ACTA NEUROBIOLOGIAE EXPERIMENTALIS

Research paper Acta Neurobiol Exp 2009, 69: 11–23

Defined serum-free culturing conditions for neural tissue engineering of human cord blood stem cells

Hamad Ali[1,2], Marcin Jurga[1], Kristina Kurgonaite[2], Nico Forraz[1], and Colin McGuckin[1]*

[1]Cell Therapy Research Institute CTI-LYON, Parc Technologique de Lyon-Saint Priest; Saint Priest; France, *Email: c.mcguckin@conoworld.com; [2]Newcastle Centre for Cord Blood, North East England Stem Cell Institute and Institute of Human Genetics, Centre for Life, Newcastle upon Tyne, UK

Taking tissue engineering applications into clinical trials requires the development of efficient and safe protocols incorporated with effective 3-dimentional cell culturing and differentiation systems in order to develop transplantable tissues that may offer a life-line for patients in the future. Cord blood, which is perhaps the most abundant world stem cell source, has shown previously practical and ethical advantages over other stem cells sources in many research and clinical applications including regenerative medicine. We previously developed a three-step protocol for isolation, expansion and sequential neuronal differentiation of cord blood pluripotent stem cells (characterized with our unique triple immunocytochemisty scheme for Oct-4, Sox-2 and Nanog) in defined serum-free culturing conditions. In this study we incorporated this protocol with 3-dimentional culturing systems which produced artificial neuronal tissues expressing Nestin, NF-200, TUJ1, PSD-95 and NeuN. We showed that cord blood pluripotent stem cells are a potential and promising candidate for future neural tissue engineering and regenerative medicine.

Key words: umbilical cord blood, adult stem cells, neural differentiation, tissue engineering

INTRODUCTION

Human umbilical cord blood is considered as a rich source of pluripotent stem cells with practical and ethical advantages over other stem cells sources. Its clinical practices started in 1972 by the pioneer doctors in the United States, Ende and Ende, to treat a lymphoblastic leukemia (Ende and Ende 1972). Further developed toward a regular transplant in the hematology setting for bone marrow replacement, following either hematological malignancy or bone marrow failure after any chemotherapy side effect, umbilical cord blood was, for many years, considered to be restricted to blood disease therapy (Gluckman et al. 1989, Slatter et al. 2006). However, advances in the production of about 20 organ-based tissue groups has highlighted the further potential of umbilical cord blood the most abundant world stem cell source, not only in treatment of hematological disorders but stretched its impact to cover vast number of pathological disorders and medical applications including regenerative medicine and tissue engineering (Watt and Contreras 2005, McGuckin et al. 2006, Denner et al. 2007, McGuckin and Forraz 2008).

Umbilical cord blood stem cells have been shown previously to have a high potential for neuronal differentiation (Buzanska et al. 2002, McGuckin et al. 2004, Jurga et al. 2006). This differentiation capability made umbilical cord blood stem cells a potential candidate for neuronal tissue engineering applications.

Moving into regenerative medicine clinical application requires the development of efficient 3-dimentional tissue engineering systems including bio-compatible scaffolds. These systems must be accompanied with highly efficient and effective isolation, expansion and differentiation protocols in order to generate clinically functional tissues that can be transplanted into patients (McGuckin and Forraz 2008).

However, before considering taking the 3-dimentioal tissue engineering protocols into clinical trials, many important issues need to be considered to

Correspondence should be addressed to C. McGuckin, Email: c.mcguckin@conoworld.com

Received 16 October 2008, accepted 28 November 2008

achieve the targeted outcomes. One of the most important issues that need to be taken into consideration is the protocol safety issue; therefore it is necessary to design culturing conditions free of animal products in order to reduce contamination and possible infections. It is also important to use defined media (serum-free) in order to avoid any effect serum proteins might have on differentiation pathways (Xu et al. 2005, Corselli et al. 2008). It is important as well to keep the *in-vitro* culturing time short to reduce the risk of infections and chromosomal aberrations common in other stem cell populations including embryonic stem cells (Corselli et al. 2008, McGuckin et al. 2008).

Moving toward effective clinical applications requires a readily and abundant supply of stem cells, first to provide the needed amounts of stem cells and second to provide clinicians with a more accessible and compatible supply of stem cells. Umbilical cord blood can be stored and cryopreserved in cord blood banks for later clinical uses which provide clinicians with the opportunity to choose the most compatible HLA type for the patient which reduce the chance of tissue rejection. (Watt and Contreras 2005, McGuckin et al. 2006, Lee et al. 2007).

Many research groups have successfully differentiated umbilical cord blood stem cells into neural cells (Chen et al. 2005, Domanska-Janik et al. 2006, Lee et al. 2007), however these groups used un-defined media containing animal serum and their *in-vitro* culturing periods were relatively long, thus delay any potential clinical applications.

We have developed a three-step protocol for isolation of cord blood stem cells from umbilical cord blood and the onward differentiation toward neural cells (McGuckin

Table I

Media components and their final concentrations in harvesting and expanding media

Serum free-Harvesting	Serum-Harvesting	Serum-free Expanding	Serum-Expanding
DMEM/F12 GIBCO, cat. no. 31331-093	DMEM/F12	DMEM/F12	DMEM/F12
B27 (1:100) GIBCO, cat. no. 17504-044	10% FBSGIBCO cat. no. 16010-167	B27 (1:100)	10% FBS
N2 (1:50) GIBCO, cat. no. 17502-048	ITS (1:100)Sigma, cat. no. I2771-5ML	N2 (1:50)	ITS (1:100)
SCF (20 ng/ml) ImmunoTools, cat. no. 11343325	SCF (20 ng/ml)	SCF (5 ng/ml)	SCF (5 ng/ml)
TPO (10 ng/ml) ImmunoTools, cat. no. 11343615	TPO (10 ng/ml)	EGF (20 ng/ml) ImmunoTools, cat. no. 11343407	EGF (20 ng/ml)
Flt3 (50 ng/ml) ImmunoTools, cat. no. 11343305	Flt3 (50 ng/ml)	bFGF (20 ng/ml) ImmunoTools, cat. no. 11343627	bFGF (20 ng/ml)
Fibronectin (5 μg/ml) Sigma, cat. no. 86088-83-7	Fibronectin (5 μl/ml)	Heparin (5 μg/ml) Sigma, cat. no. H4784-1G	Heparin (5 μg/ml)
Pen/Strep (1:100) GIBCO, cat. no. 15070-063	Pen/Strep (1:100)	Fibronectin (5 μg/ml)	Fibronectin (5 μg/ml)
Fungizone (1:100) GIBCO, cat. no. 15290026	Fungizone (1:100)	Pen/Strep (1:100)	Pen/Strep (1:100)
		Fungizone (1:100)	Fungizone (1:100)

Serum containing media were used for cultures served as control

et al. 2008). Our system emphasizes our philosophy of increasing adherence toward clinical grade protocols for potential clinical applications; therefore, we use defined, serum-free media and a short time of cell expansion *in vitro* to reduce risk of infections and chromosomal aberrations common in other stem cell populations including embryonic stem cells. Here we incorporate our previously described three-step protocol (McGuckin et al. 2008) with 3-dimentional culturing system to develop artificial neural tissues from umbilical cord blood stem cells as a potential candidate for future tissue engineering and regenerative medicine applications.

