Culture of embryonic-like stem cells from human umbilical cord blood and onward differentiation to neural cells *in vitro*

Colin McGuckin[1], Marcin Jurga[1], Hamad Ali[1], Marko Strbad[1,2] & Nicolas Forraz[1]

[1]Newcastle Centre for Cord Blood, North East England Stem Cell Institute and Institute of Human Genetics, Centre for Life, Central Parkway, Newcastle upon Tyne, NE1 3BZ, UK. [2]Department of Immunohaematology, Blood Transfusion Centre of Slovenia, Slajmerjeva 6, 1000 Ljubljana, Slovenia. Correspondence should be addressed to C.M. (c.mcguckin@novussanguis.org) or (c.mcguckin@newcastle.ac.uk).

Published online 29 May 2008; doi:10.1038/nprot.2008.69

This 3-week protocol produces embryonic-like stem cells from human umbilical cord blood (CBEs) for neural differentiation using a three-step system (cell isolation/expansion/differentiation). The CBE isolation produces a highly purified fraction (CD45−, CD33−, CD7−, CD235a−) of small pluripotent stem cells (2–3 μm in diameter) coexpressing embryonic stem cell markers including Oct4 and Sox2. Initial CBE expansion is performed in high density (5–10 millions per ml) in the presence of extracellular matrix proteins and epidermal growth factor. Subsequent neural differentiation of CBEs requires sequential introduction of morphogenes, retinoic acid, brain-derived neurotrophic factor and cyclic AMP. Described methods emphasize defined media and reagents at all stages of the experiment comparable to protocols described for culturing human embryonic stem cells and cells from other somatic stem cell sources. Neural progenitor and cells generated from CBEs may be used for *in vitro* drug testing and cell-based assays and potentially for clinical transplantation.

INTRODUCTION

Transplantation with cord blood stem cells (SCs) was first achieved in 1972 by the pioneer doctors in the United States, Ende and Ende. 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. However, advances in the production of ~20 organ-based tissue groups has highlighted the further potential of this—the most abundant world SC source.

We describe here a three-step protocol for production of a restricted cell population containing embryonic-like SCs from human umbilical cord blood (CBEs) and the onward differentiation toward neural cells[1,2]. Our system emphasizes our philosophy of increasing adherence toward clinical-grade protocols for potential clinical application of CBEs-derived neural cells; 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 SC populations including embryonic SCs.

Human umbilical cord blood is probably the largest, but underused, source of SCs, with the yearly global birth rate of 130 million. Cord blood SCs are easily accessible and immunologically naïve, and cord blood provides a source of such cells that is free from ethical controversies[3]. Human umbilical cord blood is used in the clinical treatment of >80 diseases, mostly related to hematopoietic and immune systems[4] (and see http://www.nationalcordbloodprogram.org).

Our experiments revealed that these cells can be effectively differentiated toward mature neural cells *in vitro*[1,5,6]. Moreover, stable neural cell lines derived from human cord blood may produce functional neuronal networks generating spontaneous action potentials (Jurga, M. *et al.*, unpublished data, see Proceedings of International Brain Research Organisations World Congress of Neuroscience-IBRO 2007) which has been confirmed by other research centers by both *in vitro* experiments[7] and *in vivo* xenotransplantations[8]. Human cord blood has also been used in a single clinical trial for regeneration of damaged spinal cord in Korea, with a major US–China clinical trial in preparation[9]. Most previously described *in vitro* and *in vivo* experiments that focused on neural differentiation and regeneration have used whole mononuclear cell fractions or mesenchymal cells present in cord blood[9,10]. In most cases, such cells were maintained in serum-containing or conditioned media. *In vivo* experiments have demonstrated migratory properties of cord blood cells and their capacity to maintain multipotency within the host brain. Despite this, long-term survival and functional integration of individual cells remains to be conclusively proven for cord blood and embryonic SCs[11,12]. It seems that more promising results regarding neural differentiation of transplanted SCs might be obtained after initial neural commitment *in vitro*[6,8]. However, more such experiments and the appropriate method to produce these cells are urgently needed.

