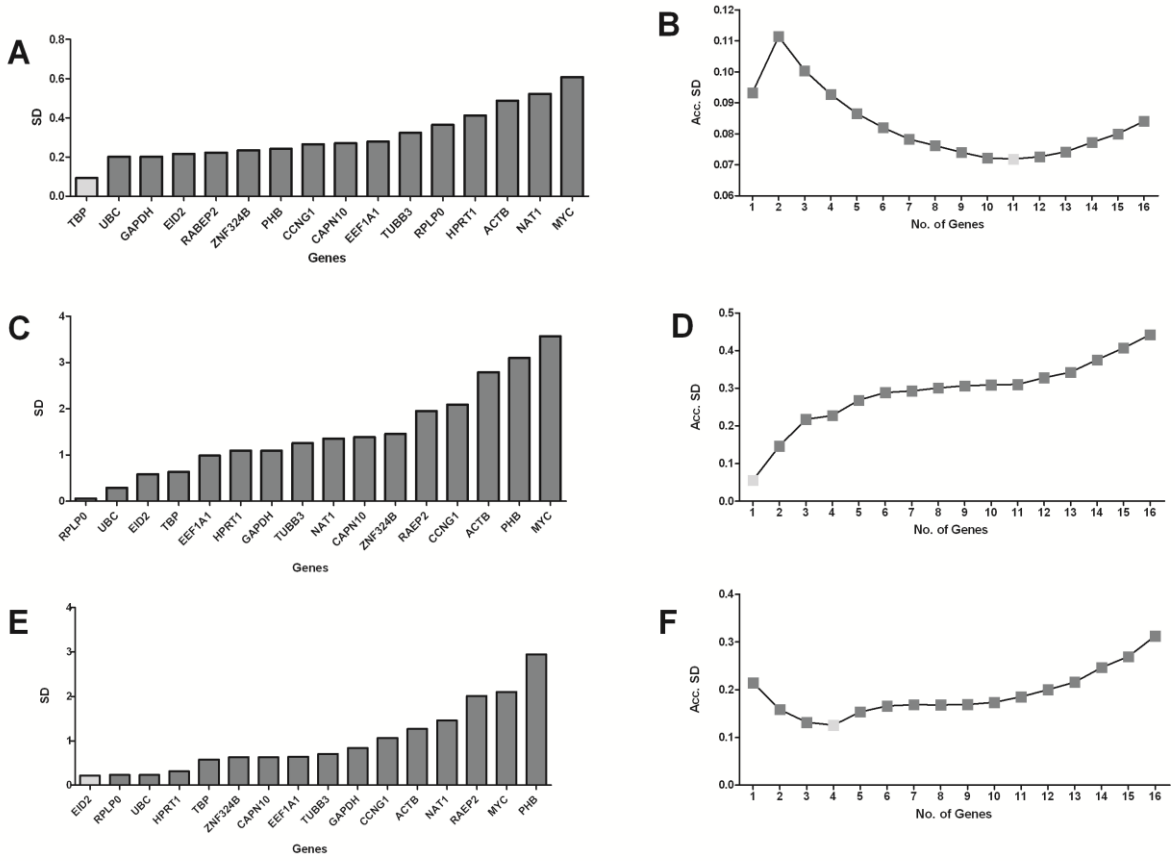

Supp.Fig.1. *Expression stability of 16 candidate reference genes in hiPSC-derived neural stem/progenitor cells after Idebenone treatment, calculated using NormFinder algorithm (a, b, e). The lowest values correspond to the most stable genes. Determination of the optimal number of reference genes for normalization for: NSC cells (b), eNP cells (d) and NP cells (f) are based on the calculation of the accumulated standard deviation Acc. S.D.*

Suppl.Tab.1. Antibodies applied for the immunocytochemistry staining of NSC, eNP, NP