METHODS

Isolation and culture of umbilical cord blood stem cells

Umbilical cord blood units were collected in the caesarean sections delivery suit of the maternity unit in the Royal Victoria Infirmary at University of Newcastle. The parents' informed consents were obtained before the delivery. All hospital ethical requirements have been fulfilled. Mononuclear cells fraction was separated from umbilical cord blood using Ficoll-Paque™ PREMIUM (GE healthcare, cat. no. 17-5442-02) on the same day of cord blood collection. No lysing buffers were used due to their negative effect on cells survival and viability.

Cells were cultured at a density of 5×10^6 cells/ml and were divided into two groups. First group were cultured and further expanded and differentiated in serum-free medium, second group in serum containing medium to serve as a control. Cells were cultured in harvesting medium (Table I) for 24 hours in 24 wells plate (Grainer Bio-One, cat. no. 662160) at 37°C at 5% CO_2. Medium was changed to expansion medium (Table I) for 9 days and incubated at 37°C at 5% CO_2. Half the medium was changed every 3 days.

Table II

Neuronal differentiation and maturation media components and final concentrations

Serum-free neuronal differentiation	Serum neuronal differentiation	Serum-free maturation	Serum maturation
DMEM/F12	DMEM/F12	DMEM/F12	DMEM/F12
B27 (1:50)	5% FBS	B27 (1:50)	5% FBS
N2 (1:25)	ITS (1:100)	N2 (1:25)	ITS (1:100)
EGF (10 ng/ml)	EGF (10 ng/ml)	EGF (10 ng/ml)	EGF (10 ng/ml)
BDNF (10 ng/ml) ImmunoTools, cat. no. 11343375	BDNF (10 ng/ml)	NGF (10 ng/ml)	NGF (10 ng/ml)
RA (50 μM) Sigma, cat. no. R2625	RA (50 μm)	BDNF (10 ng/ml)	BDNF (10 ng/ml)
Collagen IV (1 μg/ml) Sigma, cat. no. C5533	Collagen IV (1 μg/ml)	RA (50 μM)	RA (50 μM)
Fibronectin (1 μg/ml)	Fibronectin (1μg/ml)	dBcAMP (100 μM) Sigma, cat. no. D0627	dBcAMP (100 μM)
Pen/Strep (1:100)	Pen/Strep (1:100)	Collagen IV (1 μg/ml)	Collagen IV (1 μg/ml)
Fungizone (1:100)	Fungizone (1:100)	Fibronectin (1 μg/ml)	Fibronectin (1 μg/ml)
		Pen/Strep (1:100)	Pen/Strep (1:100)
		Fungizone (1:100)	Fungizone (1:100)

Serum media used for cultures served as control

Table III

Primary antibodies used for characterization of undifferentiated and differentiated cells

Primary antibody	Host	Isotype	Suggested working dilution	Manufacturers	Catalogue number
Oct-4	Mouse	IgG2b MC	1:300	SantaCruze Biotechnology	Sc8630
Sox-2	Mouse	IgG2a	1:50	R&D systems, USA	MAB2018
Nanog	Goat	IgG	1:20	R&D systems, USA	AF1997
Nestin	Mouse	IgG1	1:100	R&D systems, UK	MAB1259
TUJ1	Rabbit	IgG	1:2000	Covance, USA	PRB435P
GFAP	Rabbit	IgG	1:1000	Abcam, UK	Ab7779
NeuN	Mouse	IgG1	1:100	Chemicon, USA	MAB377
NF-200	Mouse	IgG1	1:800	Sigma, USA	N1042
PSD95	Rabbit	IgG	1:500	Abcam, UK	Ab18258

Neuronal differentiation and maturation

A sterile cover glass insert was put in each well of 24-wells plate, one cover glass per well. The cover glass was coated with collagen IV and incubated for 3 hours at 37°C in 5% CO_2 to dry the collagen. Half a million of floating cells including aggregates were placed into each well on the coated cover glass insert at day 5, cultures were kept in expansion medium for 5 more day (Table I). Differentiation medium (500 µl) was added to each well at day 10 (Table II). Cells were kept in differentiation media for one week. Half the media was changed to maturation media and half media was changed every three days (Table II). Cells were kept in maturation media for one week.

Three-dimensional neuronal differentiation

Opla scaffolds (BD, cat. no. 354614) were washed in 70% ethanol and then PBS. Washed scaffolds were placed in a well of a 24 wells plate. Expansion medium (20 µl) (Table II) was added to wet the scaffold. Concentrated cells (at day 5) (100 000–500 000 cells) were placed on the scaffold gently and incubated at 37°C in 5% CO_2 for 2–4 hours to allow cells attachment to the scaffold's matrix. Serum-free expansion medium was added to the wells containing the scaffolds. Cells were kept in expansion medium total of 10 days. Differentiation medium (Table II) was added and half medium was changed every three days for 1 week. The medium then was changed to maturation medium for another week (Table II).

Reverse transcription PCR analysis

Total RNA was isolated from cells at day-0, day-10 and day-17 of described protocol using Qiagen RNeasy® Plus mini kit following manufacturer protocol. cDNA was produced using SuperScript ® VILO kit (Invitrogen, UK) in 20 µl total volume using 1 µg of total RNA following manufacturer protocol. Samples were incubated at 25ºC for 10 minutes and then 42ºC for 60 minutes and reactions were terminated by heating the mix at 85°C for 10 minutes. PCR reactions were run using Taq PCR master mix following manufacturer protocol (Qiagen, UK). Primers used were: Oct-4a (5'-caagccctcatttcaccag-3') and (5'-cttggaagctagccaggtc-3'), Sox-2 (5'- agtctccaagcgacgaaaaa-3') and (5'-tttcacgtttgcaactgtcc-3'), Nanog (5'-ttccttcctc-

Table IV

Secondary antibodies used for characterization of undifferentiated and differentiated cells

Secondary antibody	isotype	dilution	Manufacturer
Rhodamine Red TM – X-conjugated AfiiniPure	IgG2b MC	1:100	Jackson Immuno research Lab, Inc. 112-175-207
Cy TM5conjugated AffiniPure	IgG2a	1:500	Jackson Immuno research Lab, Inc. 112-175-206
Light Green	IgG PC	1:200	Jackson Immuno research Lab, Inc. NL493
FITC Alexa 488	IgG1 MC	1:500	Ivitrogen
TxRed Alexa 594	IgG PC	1:500	Ivitrogen

catggatctg-3') and (5'-tctgctggaggctgaggtat-3'), Nestin (5'-aggatgtggaggtagtgaga-3') and (5'-tggagatctcagtg-gctctt-3'), BDNF (5'-gattagcctggagcaggttg-3') and (5'-gagcaaggcaccttcaagtc-3'), NRCAM (5'-tcagaggcac-cttctcctgt-3') and (5'-tgcactttgcattccagttc-3'), and GAPDH (5'-ccgcatcttcttttgcgt-3') and (5'- ctggaagatg-gtgatgggat-3'). We used 1 µg of cDNA per PCR reaction. Reactions were run in 100 µl reaction volumes. PCR products were separated by electrophoresis on 1.5% agrose gel in 1× TAE buffer and visualized by ethidium bromide staining.