Our protocol first focuses on the importance of selecting the correct group of cells within cord blood which have the ability to cross differentiate[1,3]. These cells are described as 'embryonic-like' SCs from human umbilical cord blood (CBEs) due to their expression of similar markers as the embryonic SCs derived from epiblasts of human blastocystes[1,13]. CBEs are a very small fraction of mononucleated cells present in human umbilical cord blood (0.1–1%). The CBE fraction is depleted in our protocol of most hematopoietic SCs that secrete chemokines and growth factors stimulating undesired hematopoietic commitment. This procedure has been established to obtain the maximum number of pluripotent cells at lower stages of their differentiation. Similar cell fractions of pluripotent SC isolated from human umbilical cord blood have also been reported by other groups[2,14]. Other cells in the CBE population also support the cells during cell growth and during neural differentiation[6]. Freshly isolated CBEs are very

sensitive; therefore, we plate them initially in a high concentration (5–10 million cells per 1 ml) in TPOFLK (thrombopoietin, flt-3 ligand and c-kit ligand) medium supplemented with extracellular matrix (ECM) proteins (collagen type IV and fibronectin) to reduce their apoptosis[14,15]. Initial high cell density and ECM proteins are crucial for CBEs survival and formation of aggregates similar to embryoid bodies. Appearance of embryoid bodies is the first phenotypic hallmark of proper *in vitro* growth of CBEs[1,14]. Such aggregation promotes cell–cell interactions and secretion of growth factors, which play a major role in cell survival. For this protocol, we highly recommend dynamic cell culture conditions based on observation of cell phenotypic changes and to adjust the timing regime for the CBE culture. The following steps are crucial for cell survival, expansion and differentiation: (i) formation of floating aggregates (16–24 h after isolation), (ii) size and number of floating cells/aggregates (1–7 d after isolation) and (iii) cell adhesion and differentiation (1–3 weeks after isolation).

The expansion step where growth factors are introduced (epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and stem cell factor (SCF)) begins just after aggregates are formed (usually 16–24 h after CBE isolation). This step is important to increase number of CBEs as the initial number present in cord blood is ~1 million (for an average cord blood volume of 80 ml). Presence of EGF is important for initial epithelial and neural commitment of CBEs. Through the expansion step, CBEs increase their size from 2–3 to >10 μm in diameter and begin to adhere spontaneously. However, it is worth emphasizing that prolonged *in vitro* expansion may trigger genetic instability (e.g., polyploidy) as reported previously[16].

Cell/aggregate adhesion and neural differentiation is further stimulated in our protocol by retinoic acid (RA—a morphogene with wide-range action) and brain-derived neurotrophic factor (BDNF—a major growth factor responsible for neural differentiation). After adhesion, most of the cells already coexpress Nestin and GFAP (markers of neural SCs). When their morphology changes to unipolar, we introduce dibutyryl cyclic AMP (dBcAMP) to stimulate their terminal differentiation[17]. We would like to stress that phenotype changes described in our protocol should be regarded as critical steps in neural cell development. These phenotypic changes may slightly vary in time depending on age and quality of CBEs[18]. However, the above-mentioned phenotypic hallmarks should be easily distinguished in simple light microscope analysis enabling progression to the next step in the protocol. Another point of note, is that expression of mRNA, proteins (e.g., transcription factors) and their intracellular localization accompany phenotypic changes and have already been investigated elsewhere[1,14,19].

The limitations of this method are the very low number of lineage-negative CBEs after their isolation (the mononuclear cells depleted of CD45, CD33, CD7 and CD235a) and the simultaneous requirement for a high cell concentration for initial plating, which is crucial to support CBEs survival and formation of aggregates. For this reason, low volumes of cord blood or those cord blood units with limited numbers of CBEs might not allow the effective isolation of a decent amount of SCs. Therefore, further experiments need to be performed to develop a protocol that allows expansion of extremely small amounts (semi-clonal) of CBEs present in some cord blood units.

For CBE isolation, expansion and differentiation, we describe defined serum-free culture media. Growth factors present in these culture media are introduced sequentially to promote CBE survival, expansion, differentiation and maturation toward neural phenotypes. The sequence of growth factors must be applied according to phenotypic changes in the cell morphology. Defined media, free from animal products, will allow the use of CBEs for drug testing and detailed analysis of factors regulating cell fates and for direct clinical transplantation, particularly for neurodegenerative diseases including stroke and also following trauma as, for instance, with spinal cord injury. But more basic and clinical research must be carried out by the scientific community worldwide to unravel this potential fully. It is important to note that beyond neural tissue production, cells of all three germ layers have been produced in our laboratory with this population. Because CBE populations can be directed to several different organ-based lineages, including hepatic, pancreatic and endothelial-like tissues as an example, it is important to be careful with regard to the choice and source of growth factors and bioscaffolds to enable translation of those discoveries into biotechnology and clinical applications.