Primary antibodies			*Secondary antibodies*		
Name	*Company that produces antibodies*	*Dilution*	*Name*	*Company that produces antibodies*	*Dilution*
β-TUBULIN III	Sigma-Aldrich	1:1000	Alexa Fluor 488 and 546	Thermo Fisher Scientific	1:1000
DCX	Cell Signaling Technology	1:500			
MAP-2	Sigma-Aldrich	1:500			
NF200	Sigma-Aldrich	1:200			
Ki67	Novocastra	1:500			

Suppl.Tab.2. Primers used for RT-qPCR

Primers	*Genbank number*	*Primers sequence*	*Amplicon length*
POU5F1 F	*NM_002701.5*	*GAGAGGGGTTGAGTAGTCCCTT*	*100*
POU5F1 R		*CGAAATCCGAAGCCAGGTGTC*	
SOX2 F	*NM_003106.3*	*CGGAAAACCAAGACGCTCAT*	*140*
SOX2 R		*TAACTGTCCATGCGCTGGTT*	
NANOG F	*NM_024865.3*	*AATAACCTTGGCTGCCGTCT*	*150*
NANOG R		*AGCCTCCCAATCCCAAACAAT*	
MAP2 F	*NM_002374.3*	*TGCCTCAGAACAGACTGTCAC*	*101*
MAP2 R		*AAGGCTCAGCTGTAGAGGGA*	
GFAP F	*NM_002055.4*	*GTGAAGACCGTGGAGATGCG*	*76*
GFAP R		*TGCCTCACATCACATCCTTGT*	
NES F	*NM_006617.1*	*CCCCGTCGGTCTCTTTTCTC*	*96*
NES R		*TCGTCTGACCCACTGAGGAT*	
NEUROD1 F	*NM_002500.4*	*ATCTTGCACAGGGAGTCACC*	*90*
NEUROD1 R		*TACTGCCGTCCAGTCCCATA*	
NEFL F	*NM_006158.4*	*AGCGTGGGAAGCATAACCAG*	*80*
NEFL R		*CTGGTCTGTAAACCGCCGTA*	
TUBB3 F	*NM_006086.3*	*CAACCAGATCGGGGCCAAGTT*	*146*
TUBB3 R		*GAGGCACGTACTTGTGAGAAGA*	
NRF1 F	*NM_001001928.2*	*CAGCCGCTCTGAGAACTTCAT*	*148*
NRF1 R		*GTCTTCATCAGCACTCAGCATACTA*	
TFAM F	*NM_005011.4*	*TGAAAGATTCCAAGAAGCTAAGGGT*	*132*
TFAM R		*TAACGAGTTTCGTCCTCTTTAGCAT*	
PPARGC1A F	*NM_003201.2*	*TAGTAAGACAGGTGCCTTCAGTTC*	*174*
PPARGC1A R		*CTCGATGTCACTCCATACAGACTC*	
ACTB F	*NM_001101.3*	*GCTCACCATGGATGATGATATCGC*	*169*
ACTB R		*CACATAGGAATCCTTCTGACCCAT*	
GAPDH F	*NM_002046.5*	*GTTCGACAGTCAGCCGCATC*	*90*
GAPDH R		*TCCGTTGACTCCGACCTTCA*	
HPRT1 F	*NM_000194.2*	*AGGCGAACCTCTCGGCTTTC*	*166*
HPRT1 R		*CTGGTTCATCATCACTAATCACGAC*	
TUBB3 F	*NM_006086.3*	*CAACCAGATCGGGGCCAAGTT*	*146*
TUBB3 R		*GAGGCACGTACTTGTGAGAAGA*	
EID2 F	*NM_153232.3*	*GGCATCGCTCTGTCCAGTTA*	*74*
EID2 R		*GCTTGGACATCTCAGACCGT*	
CAPN10 F	*NM_023083.3*	*TCTCACCGGGCTACTACCTG*	*86*
CAPN10 R		*CCCGGTAGAGAAGACTCGGA*	
RABEP2 F	*NM_024816.2*	*AGGAAGGGGCAAATGGTGAG*	*96*