Immunocytochemistry

Cells on cytospin slides and in the wells of the 24-wells plates were washed with PBS. Cells then were fixed with Accustain (Sigma, cat. no. A5472-1GAL) at room temperature for 30 minutes. Nonspecific binding sites were blocked with 5% FCS, 5% goat serum (Zymed, cat. no. 50-062Z) and 0.1% triton®x100 (Sigma, cat. co. 93443-100ML) all in PBS for 1 hour. The Primary antibodies were added to the cells after being appropriately diluted in PBS and incubated overnight at +4ºC (Table III). Cells were washed with PBS 3 times for 10 minutes and the secondary antibodies were added after being appropriately diluted in PBS and left for one hour at room temperature. After adding the second secondary antibodies for 1 hour (Table IV), cells were washed again in PBS 3 times for 10 minutes. Bis-Benzimide (Sigma, cat. no. B2883-500MG) was added to the cells at a concentration of 1 µg/ml in PBS and left for 10 minutes to visualize cells nuclei. Slides were stored in dark at 4°C. Slides were later visualized using Leica inverted confocal microscope and Zeiss laser confocal microscope.

FACS analysis of mono-nuclear cells fraction

A small fraction of isolated cells (100 000 cells) from at least 5 different cord bloods was analyzed using BD LSRII for the expression of CD14, CD133, and CD45 (Table V). The analysis was done for Cells at Day zero and day 10.

Table V

Antibodies used for FACS analysis

Antibody	Type	Cat No.	Distributor
CD14 (pacific blue)	Mouse IgG2A	558121	BD Pharmingen, USA
CD45 (APC/Cy7)	Mouse IgG1	557833	BD Pharmingen, USA
CD133/1 (APC)	Mouse IgG1	130-090-826	Miltenyi Biotech, Germany

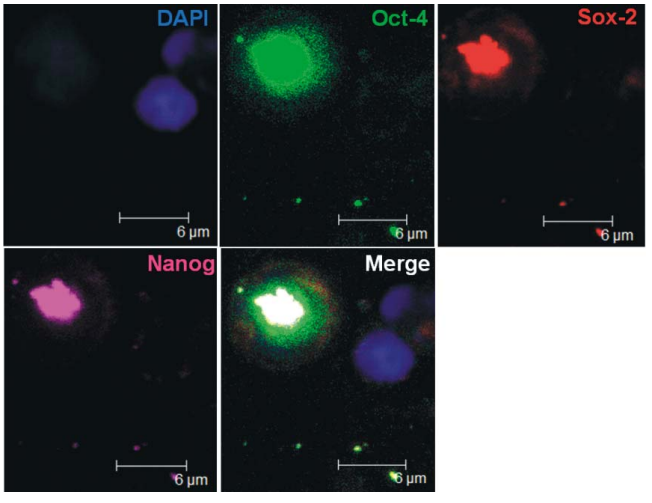

Fig. 1. Umbilical cord blood stem cells co expressed Oct-4 (green), Sox-2 (red) and Nanog (purple). All three transcription factors were expressed in the cell's nucleus.

RESULTS

Cord blood stem cells produced neuronal cells in serum-free defined cultures

The isolation process of cord blood stem cells involved the use of Ficoll-Paque™ separation which positively selected the mono-nuclear cells fraction (including cord blood stem cells). Immunocytochemistry results showed that cord blood stem cells co-expressed Oct-4, Sox-2 and Nanog in their nuclei which are key pluripotency markers right after isolation (Fig. 1). During the 9 days expansion period the cells formed floating aggregates that attached with time (Fig. 2A,B). The attachment rate increased with time. No morphological differences were noticed between the serum-free cultures and the serum-containing cultures however FACS data showed that Serum containing cultures had more cells positive for CD14 and CD45 (mature hematopoietic markers) compared to serum-free cultures at day 10 of expansion (Fig. 3). After the expansion period the cells co-expressed Nestin and GFAP (Fig. 2G–J). The expression was higher in cells aggregates rather than single cells (Fig. 2J). After 7–10 days of expansion cells were moved into differentiation medium containing RA and BDNF (serum and serum-free), within 48 hours, cells lost their perfect round shape and small projections start to grow from these cells. After a week of differentiation in serum-free cultures, cells showed polar morphology, axons and dendrites structures were clearly noticed and potential simple neural network was noticed (Fig. 4A). A cell with a