MATERIALS

REAGENTS

- Umbilical cord blood unit ! **CAUTION** Parents' full and informed consents, in adherence with the law, must be obtained before the delivery. All the hospital ethical requirements must be fulfilled.
- Ficoll-Paque PREMIUM (GE Healthcare, cat. no. 17-5442-02)
- DMEM/F-12 + GlutaMax (GIBCO, cat. no. 31331093)
- B27 supplement (GIBCO, cat. no. 17504044)
- N2 supplement (GIBCO, cat. no. 17502048)
- Recombinant human SCF (rh SCF; ImmunoTools, cat. no. 11343325) ▲ **CRITICAL** Growth factors and morphogens adhere to walls of containers and denature at low concentrations. Store them in appropriate aliquots according to the manufacturer's recommendations at −20 °C.
- Recombinant human thrombopoietin (rh TPO; ImmunoTools, cat. no. 11343615)
- Recombinant human Flt3-ligand (rh Flt3; ImmunoTools, cat. no. 11343305)
- Recombinant human SCF (rh SCF; ImmunoTools, cat. no. 11343322)
- Recombinant human FGF, basic (rh FGF-basic; ImmunoTools, cat. no. 11343627)
- Recombinant human EGF (rh EGF; ImmunoTools, cat. no. 11343407)
- Fibronectin from human plasma (Sigma-Aldrich, cat. no. 86088-83-7) (see REAGENT SETUP)
- Collagen type IV (Sigma-Aldrich, cat. no. C5533) (see REAGENT SETUP)
- Heparin sodium salt (Sigma-Aldrich, cat. no. H4784-1G) (see REAGENT SETUP)
- Penicillin–streptomycin (GIBCO, cat. no. 15070063)
- Fungizone (GIBCO, cat. no. 15290026)
- BDNF (ImmunoTools, cat. no. 11343375) (see REAGENT SETUP)
- RA (Sigma-Aldrich, cat. no. R2625) (see REAGENT SETUP)
- dBcAMP (Sigma-Aldrich, cat. no. D0627) ▲ **CRITICAL** Only in the form of dBcAMP can the compound penetrate through cell membranes appropriately.
- DMSO (Sigma-Aldrich, cat. no. D8418)
- Deionized sterile water
- 10× PBS (Sigma-Aldrich, cat. no.P5493-1L)
- 1× Hanks' balanced salt solution (HBSS) without $CaCl_2$ and $MgCl_2$ (GIBCO–Invitrogen, cat. no. 14175-053)
- ZENLAB 4.5—human albumin 4.5% solution Ph.Eur (Bio Products Laboratory, cat. no. ADAN7459)

TABLE 1 | Abs used for flow cytometry (fluorescence-activated cell sorting) estimation of purity of isolated CBEs.

Ab	Type	Volume used (μl)	Cat. no.	Distributor
CD14 (pacific blue)	Mouse IgG_{2A}	5	558121	BD Pharmingen
CD34 (PE/Cy7)	Mouse IgG_1	5	348811	BD
CD45 (APC/Cy7)	Mouse IgG_1	5	557833	BD Pharmingen
CD90 (FITC)	Mouse IgG_1	2	555595	BD Pharmingen
CD133/1 (APC)	Mouse IgG_1	10	130-090-826	Miltenyi Biotech
SSEA-4 (PE)	Mouse IgG_3	10	FAB1435P	R & D Systems

- Gentran 40–Dextran 40 intravenous infusion BP 10% wt/vol in sodium chloride intravenous infusion BP 0.9% wt/vol (Baxter Healthcare Ltd, cat. no. B5043)
- Mouse anti-human anti-GLyA/CD235a (DakoCytomation, cat. no. M0819)
- Mouse IgG1 anti-human CD45 (Autogen Bioclear, cat. no. ABX252)
- Mouse IgG1 anti-human CD33 (Autogen Bioclear, cat. no. AB163)
- Mouse IgG3 anti-human CD7 (Autogen Bioclear, cat. no. AB164)
- γ-Globulin from human blood (Fluka Chemie GmbH, cat. no. 49000-5g)
- Dynabeads PanMouse IgG (Dynal Biotech ASA, cat. no. 11042)
- Abs for fluorescence-activated cell sorting (FACS) analysis: see **Table 1**
- Primary Abs to provide markers for confocal analysis of stages of neural differentiation: see **Table 2**
- Secondary Abs: Alexa 594 rabbit anti-goat (IgG H+L) (Invitrogen, cat. no. A11080), Alexa 594 goat anti-rabbit (IgG H+L) (Invitrogen, cat. no. A-11012), Alexa 488 rabbit anti-mouse (IgG H+L) (Invitrogen, cat. no. A-11059) and Alexa 488 goat anti-mouse (IgG1) (Invitrogen, cat. no. A-21121)
- Washing buffer (see REAGENT SETUP)
- Working buffer (see REAGENT SETUP)