RABEP2 R		*CAGCCTTCATGGTTTCCATTTCTG*	
ZNF324B F	*NM_207395.2*	*CATTGGAAGGACAAACCTAGGATGATG*	*164*
ZNF324B R		*CTTATCTGCTCCAAAGCTATCACTGTC*	
NAT1 F	*NM_001160170.3*	*TGGTTGCCGGCTGAAATAAC*	*93*
NAT1 R		*TCTGTCTAGGCCAGTCTCCT*	
TBP F	*NM_003194.4*	*GCAAGGGTTTCTGGTTTGCC*	*80*
TBP R		*CAAGCCCTGAGCGTAAGGTG*	
PHB F	*NM_001281496.1*	*TGGAAGCAGGTGAGAATGGAG*	*76*
PHB R		*ATCATGGAGCAGAGGAGGACT*	
UBC F	*NM_021009.6*	*ACGGGACTTGGGTGACTCTA*	*82*
UBC R		*ATCGCCGAGAAGGGACTACT*	
CCNG1 F	*NM_004060.3*	*GCCTCTCGGATCTGATATCGT*	*138*
CCNG1 R		*CATTCAGCTGGTGTAGCAGT*	
MYC F	*NM_002467.4*	*CCCTCCACTCGGAAGGACTA*	*96*
MYC R		*GCTGGTGCATTTTCGGTTGT*	
EEF1A1 F	*NM_001402.5*	*TGTTCCTTTGGTCAACACCGA*	*122*
EEF1A1 R		*ACAACCCTATTCTCCACCCA*	
RPLP0 F	*NM_001002.3*	*CCTCGTGGAAGTGACATCGT*	*76*
RPLP0 R		*CTGTCTTCCCTGGGCATCAC*	

F- forward; R-reverse

Suppl.Tab.3. Primers used for qPCR

Gene symbol	*Genbank number*	*Primers sequence*	*Amplicon length (bp)*
SERPINA1 F	NM_000295.4	CAGTGAATAAATGAGGCGTACATCC	89
SERPINA1 R		GACTGTTTCTCATGCCTCTGGAAAG	
SLCO2B1 F	NM_007256.4	CCTGATGCCTAGGTTTCTTTTCTTG	85
SLCO2B1 R		GGTCATCTGCCTACCCTAGAAC	
mt-ND1 F	NC_012920.1	TACGGGCTACTACAACCCTTC	77
mt-ND1 R		ATGGTAGATGTGGCGGGTTT	
mt-ND5 F	NC_011137.1	CATTACTAACAACATTTCCCCCGC	70
mt-ND5 R		GGCTGTGAGTTTTAGGTAGAGGG	

F- forward; R-reverse

11. Pisemne oświadczenia autorów prac tworzących zbiór

11.1. Oświadczenia kandydata określające jego indywidualny w skład w powstanie każdej z prac tworzących zbiór

Warszawa, 29.11.2017

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Oświadczenie

Oświadczam, że w pracy: „Augustyniak J, Zychowicz M, Podobinska M, Barta T, Buzanska L.; Reprogramming of somatic cells: possible methods to derive safe, clinical-grade human induced pluripotent stem cells.; Acta Neurobiol Exp (Wars). 2014;74(4):373-82. Review" mój udział polegał na przygotowaniu opisu metod reprogramowania: mRNA; mikroRNA; transdukcji białkami oraz redakcji manuskryptu.