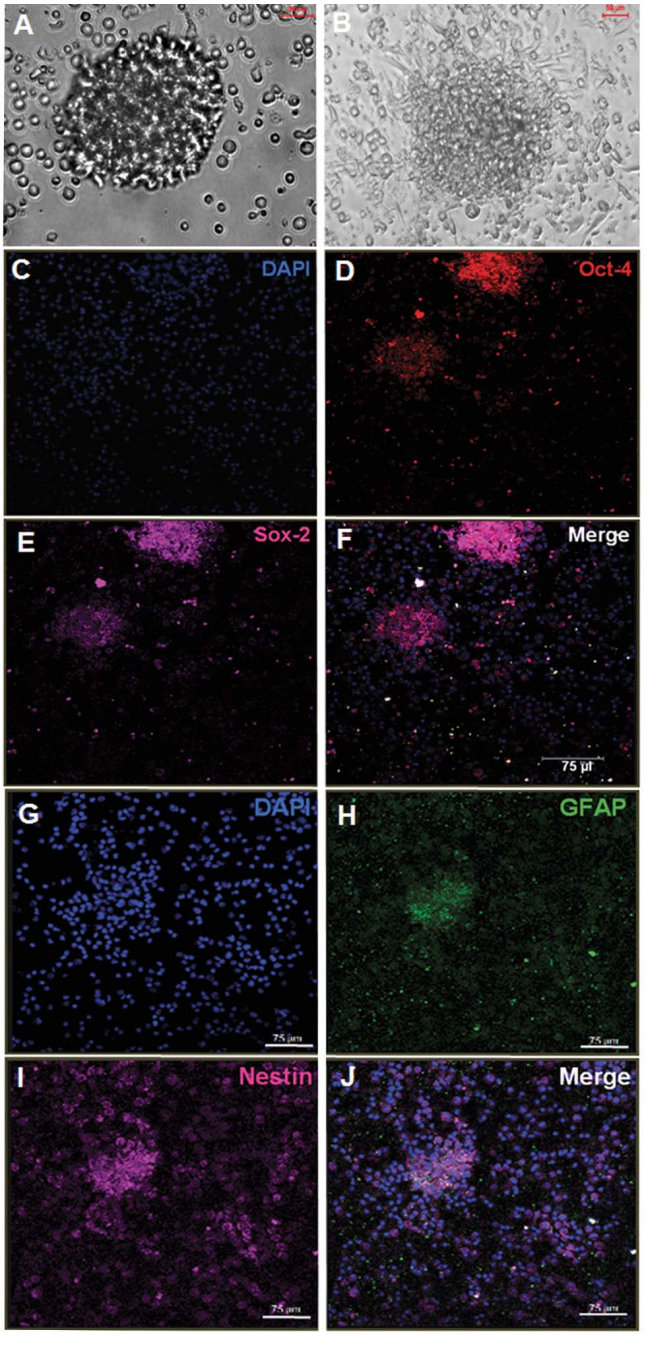

Fig. 2. Cord blood stem cells formed floating cells aggregates in serum-free medium 16–48 hours after isolation (A). These aggregates started to attach to collagen coated cover glasses after 48 hours of initial culturing (B). The attachment rate to the collagen coated cover glasses increased with time. Cells within aggregates highly co-expressed pluripotent markers (C–F) such as Oct-4 (D) and Sox-2 (E) unlike single cells where the expression was too weak. They also expressed Nestin (I) and GFAP (H) in a similar pattern. Immunocytochemistry pictures taken from serum-free cultures at day one (C–F) and day 10 (G–J).

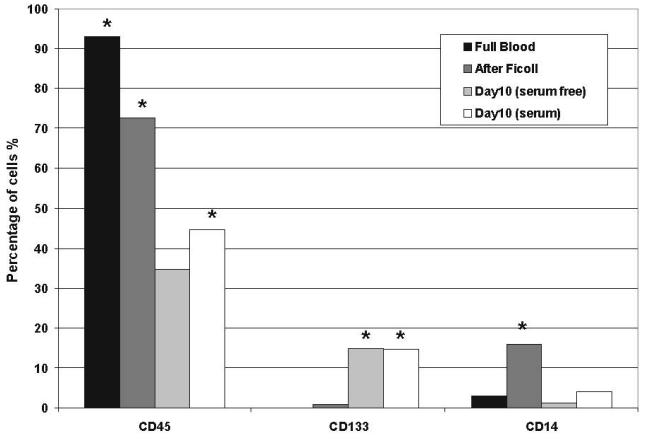

Fig. 3. FACS analysis for immature and mature hematopoietic markers in serum and serum-free cultures. Although both serum and serum-free cultures had similar percentage of cells expressing immature hematopoietic marker (CD133). FACS data shows that serum-free cultures had lower percentage of hematopoietic committed cells (CD45+ and CD14+) after 5 days in culture compared to serum cultures.

neuronal morphology showed physical connections with neighboring cell through a possible synaptic joints (Fig. 4A). Differentiating cells from aggregates were more likely to form neuronal networks due to their close cell-cell interaction (Fig. 4B). The differentiation process was accompanied with apoptosis of some differentiation cells. Apoptosis rate was higher in control cultures (serum cultures) compared to serum free cultures (Fig. 5).

After differentiation, medium was changed to serum-free maturation medium containing cAMP and NGF for 7 days. The introduction of maturation medium induced morphological maturation for the differentiating cells. The cells formed a much complex potential neural networks (Fig. 4B) and highly expressed NF-200 (neurofilament-200) (Fig. 4C). The NF-200 was highly concentrated along the axon structures of the neuronal cells (Fig. 4C).

Similar apoptosis action as in differentiation was noticed during maturation. Serum cultures had higher apoptosis rate than serum-free cultures (Fig. 5).

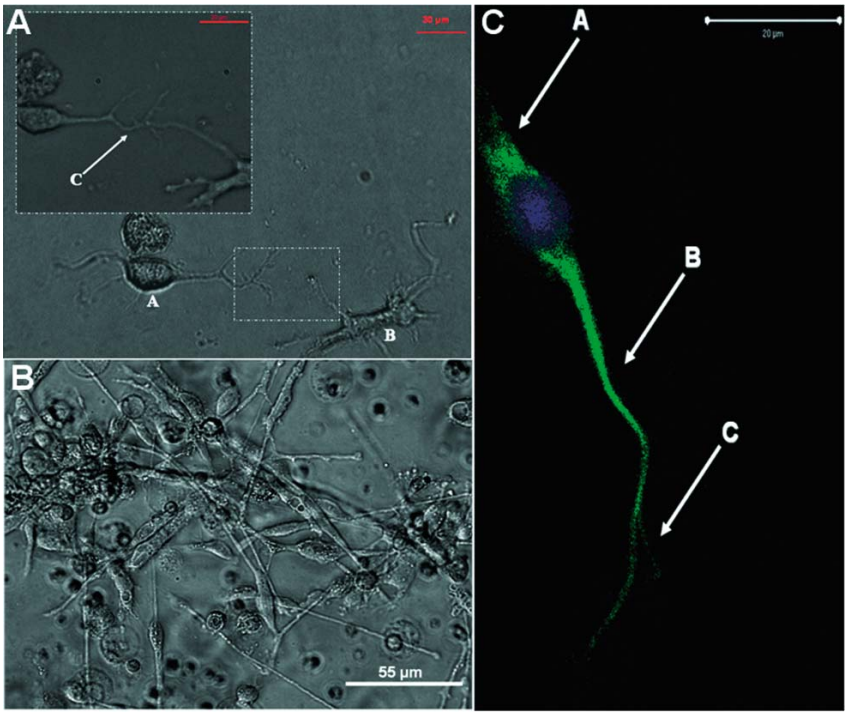

Fig. 4. Neuronal differentiated and maturated cells. (A) A potential synapses-like structure, a characteristic of neuronal cells, was observed in the differentiated cells after a week in serum-free differentiation medium. The structure seemed to form a junction between an axon-like structure of cell (AA) and dendrites-like structure of cell (AB). Arrow (AC) shows the synapses-like structure using higher magnification. (B) Potential neural networks were observed in serum-free cultures after maturation. The cells acquired advanced neuronal phenotypes with long axons structures connecting cells to each other forming potential complex neural networks. (C) Cells with neuronal morphology after differentiation and maturation highly expressed NF-200 (green). The expression was high in axon (CB) moderate in dendrites (CA) and terminal branching (CC). Pictures were taken after maturation in serum-free cultures.

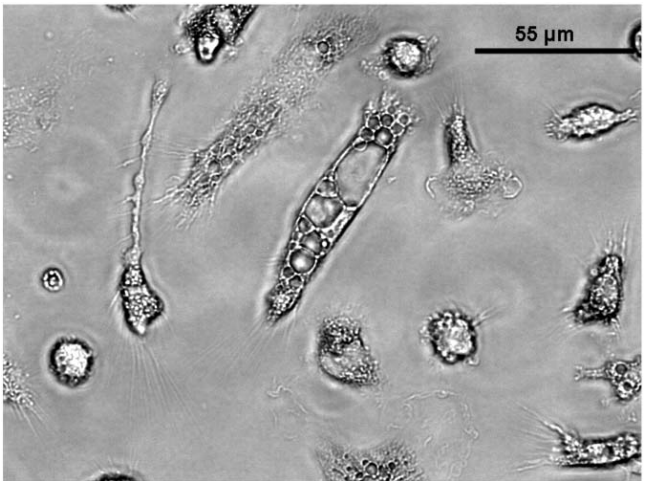

Fig. 5. Apoptotic cells after differentiation in serum cultures. The apoptosis rate in serum cultures was higher than serum-free cultures after differentiation and maturation.