EQUIPMENT

- 15-ml Universal tubes (Greiner Bio-One, cat. no. 118271)
- 50-ml Universal tubes (Greiner Bio-One, cat. no. 210261)
- 10-ml Plastic pipettes (Greiner Bio-One, cat. no. 607180)
- 24-Well plate (Greiner Bio-One, cat. no. 662160)
- 96-Well plate, U-shape (Greiner Bio-One, cat. no. 650180)
- Plastic pipettes (Delta Lab, cat. no. 200037)
- Pipettes and tips (Jencons)
- Shandon-thermo centrifuge (Thermo Scientific)
- Syringes, 50 ml (Terumo Europe N.V.)
- Syringe filters, 0.22 μm (PALL Life Sciences, cat. no. PN 4612)
- Magnetic Particle Concentrator (MPC) (Dynal MPC-I; Dynal AS, cat. no. 120-01)
- MPC (Dynal MPC-S; Dynal AS, cat. no. 120-00)
- Cover glass (VWR, cat. no. 613-0149)
- Microscope glass slides (VWR, cat. no. 631-0149)
- Centrifuge (Rotanta 460R; Zentrifugen)
- CO_2 incubator Hera Cell 240 (Heraeus)
- Millipore Direct-Q (Millipore, cat. no. ZRQS5005Y)
- Cell counter Coulter A^C T diff2 (Becton Coulter)
- Trypan Blue solution 0.4% (Sigma-Aldrich, cat. no. T8154)
- Chamber for cell counting—Hemacytometer (Hauser Scientific, cat. no. 1492)

REAGENTS SETUP

See **Table 3** for reagents used to prepare serum-free harvesting (SF-TPO) and expansion (SF-EFS) media.

See **Table 4** for the reagents used to prepare serum-free media for neural differentiation (SF-NEURO/D) and maturation (SF-NEURO/M). ▲ **CRITICAL** Both neural differentiation media and neural maturation media tubes should be wrapped with aluminum foil because RA is light sensitive. All media should be stored at 4 °C for no >2 weeks. ! **CAUTION** All media should be filtered before use using 0.22-μm filter to reduce contamination risks with pathogens.

Fibronectin Reconstitute the powder by properly dissolving it in sterile water to give up a stock concentration of 1 mg ml^{-1}.

Collagen type IV Reconstitute the powder by properly dissolving it in 0.25% acetic acid to give a stock concentration of 1 mg ml^{-1}.

Heparin Prepare a stock concentration of 1 mg ml^{-1} by dissolving heparin in DMEM/F12. ▲ **CRITICAL** The solution must be filtered using 0.22-μm filter to reduce contamination risks with pathogens.

TABLE 2 | Abs used for confocal analysis of subsequent stages of neural differentiation.

Primary Ab	Host	Isotype	Suggested working dilution	Manufacturer	Cat. no.
Undifferentiated cells					
Oct4	Goat	IgG	1:500	Abcam	ab27985
Sox2	Mouse	IgG2a	1:50	R & D Systems	MAB2018
Nanog	Goat	IgG	1:20	R & D Systems	AF1997
Ki67	Mouse	IgG1	1:100	Novocastra	L111853
Neural progenitors					
Nestin	Mouse	IgG1	1:100	R & D Systems	MAB1259
GFAP*	Rabbit	IgG	1:1,000	Abcam	Ab7779
Neuroblasts and mature neurons					
NF-200	Mouse	IgG1	1:800	Sigma-Aldrich	N1042
Doublecortin	Rabbit	IgG	1:500	Abcam	Ab28941
MAP2	Mouse	IgG	1:1,000	Sigma-Aldrich	M4403
TUJ1	Rabbit	IgG	1:2,000	Covance	PRB435P
NeuN	Mouse	IgG1	1:100	Chemicon	MAB377
PSD95	Rabbit	IgG	1:500	Abcam	Ab18258
Astroglial cells					
S100β	Rabbit	IgG	1:5,000	Swant	37A

*Expression of GFAP is expressed by both astroglial cells and neural progenitors and should be considered a marker of undifferentiated neurons when coexpressed with Nestin.