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Oświadczenie

Oświadczam, że w pracy: „Augustyniak J, Lenart J, Zychowicz M, Lipka G, Gaj P, Kolanowska M, Stepien PP, Buzanska L.; Sensitivity of hiPSC-derived neural stem cells (NSC) to Pyrroloquinoline quinone depends on their developmental stage.; Toxicol In Vitro. 2017 May 31. pii: S0887-2333(17)30133-9. doi: 10.1016/j.tiv.2017.05.017" mój udział polegał na uczestniczeniu w koncepcji projektu, prowadzeniu eksperymentów i przygotowaniu publikacji, szczegółowo: 1) różnicowaniu komórek hiPSC w kierunku neuralnym oraz prowadzeniu hodowli komórek: hiPSC, NSC, eNP, NP; 2) przygotowaniu i przeprowadzeniu testów komórkowych; 3) przygotowaniu próbek do izolacji kwasów nukleinowych; 4) zaprojektowaniu starterów do analiz metodą: RT-PCR, qPCR, qRT-PCR oraz udział w wykonywaniu analiz tymi metodami; 5) zaprojektowanie panelu do wyznaczenia genów referencyjnych w komórkach: NSC, eNP, NP; 6) wyborze analizowanych genów; 7) detekcji w żelu agarozowym produktów reakcji; 8) analizie statystycznej otrzymanych wyników w programie Graphpad prism 5.0; 9) przygotowaniu figur i tabel; 10) udziale w przygotowaniu manuskryptu w tym: przygotowaniu opisu wyników i metod.

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Oświadczenie

Oświadczam, że w pracy: „Augustyniak J, Lenart J, Zychowicz M, Stepien PP, Buzanska L.; Mitochondrial biogenesis and neural differentiation of human iPSC is modulated by idebenone in a developmental stage-dependent manner.; Biogerontology. 2017 Aug;18(4):665-677. doi: 10.1007/s10522-017-9718-4" mój udział polegał na uczestniczeniu w koncepcji projektu, prowadzeniu eksperymentów i przygotowaniu publikacji, szczegółowo: 1) różnicowaniu komórek hiPSC w kierunku neuralnym oraz prowadzeniu hodowli komórek: hiPSC, NSC, eNP i NP; 2) przygotowaniu i przeprowadzeniu testów komórkowych; 3) przygotowaniu próbek do izolacji kwasów nukleinowych; 4) zaprojektowaniu starterów do analiz metodą: RT-PCR, qPCR, qRT-PCR oraz udział w wykonywaniu analiz tymi metodami; 5) zaprojektowanie panelu do wyznaczenia genów referencyjnych w komórkach: NSC, eNP i NP; 6) wyborze analizowanych genów; 7) analizie statystycznej otrzymanych wyników w programie Graphpad prism 5.0; 8) przygotowaniu figur i tabel; opisu metod i wyników, 9) redakcji całego manuskryptu.

Justyna Augustyniak

11.2. Oświadczenia pozostałych współautorów prac tworzących zbiór, w których wyrażają oni zgodę na wykorzystanie wspólnych publikacji w przewodzie doktorskim kandydata oraz określają swój indywidualny wkład w ich powstanie

Warszawa, 29.11.2017

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Oświadczenie

Oświadczam, że w pracy: „Augustyniak J, Zychowicz M, Podobinska M, Barta T, Buzanska L.; Reprogramming of somatic cells: possible methods to derive safe, clinical-grade human induced pluripotent stem cells.; Acta Neurobiol Exp (Wars). 2014;74(4):373-82. Review" mój udział polegał na uczestnictwie w przygotowaniu manuskryptu oraz jego dyskusji merytorycznej. Wyrażam zgodę na wykorzystanie publikacji w przewodzie doktorskim mgr inż. Justyny Augustyniak.

Marzena Zychowicz

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Oświadczenie

Oświadczam, że w pracy: „Augustyniak J, Zychowicz M, Podobinska M, Barta T, Buzanska L.; Reprogramming of somatic cells: possible methods to derive safe, clinical-grade human induced pluripotent stem cells.; Acta Neurobiol Exp (Wars). 2014;74(4):373-82. Review" mój udział w przygotowaniu manuskryptu polegał na opracowaniu rycin, formatowaniu tekstu oraz przypisów.

Wyrażam zgodę na wykorzystanie publikacji w przewodzie doktorskim mgr inż. Justyny Augustyniak.