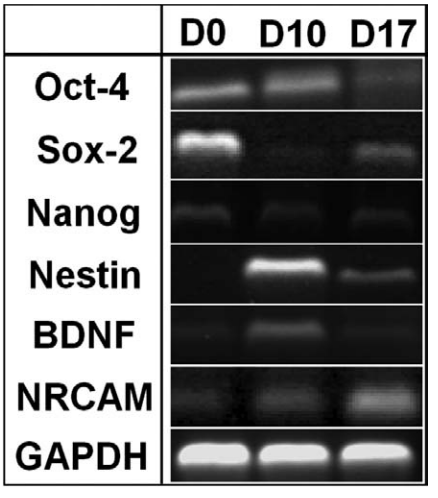

Fig. 6. Reverse transcription-PCR analysis for umbilical cord blood stem cells cultured *in vitro* from day 0 to day 14 (after neuronal differentiation).

Expression pattern of mRNA for selected markers suggested proper neuronal commitment and differentiation process

At Day-0 (D0) cells transcribed Oct-4a, Sox-2 and weakly Nanog. They also showed negative expression for early neural marker such as Nestin and later markers such as BDNF and NRCAM (Fig. 6). At Day-10 (D10) Cells became negative for Sox-2 in comparison with (D0). However, cells expressed Nestin, BDNF and weakly NRCAM at (D10). After Differentiation (D17), cells gene expression became negative for Oct-4a and very weak for Sox-2. In comparison with D10, cells at D17 had weaker Nestin gene expression and negative BDNF expression but showed positive results for NRCAM at D17 (Fig. 6).

Proper cell-cell interaction is essential for cells survival and proper neuronal differentiation

In our experiment we have plated the cells at an initial high concentration (Fig. 7B). To emphasis the importance of proper cell concentration for cells survival and differentiation, we have plated cells at a very low initial concentration (100×10^3 cells/ml) as a control (Fig. 7A). These control cultures had no cells

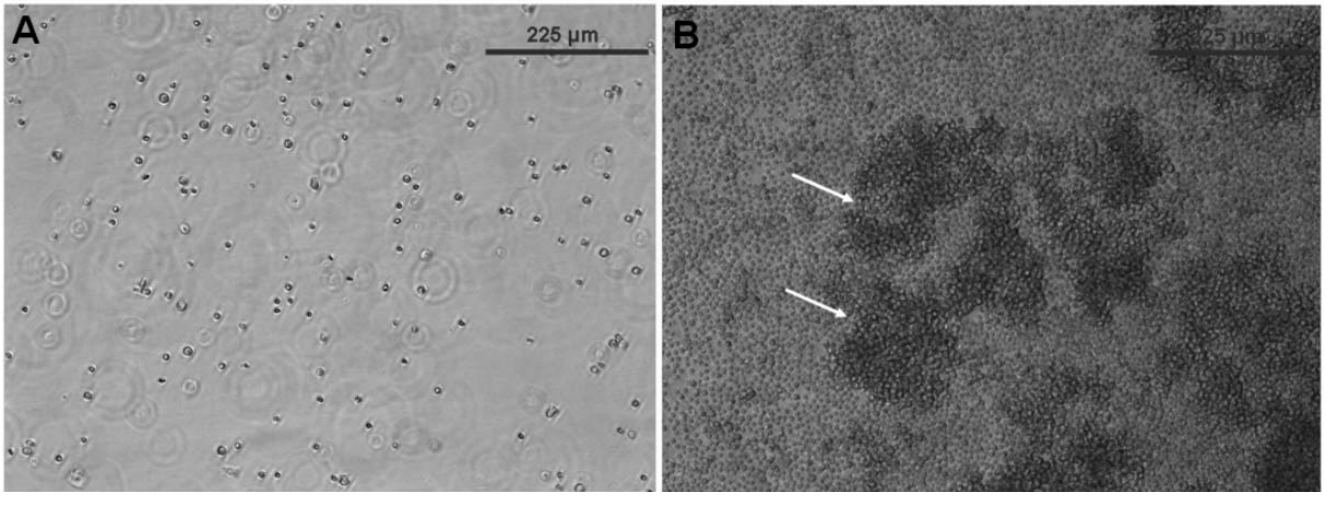

Fig. 7. Proper cell concentration induces aggregates formation and cells survival. Cells in (A) were plated at 100×10^3 cells/ml. No aggregates were observed during the 10 days culturing period. On the other hand, cells in (B) were plated at 5×10^6 cells/ml. Many cells aggregates were observed after around 48 hours of initial culturing (Arrows). Pictures from serum-free cultures.

aggregates formation and the single cells did not survive and died before the time of differentiation. The aggregates formation is strongly influenced by the cells concentration and cell-cell interaction (Fig.2 A,B). We found that the majority of cells within aggregates expressed oct-4 and sox-2 unlike the single cells where only a small number of them were positive for these markers at day 1 (Fig. 2C-F). Similar pattern was found for Nestin and GFAP expression at day 7 (Fig. 2G–J).

3D scaffold-based neural differentiation in serum-free defined conditions

Confocal scanning revealed advanced neuronal network inside the scaffold. Majority of the cells present inside the scaffold were positive for (NF200) (Fig. 8 and 9B). Neural cells were equally distributed inside the 3D scaffold (Fig. 8D). The cells are connected with each other and establish the 3D network (Fig. 8D). Single nerve-like fibers are up to 500 μm long (Fig. 8C). Moreover neuroblasts formed ganglia-like aggregates in outer regions of the scaffold (Fig. 8A,B). Maturated cells also expressed TUJ1 (Fig. 6C), NeuN (Fig. 6F) and post synaptic density-95 (PSD-95) (Fig. 6G). No signs of apoptosis were noticed in the scaffolds compared to the 2-dimintional cultures.

DISCUSSION

Umbilical cord blood contains a population of pluripotent stem cells with potential clinical advantages. In this study we used optimized serum-free culturing conditions for cells recovery, expansion and neural differentiation (McGuckin et al. 2008). We showed that our method can be incorporated with 3-dimentional tissue engineering systems to engineer neural tissues for potential future regenerative medicine clinical applications.