BDNF Reconstitute the powder by dissolving it in sterile water to give a stock concentration of 10 μg ml^{-1}.
RA Reconstitute the powder by dissolving it in DMSO to give a stock concentration of 0.05 M. ! CAUTION All vials containing RA should be wrapped with aluminum foil because RA is light sensitive. Degradation is a potential reason for failure of the protocol.
Sterile water Water is deionized and filtered using Millipore Direct-Q with Millipak Express 20 filter—0.22 μm (Millipore, cat. no. 02001) and sterilized using dry heat autoclave. ! CAUTION After autoclaving, check the sterility indicator.
Washing buffer: PBS/0.2% Albumin/Dextran Take 45.6 ml of PBS, mix it with the 4.4 ml of human albumin solution and add 50 ml of Dextran 40. Mix well and pass the buffer using a syringe (Terumo syringe 50 ml; Terumo Europe N.V.) equipped with sterile PALL Acrodisc syringe filter, 0.22 μm (PALL Life Sciences).
Note: To prepare 1× PBS: dilute 10 ml of 10× PBS without calcium ($CaCl_2$) and magnesium ($MgCl_2$) in 90 ml sterile deionized water.
Working buffer: HBSS/0.2% Albumin Add 2.2 ml of human albumin to 47.8 ml HBSS. Mix well and pass the buffer using a syringe (Terumo syringe 50 ml; Terumo Europe N.V.) equipped with sterile PALL Acrodisc syringe filter, 0.22 μm (PALL Life Sciences). ▲ CRITICAL pH of working and washing buffers should range between 7.2 and 7.4 to maintain cellular homeostasis.

TABLE 3 | Serum-free media for CBEs harvesting and expansion.

Harvesting medium (SF-TPO 50 ml)	
DMEM/F12	47 ml
B27 (1:100)	0.5 ml
N2 (1:50)	1 ml
SCF (20 ng ml^{-1})	10 μl
TPO (10 ng ml^{-1})	5 μl
Flt3 (50 ng ml^{-1})	25 μl
Fibronectin (5 μg ml^{-1})	250 μl
Pen/Strep (1:100)	500 μl
Fungizone (1:100)	500 μl
Expansion medium (SF-EFS 50 ml)	
DMEM/F12	47 ml
B27 (1:100)	0.5 ml
N2 (1:50)	1 ml
SCF (5 ng ml^{-1})	2.5 μl
EGF (20 ng ml^{-1})	10 μl
bFGF (20 ng ml^{-1})	10 μl
Heparin (5 μg ml^{-1})	250 μl
Fibronectin (5 μg ml^{-1})	250 μl
Pen/Strep (1:100)	500 μl
Fungizone (1:100)	500 μl

bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; SCF, stem cell factor; TPO, thrombopoietin.

PROCEDURE

Umbilical cord blood collection ● TIMING ~5 min

1| Collect umbilical cord blood units.
▲ CRITICAL STEP Parents' informed consents must be obtained before the delivery, and all the hospital ethical requirements must be fulfilled.
▲ CRITICAL STEP During and after isolation, mix the collection bag gently to prevent coagulation of the cord blood. Processing of the cord blood should be carried out as quickly as possible to prevent microreactions within the pack and/or hemolysis.

2| Count the cells in the blood sample, using 80 μl of blood (see **Box 1**).

TABLE 4 | Serum-free media for neural differentiation and maturation.