Martyna Podobińska

Brno, 29.11.2017

PhD Tomáš Bárta
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Statement

I declare, that in the review paper: „Augustyniak J, Zychowicz M, Podobinska M, Barta T, Buzanska L.; Reprogramming of somatic cells: possible methods to derive safe, clinical-grade human induced pluripotent stem cells.; Acta Neurobiol Exp (Wars). 2014;74(4):373-82. Review" my contribution was the part of the design and drafting the work as well as final approval of the version to be published. I agree that Ms Justyna Augustyniak will use this paper in her PhD thesis.

Tomáš Bárta, Ph.D.

Warszawa, 29.11.2017

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Oświadczenie

Oświadczam, że w pracy: „Augustyniak J, Zychowicz M, Podobinska M, Barta T, Buzanska L.; Reprogramming of somatic cells: possible methods to derive safe, clinical-grade human induced pluripotent stem cells.; Acta Neurobiol Exp (Wars). 2014;74(4):373-82. Review" mój udział polegał na przygotowaniu koncepcji merytorycznej, nadzorowaniu przygotowania manuskryptu oraz redakcji końcowej manuskryptu. Wyrażam zgodę na wykorzystanie publikacji w przewodzie doktorskim mgr inż. Justyny Augustyniak

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Oświadczenie

Oświadczam, że w pracy: „Augustyniak J, Lenart J, Zychowicz M, Lipka G, Gaj P, Kolanowska M, Stepien PP, Buzanska L.; Sensitivity of hiPSC-derived neural stem cells (NSC) to Pyrroloquinoline quinone depends on their developmental stage.; Toxicol In Vitro. 2017 May 31. pii: S0887-2333(17)30133-9. doi: 10.1016/j.tiv.2017.05.017" mój udział polegał na sprawowaniu opieki merytorycznej nad prawidłowością wykonania eksperymentów z zakresu biologii molekularnej. Wyrażam zgodę na wykorzystanie publikacji w przewodzie doktorskim mgr inż. Justyny Augustyniak

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Oświadczenie

Oświadczam, że w pracy: „Augustyniak J, Lenart J, Zychowicz M, Lipka G, Gaj P, Kolanowska M, Stepien PP, Buzanska L.; Sensitivity of hiPSC-derived neural stem cells (NSC) to Pyrroloquinoline quinone depends on their developmental stage.; Toxicol In Vitro. 2017 May 31. pii: S0887-2333(17)30133-9. doi: 10.1016/j.tiv.2017.05.017" mój udział polegał na przygotowaniu preparatów immunocytochemicznych: neuralnych komórek macierzystych (NSC); wczesnych progenitorów neuralnych (eNP); progenitorów neuralnych (NP) a także analizy jakościowej oraz ilościowej preparatów immunocytochemicznych pod kątem głównych markerów neuralnych. Wyrażam zgodę na wykorzystanie publikacji w przewodzie doktorskim mgr inż. Justyny Augustyniak.

Marzena Zychowicz

Warszawa, 29.11.2017

Prof. dr hab. Piotr P. Stępień
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Oświadczenie

Oświadczam, że w pracy: „Augustyniak J, Lenart J, Zychowicz M, Lipka G, Gaj P, Kolanowska M, Stepien PP, Buzanska L.; Sensitivity of hiPSC-derived neural stem cells (NSC) to Pyrroloquinoline quinone depends on their developmental stage.; Toxicol In Vitro. 2017 May 31. pii: S0887-2333(17)30133-9. doi: 10.1016/j.tiv.2017.05.017" mój udział polegał na uczestnictwie w planowaniu eksperymentów oraz redagowaniu i końcowej akceptacji manuskryptu. Wyrażam zgodę na wykorzystanie publikacji w przewodzie doktorskim mgr inż. Justyny Augustyniak

prof. dr hab. Piotr Stępień

Warszawa, 29.11.2017

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Wyrażam zgodę na wykorzystanie publikacji w przewodzie doktorskim mgr inż. Justyny Augustyniak