Umbilical cord blood pluripotent stem cells expressed Oct-4, Sox-2 and Nanog at day-0 (Fig. 6). All three markers showed nuclear localization (Fig. 1). These

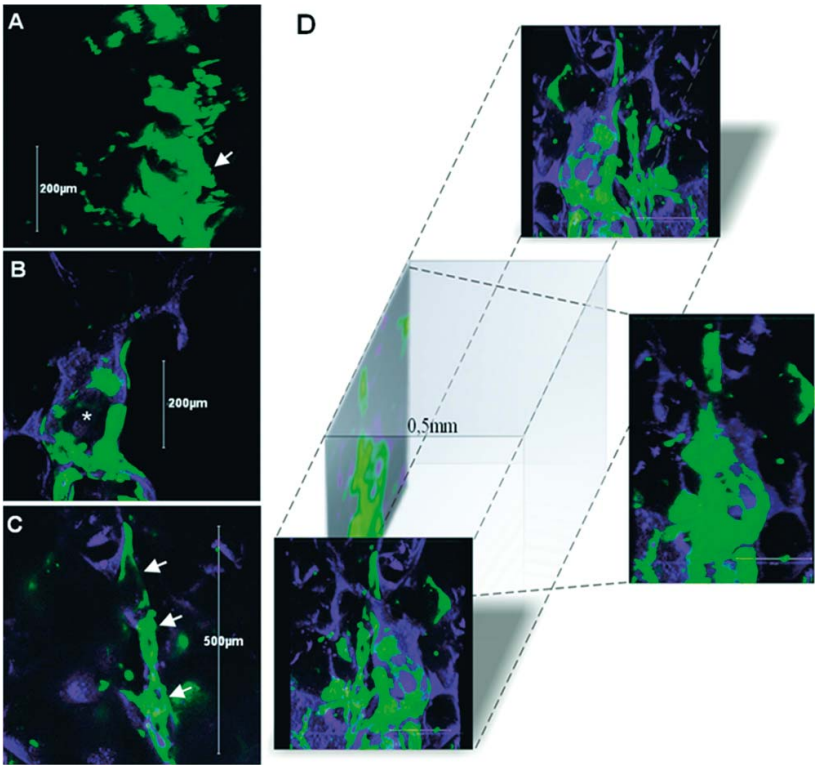

Fig. 8. Three-dimensional neuronal tissue engineering. (A–C) NF200 represented in green. Scaffold structure in purple. 3D confocal reconstruction of ganglia-like structure composed with several neurons (A – arrow). Confocal scanning through the ganglia-like structure (B – asterisk). Neuroblasts which formed nerve-like fiber along the scaffold path (C – arrows). (D) NF200 represented in green. Scaffold structure in purple. 3D confocal reconstruction of neuronal network established within the OPLA polymer scaffold. Pictures taken from serum-free cultures after maturation.

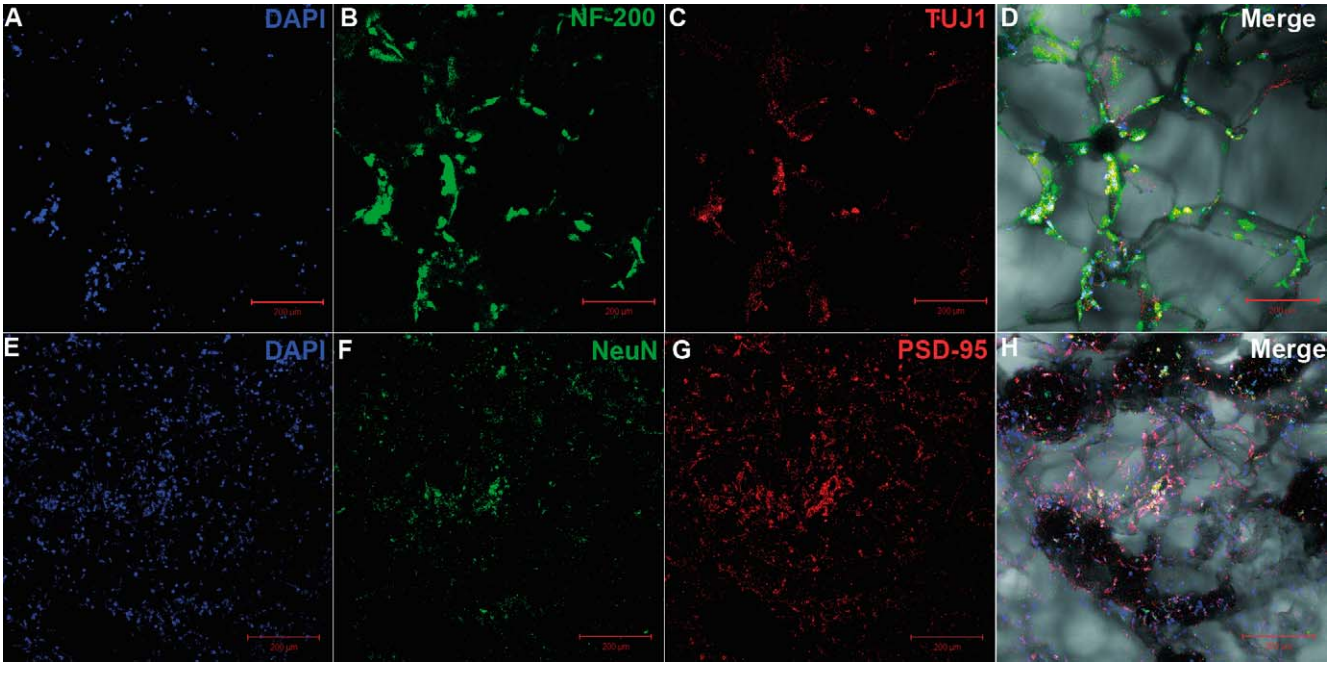

Fig. 9. Expression of mature neuronal markers after neuronal differentiation and maturation in 3-dimentional conditions. In addition to NF200 (B) cells also expressed TUJ1 (C). DIC image (D) showed that the scaffold provided cells with proper mechanical and organizational support that enhanced the formation of neuronal networks inside the scaffold. Cells also expressed mature neuronal markers, NeuN and PSD-95 (E–H).

three transcription factors are responsible for the establishment and maintenance of the pluripotent undifferentiated state of stem cells (Boyer et al. 2005, Boyer et al. 2006, Yamanaka 2008). Majority of research groups have previously characterized Oct-4 expression in stem cells without taking into consideration that Oct-4 has different isoforms (POU5F1_iA and POU5F1_iB) with different expression patterns (Cauffman et al. 2006). Cauffman and others (2006) showed that POU5F1_iA and not POU5F1_iB is expressed in human embryonic stem cells. Therefore, we used POU5F1_iA along side with Sox-2 and Nanog to characterize our umbilical cord blood pluripotent cells utilizing our triple immunocytochemistry staining technique.

We have seeded our cells at initial high concentration in order to induce cells aggregates formation necessary for proper cells propagation and differentiation (Fig. 2A,B). We have found that cells seeded at a very low concentration (Fig. 7A), where the cell-cell physical interaction is minimal, had a very low surviving rate and poor differentiation outcome (data not shown). These aggregates provide the cells with physical support and promote proper cell-cell interaction crucial for proper cell proliferation, development and survival (Watt and Hogan 2000). We found that cells within aggregates highly expressed Oct-4 and Sox-2 unlike the weak expression in the single cells surrounding the aggregates (Fig. 2C–F). Similar pattern was noticed in the Nestin and GFAP expression at day 10 (Fig. 2G–J). These results clearly suggest that proper cell-cell interaction is important for proper cells proliferation and differentiation.