Differentiation medium (SF-NEURO/D 50 ml)	
DMEM/F12	45 ml
B27 (1:50)	1 ml
N2 (1:25)	2 ml
EGF (10 ng ml^{-1})	5 μl
BDNF (10 ng ml^{-1})	50 μl
RA (5 μM)	5 μl
Collagen IV (1 μg ml^{-1})	50 μl
Fibronectin (1 μg ml^{-1})	50 μl
Pen/Strep (1:100)	500 μl
Fungizone (1:100)	500 μl
Maturation medium (SF-NEURO/M 50 ml)	
DMEM/F12	46 ml
B27 (1:50)	1 ml
N2 (1:25)	2 ml
EGF (10 ng ml^{-1})	5 μl
NGF (10 ng ml^{-1})	25 μl
BDNF (10 ng ml^{-1})	50 μl
RA (5 μM)	5 μl
dBcAMP (100 μM)	50 μl
Collagen IV (1 μg ml^{-1})	50 μl
Fibronectin (1 μg ml^{-1})	50 μl
Pen/Strep (1:100)	500 μl
Fungizone (1:100)	500 μl

BDNF, brain-derived neurotrophic factor; dBcAMP, dibutyryl cyclic AMP; EGF, epidermal growth factor; NGF, nerve growth factor; RA, retinoic acid.

3| According to the cell count, calculate appropriate volume to collect 100,000 of cells for flow cytometry (FACS) immunostaining and analysis (see **Box 2**, and **Table 1**).

Isolation of umbilical cord blood mononuclear cells using Ficoll density gradient ● TIMING ~2 h

4| Dilute the blood from Step 1 (1:1) with sterile 1× PBS in a universal 50-ml tube. Recommended minimal volume is 20 ml.

5| Layer the diluted blood slowly on 10 ml of Ficoll layer (ratio 4:1) in another 50-ml tube.
▲ CRITICAL STEP Do not mix the tube; make sure the layers are not disturbed.

6| Centrifuge the layered blood samples at 400*g* for 30 min at room temperature (20–22 °C). Acceleration and de-acceleration should be kept as low as possible to avoid any disturbance to cells in the white layer (**Fig. 1**).

7| Collect the white layer using a plastic pipette in a new universal tube. Some cells might attach to the side of the tube in a form of a pellet, 'rub' them gently using the end of the pipette and collect them (**Fig. 1**).
! CAUTION Collecting too much of plasma might increase the chance of platelet contamination. Platelets can release significant levels of chemokines which (patient dependent) can change the stimulation pattern of the cells.

BOX 1 | CELL COUNTING

One part of the blood sample (60 μl) is used for counting the cells with automatic hematological cell counter Coulter A^C T diff2 (Beckton Coulter), where all blood cells are counted including mononuclear cells, red blood cells and platelets.
Second part of the sample (20 μl) is used for assessing the viability of the cells using Trypan Blue exclusion method and hemacyometer, as follows:
1. Place a suitable volume of a cell suspension (10–20 μl) in an appropriate tube and add an equal volume of 0.4% Trypan Blue and gently mix.
2. Allow to stand for 5 min at room temperature.
3. Place 10 μl of stained cells in a hemacytometer and count the number of viable (unstained) and dead cells stained blue.
4. Calculate the average number of unstained cells in each quadrant and multiply by 2×10^4 to find number of cells per ml. The percentage of viable cells is the number of viable cells divided by the number of dead and viable cells.

8| Centrifuge the cell suspension at 630*g* for 7 min at room temperature. Acceleration should be maximum with medium de-acceleration.

9| Resuspend the pellet in ~2 ml of 1× PBS.

10| Centrifuge the sample as described in Step 8.

11| Take as much supernatant as possible without disturbing the pellet.

12| Resuspend the pellet in 1 ml of 1× PBS.

13| Count the cells using hematological cell counter and hemacytometer and asses the viability using Trypan Blue exclusion method (as described in **Box 1**).

14| Check for abnormal levels of cell aggregation and possible platelet activation, which may disturb further isolation (**Fig. 2**).
▲ CRITICAL STEP Platelets, when activated, form a fibrin net where mononuclear cells are trapped. That can be visible as fibrils in supernatant or as aggregates under the microscope.
? TROUBLESHOOTING

15| According to the cell count, calculate the appropriate volume to collect 10^5 cells for later comparison and troubleshooting by flow cytometry (FACS) immunostaining and analysis (as described in **Box 2** and **Table 1**).

Depletion of lineage positive cells ● TIMING ~**4 h**

16| To the remaining cell suspension volume (between 900 and 1,000 μl working volume), add 500 μl of 2% human γ-globulins diluted in PBS or working buffer.
▲ CRITICAL STEP All operations must be performed in a laminar flow sterile hood unless stated otherwise. All reagents (Abs, working buffer, γ-globulins, Dynabeads) and operations (e.g., centrifuging) should be used cooled at 4 °C or on ice.