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Lp.	Imię i nazwisko	Wyszczególnienie
1.	mgr Gabryela Lipka Pracownia Bioinżynierii Komórek Macierzystych Instytut Medycyny Doświadczalnej i Klinicznej Im. M. Mossakowskiego, PAN ul. A. Pawińskiego 5 02-106 Warszawa	- przygotowanie pożywek hodowlanych; pomoc w izolacji RNA oraz DNA;
2.	dr n. med. Monika Kolanowska Laboratorium Genetyki Nowotworów Człowieka Centrum Nowych Technologii UW ul. S. Banacha 2c 02-097 Warszawa	- przygotowanie reakcji sekwencjonowania transkryptomu (RNA-seq);
3.	dr n. med. Paweł Gaj Laboratorium Genetyki Nowotworów Człowieka Centrum Nowych Technologii UW ul. S. Banacha 2c 02-097 Warszawa	- opracowanie wyników z sekwencjonowania transkryptomu (RNA-seq);

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Oświadczenie

Oświadczam, że w pracy: „Augustyniak J, Lenart J, Zychowicz M, Stepien PP, Buzanska L.; Mitochondrial biogenesis and neural differentiation of human iPSC is modulated by idebenone in a developmental stage-dependent manner.; Biogerontology. 2017 Aug;18(4):665-677. doi: 10.1007/s10522-017-9718-4" mój udział polegał na sprawowaniu opieki merytorycznej nad prawidłowością wykonania eksperymentów z zakresu biologii molekularnej. Wyrażam zgodę na wykorzystanie publikacji w przewodzie doktorskim mgr inż. Justyny Augustyniak

Warszawa, 29.11.2017

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Oświadczenie

Oświadczam, że w pracy: „Augustyniak J, Lenart J, Zychowicz M, Stepien PP, Buzanska L.; Mitochondrial biogenesis and neural differentiation of human iPSC is modulated by idebenone in a developmental stage-dependent manner.; Biogerontology. 2017 Aug;18(4):665-677. doi: 10.1007/s10522-017-9718-4" mój udział polegał na przygotowaniu preparatów immunocytochemicznych: neuralnych komórek macierzystych (NSC); wczesnych progenitorów neuralnych (eNP); progenitorów neuralnych (NP) oraz panelu zdjęciowego do manuskryptu przedstawiającego zmiany markerów neuralnych w komórkach: NSC; eNP; NP podczas różnicowania. Wyrażam zgodę na wykorzystanie publikacji w przewodzie doktorskim mgr inż. Justyny Augustyniak.

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Oświadczenie

Oświadczam, że w pracy: „Augustyniak J, Lenart J, Zychowicz M, Stepien PP, Buzanska L.; Mitochondrial biogenesis and neural differentiation of human iPSC is modulated by idebenone in a developmental stage-dependent manner.; Biogerontology. 2017 Aug;18(4):665-677. doi: 10.1007/s10522-017-9718-4" mój udział polegał na uczestnictwie w planowaniu eksperymentów oraz redagowaniu i końcowej akceptacji manuskryptu. Wyrażam zgodę na wykorzystanie publikacji w przewodzie doktorskim mgr inż. Justyny Augustyniak

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Oświadczenie

Oświadczam, że w pracy: „Augustyniak J, Lenart J, Zychowicz M, Stepien PP, Buzanska L.; Mitochondrial biogenesis and neural differentiation of human iPSC is modulated by idebenone in a developmental stage-dependent manner.; Biogerontology. 2017 Aug;18(4):665-677. doi: 10.1007/s10522-017-9718-4" mój udział polegał na uczestniczeniu w koncepcji i sprawowaniu nadzoru merytorycznego nad wykonaniem projektu, interpretacją wyników oraz pisaniem i redakcją końcową manuskryptu. Wyrażam zgodę na wykorzystanie publikacji w przewodzie doktorskim mgr inż. Justyny Augustyniak

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