In this study, we have used serum-free media for harvesting, expansion, neuronal differentiation and maturation of cord blood stem cells. We have designed our expansion medium to induce both stem cells proliferation and neural commitment over the 9 days culturing period. FACS results showed that after 10 days in expansion, serum cultures had more cells expressing mature hematopoietic markers such as CD45 and CD14 compared to serum-free cultures (Fig. 3). This result suggests that our serum-free expansion medium do not stimulate hematopoietic commitment of cord blood stem cells which is important for efficient neuronal differentiation protocol.

Umbilical cord blood stem cells co-expressed Nestin and GFAP after the expansion period (D10) at protein level (Fig. 2G–J), an expression pattern unique for neural stem cells (Messam et al. 2002, Suh et al. 2007,

Zhao et al. 2008), and also expressed Nestin at the mRNA level at D10 (Fig. 6). Basic-FGF along with EGF in expansion medium served more as mitogens regulating stem cells proliferation and stimulators for neural commitment (without triggering major morphological differentiation) which explains the co-expression pattern of Nestin and GFAP (Gremo and Presta 2000, Nelson et al. 2008, Zhao et al. 2008) and the shift in Nestin transcription between D0 and D10 (Fig. 6).

In our neuronal differentiation protocol we used a combination of Retinoic acid (RA) and brain-derived neurotrophic factor (BDNF) to specify the neuronal fate of cord blood stem cells. RA is a general neural morphogen, it regulates and induces the differentiation of astrocytes (Wuarin et al. 1990), oligodendrocytes (Noll and Miller 1994) and neurons (Henion and Weston 1994). BDNF on the other hand, is mainly expressed in neurons and can be used to induce neuronal differentiation (Murer et al. 2001). We induced neuronal commitment and differentiation of Nestin and GFAP positive cells using a combination of RA and BDNF and the cells started to acquire a neuronal morphology 48 hours after applying the neuronal medium. Cells after expansion showed positive gene expression result for BDNF and negative results for the same gene after differentiation (Fig. 6). This indicates that the cells were stimulated to produce their own BDNF bearing in mind that our expansion medium is not supplied with BDNF. But after differentiation (D17) the cells stop producing BDNF although they acquired a neuronal morphology and expressed neuronal proteins because differentiation medium is supplied with BDNF. Differentiated cells also had positive gene expression of neuronal cell adhesion molecule (NRCAM) (Fig. 6) a sign of neuronal commitment.

The neuronal differentiation was further induced using maturation medium supplied with nerve growth factor (NGF) and cAMP which are essential for proper regulation of neurogenesis (Nakagawa et al. 2002) as well as induction of neuronal morphological maturation (Fig. 4B) (Fujioka et al. 2004). Our developed neuronal cells acquired a neuronal morphology, (Fig. 4B and 9D) and expressed NF-200, an intermediate filament found specifically in neurons (Fig. 4C), which indicates effective and efficient differentiation and maturation protocols.

Proper timing of neural morphogens introduction is very crucial to obtain effective and successful differentiation. Stem cells need to be competent before applying morphogens. In separate experiment (data not shown), we applied differentiation medium for cells right after isolation and massive apoptosis and weak differentiation was observed which stress the importance of proper timing. Therefore, we developed our sequential neuronal induction consisting of expansion, differentiation and maturation to properly induce neuronal commitment.

The differentiation and maturation processes were accompanied with apoptosis of some differentiating cells (Fig. 5). However the apoptosis rate was higher in the serum cultures compared to serum-free cultures. Serum contains undefined population of proteins and factors. These factors might have activated unwanted apoptosis signaling pathways which explains that higher apoptosis rate recorded (Martin et al. 2005).

We previously developed defined expansion and differentiation protocols with short *in-vitro* culturing time in order to increase adherence toward clinical grade protocols (McGuckin et al. 2008). We applied our differentiation protocol in 3-dimentional neural tissue engineering applications. Our results showed that umbilical cord blood stem cells differentiated successfully into neuronal cells and were equally distributed inside the scaffolds indicating that the scaffolds provided mechanical support that allow proper cells organization and 3D cells growth and differentiation (Fig. 8, 9D and 9H). The cells highly expressed NF-200 and we observed neuronal networks within the scaffolds (Fig. 8 and 9D). They also expressed TUJ1, a microtubule protein specific for neurons and neuronal tissues (Xiao et al. 2006), NeuN, a DNA-binding neuron-specific protein that is specific for mature neurons (Mullen et al. 1992) and PSD-95, a post synaptic density protein that is associated with receptors and cytoskeletal elements at the synapses and pre-synaptic terminals (Hata and Takai 1999) which indicate mature neuronal developmental stage (Fig. 6)

Our results showed no sign of apoptosis which suggest that 3-dimentional cultures enhance neuronal differentiation and survival compared with 2D cultures (Brannvall et al. 2007, Ju et al. 2007).

The umbilical cord blood mononuclear cells fraction contains, beside our wanted pluripotent stem cells, many mature hematopoietic cells. Therefore, as a future study we will investigate the effect of purifying our wanted cells population before applying our expansion and differentiation protocols on differentiation outcome. We also intend to investigate the effect

of using perfused Rotary culture system with our culturing protocol in an attempt to increase the efficiency and quality of the tissue developed.

CONCLUSION

Clinical therapies for stoke, spinal cord damage and neural degeneration require novel tissue culture systems to re-establish function. 3-dimentional integrative scaffolding is an important step towards therapies for these difficult clinical indications.

ACKNOWLEDGEMENTS

We would like to thank Kuwait University, Department of Pathology and the State of Kuwait for their postgraduate scholarship to Mr. Hamad Ali. We also gratefully acknowledge the support of the Le Jeune Foundation, Paris for their contribution to our neural diseases research program. Finally we would like to thank Mr. Jonathan Terrell for his philanthropic support of our adult stem cells research work.

REFRENCES

Boyer LA, Lee TI, Cole MF, Johnstone SE, Levine SS, Zucker JP, Guenther MG, Kumar RM, Murray HL, Jenner RG, Gifford DK, Melton DA, Jaenisch R, Young RA (2005) Core transcriptional regulatory circuitry in human embryonic stem cells. Cell 122: 947–956.

Boyer LA, Mathur D, Jaenisch R (2006) Molecular control of pluripotency. Curr Opin Genet Dev 16: 455–462.

Brannvall K, Bergman K, Wallenquist U, Svahn S, Bowden T, Hilborn J, Forsberg-Nilsson K (2007) Enhanced neuronal differentiation in a three-dimensional collagen-hyaluronan matrix. J Neurosci Res 85: 2138–2146.

Buzanska L, Machaj EK, Zablocka B, Pojda Z, Domanska-Janik K (2002) Human cord blood-derived cells attain neuronal and glial features in vitro. J Cell Sci 115: 2131–2138.

Cauffman G, Liebaers I, Van Steirteghem A, Van de Velde H (2006) POU5F1 isoforms show different expression patterns in human embryonic stem cells and preimplantation embryos. Stem Cells 24: 2685–2691.