17| Incubate cells for 20 min at 4 °C or on ice to block nonspecific Fc receptors (during the incubation, gently rock or rotate the tube to achieve homogenous binding).
! CAUTION If automated rotator is not available, gently rotate tube manually every few minutes to avoid clumping and cellular adherence.

18| Add mouse monoclonal anti-human anti-GlyA (CD235a); use 0.22 μg of Ab per 10^6 cells, and mouse monoclonal anti-human anti-CD45; use 0.5 μg Ab per 10^6 cells. Incubate cells for 30 min at 4 °C or on ice with gentle rocking or rotating.

19| During the 30-min incubation, prepare the beads—monoclonal anti-mouse IgG Ab (PanMouse Dynabeads): vortex the beads before use and use 50 μl of beads per 10^7 cells.
! CAUTION Be sure you are using mouse anti-human primary Abs and beads coupled to them (panmouse Abs conjugated with beads).

BOX 2 | FLUORESCENCE-ACTIVATED CELL SORTING (FACS) ANALYSIS

Flow cytometric analysis of cells taken at various stages of this procedure is an optional step, but it can be helpful in estimating the number of undifferentiated stem cells and can provide further information about the purity of the isolated fraction.

To prepare cells for fluorescence-activated cell sorting (FACS) analysis, add an appropriate volume of Abs (see **Table 1**) into cell suspension (10^5 cells in 100 μl), mix them and incubate at room temperature in dark for 20 min. Wash the cells according to the manufacturer's recommendation or using BD wash/lyse assistant and perform the FACS analysis[18]. FACS analysis gives the indication of purity of CBE fraction (expected negative signal from CD45 and CD34 antibodies) and presence of embryonic-like cells (expected positive signal from SSEA-4 and CD133 antibodies) (see **Table 1**).

20| Wash the beads with the same volume or at least 1 ml of washing buffer and mix.
! CAUTION Avoid forming bubble/foam. Formation of bubbles/foam can disturb migration/connection of beads to the well of the tube and decrease final cell yield at the end of the procedure.

21| Apply the tube to a Dynal MPC for 2 min. Allow the beads to attach to the wall of the tube. Discard the supernatant.

22| Take the tube from magnet and repeat the washing Steps 19 and 20 two times (total number of washes is three).

23| After the 30-min incubation, wash excess of Ab from sample (from Step 18) using 10 ml working buffer.

24| Centrifuge the cell suspension at 630*g* for 7 min at 4 °C. The centrifuge should be chilled before use; acceleration should be maximum with medium de-acceleration.

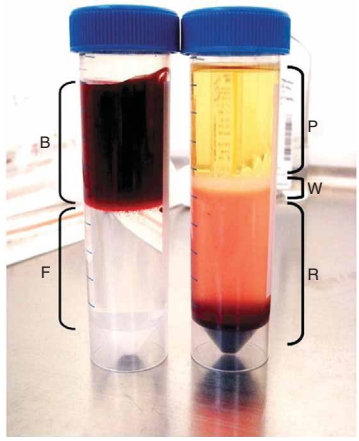

Figure 1 | Separation of cord blood with Ficoll before (left tube) and after (right tube) centrifugation. Left tube shows distinct layer of diluted cord blood (B) over the Ficoll layer (F). After centrifugation, there should be distinct layers as shown in right tube (from bottom to the top: red blood cells and Ficoll (R), mononuclear cells with stem cell population (white layer) (W), plasma with platelets (P)).

25| Discard supernatant and resuspend the cells using 500 µl working buffer per 10^8 cells.
! CAUTION Avoid forming bubble/foam. Formation of bubbles/foam can disturb migration/connection of beads to the well of the tube and decrease final cell yield at the end of the procedure.

26| Add washed beads (from Step 22) to the sample and incubate for 30 min at 4 °C or on ice with gentle rocking or rotating.

27| Apply tube to MPC for 2 min.

28| Collect the supernatant in a new sterile tube and mark as GlyA/CD45 negative cells.
▲ CRITICAL STEP Do not discard supernatant.

29| Rinse the positive fraction (beads remaining in the tube from Step 27) two times by adding 4 ml working buffer and reapplying the tube to MPC, and each time add the supernatant obtained to the tube containing GlyA/CD45 negative cells (from Step 28).
▲ CRITICAL STEP Reapply the tube to MPC at least three times to remove all positively marked cells completely.

30| Centrifuge the GlyA/CD45 negative cells as described in Step 24.