Chen N, Hudson JE, Walczak P, Misiuta I, Garbuzova-Davis S, Jiang L, Sanchez-Ramos J, Sanberg PR, Zigova T, Willing AE (2005) Human umbilical cord blood progenitors: the potential of these hematopoietic cells to become neural. Stem Cells 23: 1560–1570.

Corselli M, Parodi A, Mogni M, Sessarego N, Kunkl A, Dagna-Bricarelli F, Ibatici A, Pozzi S, Bacigalupo A, Frassoni F, Piaggio G (2008) Clinical scale ex vivo expansion of cord blood-derived outgrowth endothelial progenitor cells is associated with high incidence of karyotype aberrations. Exp Hematol 36: 340–349.

Denner L, Bodenburg Y, Zhao JG, Howe M, Cappo J, Tilton RG, Copland JA, Forraz N, McGuckin C, Urban R (2007) Directed engineering of umbilical cord blood stem cells to produce C-peptide and insulin. Cell Prolif 40: 367–380.

Domanska-Janik K, Habich A, Sarnowska A, Janowski M (2006) Neural commitment of cord blood stem cells (HUCB-NSC/NP): therapeutic perspectives. Acta Neurobiol Exp (Wars) 66: 279–291.

Ende M, Ende N (1972) Hematopoietic transplantation by means of fetal (cord) blood. A new method. Va Med Mon (1918) 99: 276–280.

Fujioka T, Fujioka A, Duman RS (2004) Activation of cAMP signaling facilitates the morphological maturation of newborn neurons in adult hippocampus. J Neurosci 24: 319–328.

Gluckman E, Broxmeyer HA, Auerbach AD, Friedman HS, Douglas GW, Devergie A, Esperou H, Thierry D, Socie G, Lehn P, et al. (1989) Hematopoietic reconstitution in a patient with Fanconi's anemia by means of umbilical-cord blood from an HLA-identical sibling. N Engl J Med 321: 1174–1178.

Gremo F, Presta M (2000) Role of fibroblast growth factor-2 in human brain: a focus on development. Int J Dev Neurosci 18: 271–279.

Hata Y, Takai Y (1999) Roles of postsynaptic density-95/synapse-associated protein 90 and its interacting proteins in the organization of synapses. Cell Mol Life Sci 56: 5–6.

Henion PD, Weston JA (1994) Retinoic acid selectively promotes the survival and proliferation of neurogenic precursors in cultured neural crest cell populations. Dev Biol 161: 243–250.

Ju YE, Janmey PA, McCormick ME, Sawyer ES, Flanagan LA (2007) Enhanced neurite growth from mammalian neurons in three-dimensional salmon fibrin gels. Biomaterials 28: 2097–2108.

Jurga M, Markiewicz I, Sarnowska A, Habich A, Kozlowska H, Lukomska B, Buzanska L, Domanska-Janik K (2006) Neurogenic potential of human umbilical cord blood: neural-like stem cells depend on previous long-term culture conditions. J Neurosci Res 83: 627–637.

Lee MW, Moon YJ, Yang MS, Kim SK, Jang IK, Eom YW, Park JS, Kim HC, Song KY, Park SC, Lim HS, Kim YJ (2007) Neural differentiation of novel multipotent progenitor cells from cryopreserved human umbilical cord blood. Biochem Biophys Res Commun 358: 637–643.

Martin MJ, Muotri A, Gage F, Varki A (2005) Human embryonic stem cells express an immunogenic nonhuman sialic acid. Nat Med 11: 228–232.

McGuckin C, Forraz N, Baradez MO, Basford C, Dickinson AM, Navran S, Hartgerink JD (2006) Embryonic-like stem cells from umbilical cord blood and potential for neural modeling. Acta Neurobiol Exp (Wars) 66: 321–329.

McGuckin C, Jurga M, Ali H, Strbad M, Forraz N (2008) Culture of embryonic-like stem cells from human umbilical cord blood and onward differentiation to neural cells in vitro. Nat Protoc 3: 1046–1055.

McGuckin CP, Forraz N (2008) Potential for access to embryonic-like cells from human umbilical cord blood. Cell Prolif 41 (Suppl 1): 31–40.

McGuckin CP, Forraz N, Allouard Q, Pettengell R (2004) Umbilical cord blood stem cells can expand hematopoietic and neuroglial progenitors in vitro. Exp Cell Res 295: 350–359.

Messam CA, Hou J, Berman JW, Major EO (2002) Analysis of the temporal expression of nestin in human fetal brain derived neuronal and glial progenitor cells. Brain Res Dev Brain Res 134: 87–92.

Mullen RJ, Buck CR, Smith AM (1992) NeuN, a neuronal specific nuclear protein in vertebrates. Development 116: 201–211.

Murer MG, Yan Q, Raisman-Vozari R (2001) Brain-derived neurotrophic factor in the control human brain, and in Alzheimer's disease and Parkinson's disease. Prog Neurobiol 63: 71–124.

Nakagawa S, Kim JE, Lee R, Malberg JE, Chen J, Steffen C, Zhang YJ, Nestler EJ, Duman RS (2002) Regulation of neurogenesis in adult mouse hippocampus by cAMP and the cAMP response element-binding protein. J Neurosci 22: 3673–3682.

Nelson AD, Suzuki M, Svendsen CN (2008) A high concentration of epidermal growth factor increases the growth and survival of neurogenic radial glial cells within human neurosphere cultures. Stem Cells 26: 348–355.

Noll E, Miller RH (1994) Regulation of oligodendrocyte differentiation: a role for retinoic acid in the spinal cord. Development 120: 649–660.

Slatter MA, Bhattacharya A, Flood TJ, Abinun M, Cant AJ, Gennery AR (2006) Use of two unrelated umbilical cord stem cell units in stem cell transplantation for Wiskott-Aldrich syndrome. Pediatr Blood Cancer 47: 332–334.

Suh H, Consiglio A, Ray J, Sawai T, D'Amour KA, Gage FH (2007) In Vivo Fate Analysis Reveals the Multipotent and Self-Renewal Capacities of Sox2(+) Neural Stem Cells in the Adult Hippocampus. Cell Stem Cell 1: 515–528.

Watt FM, Hogan BL (2000) Out of Eden: stem cells and their niches. Science 287: 1427–1430.

Watt SM, Contreras M (2005) Stem cell medicine: umbilical cord blood and its stem cell potential. Semin Fetal Neonatal Med 10: 209–220.

Wuarin L, Sidell N, de Vellis J (1990) Retinoids increase perinatal spinal cord neuronal survival and astroglial differentiation. Int J Dev Neurosci 8: 317–326.

Xiao S, McLean J, Robertson J (2006) Neuronal intermediate filaments and ALS: a new look at an old question. Biochim Biophys Acta 1762: 1001–1012.

Xu RH, Peck RM, Li DS, Feng X, Ludwig T, Thomson JA (2005) Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. Nat Methods 2: 185–190.

Yamanaka S (2008) Pluripotency and nuclear reprogramming. Philos Trans R Soc Lond B Biol Sci 363: 2079–2087.

Zhao C, Deng W, Gage FH (2008) Mechanisms and functional implications of adult neurogenesis. Cell 132: 645–660.