31| Remove supernatant and resuspend the cells using 500 µl working buffer.

32| Enumerate the cells and asses viability using Trypan Blue exclusion method (as described in **Box 1**).
▲ CRITICAL STEP Check the aggregation of cells and possible platelet activation, which may disturb further isolation (as described in Step 14).
? TROUBLESHOOTING

33| Incubate sample for 30 min at 4 °C or on ice simultaneously with mouse monoclonal anti-human CD45/CD33/CD7 Ab. Use 1.0 µg of Ab per 10^6 cells.

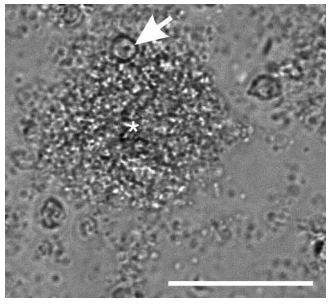

Figure 2 | Contamination of platelets in culture. The asterisk shows an aggregate of activated platelets, and arrow indicates clamped stem cell within. Scale bar, 10 µm.

34| During the incubation, prepare beads as described in Steps 19–22.

35| Wash excess of Ab from sample from Step 33 by adding 10 ml of working buffer.

36| Centrifuge the sample as described in Step 24.

37| Remove supernatant and resuspend the cells using 500 µl working buffer.
! CAUTION Avoid forming bubble/foam. Formation of bubbles/foam can disturb migration/connection of beads to the well of the tube and decrease final cell yield at the end of the procedure.

38| Apply washed beads (from Step 35) to the sample and incubate for 30 min at 4 °C or on ice with gentle rocking or rotating.

39| Apply tube to MPC for 2 min.

40| Collect the supernatant in a new sterile tube and mark as lineage-negative cells.
▲ CRITICAL STEP Do not discard supernatant.

41| Rinse the positive fraction (beads remaining in the tube from Step 37) two times by adding 4 ml working buffer and reapplying the tube to MPC, and each time add the supernatant obtained to the tube containing lineage-negative cells (from Step 40).
▲ CRITICAL STEP Reapply the tube to MPC at least three times to remove all positively marked cells completely.

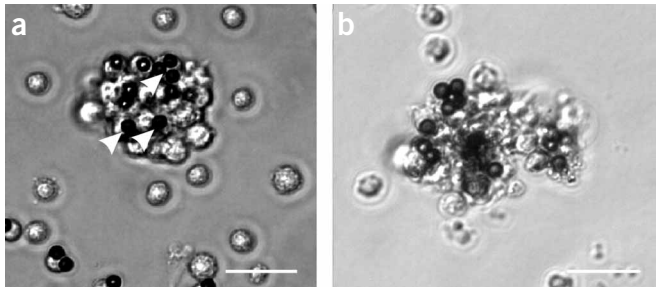

Figure 3 | CBEs contaminated with magnetic beads. (**a**) Magnetic beads (arrows) attached on stem cell aggregate of lineage-negative cells after isolation. (**b**) If those cells are cultured, the beads could affect cell viability—cells undergo apoptosis and die. Scale bar, 10 μm.

42| Take a small sample of cell suspension (10–25 μl), and under the microscope check for the presence of magnetic beads using a glass slide or hemacytometer (**Fig. 3a**). Magnetic beads will affect cell viability (**Fig. 3b**). If necessary, repeat Step 37 until no magnetic beads are observed under the microscope.
? TROUBLESHOOTING

43| Centrifuge the lineage-negative cells as described in Step 24.

44| Remove supernatant and resuspend the cells in 500 μl chilled harvesting culture medium SF-TPO (see **Table 3** and **Fig. 4**).
▲ CRITICAL STEP Avoid rapid changes in temperature—adjust the temperature of culture medium and introduce it by dropping to cell pellet.

45| Enumerate the cells and assess viability using Trypan Blue exclusion method, as described in **Box 1**.
▲ CRITICAL STEP After this step, a restricted cell population containing CBEs (2–3 μm in diameter) should be obtained. This cell population will require expansion.

46| Take 10^5 cells and perform the flow cytometry (FACS) immunostaining and analysis for characterization and potential troubleshooting (as described in **Box 2** and **Table 1**).

Culture and expansion of lineage-negative cells in serum-free media ● TIMING ~3–9 d

47| Take 60,000 cells and prepare cytospin slides. Cell number on each slide should be ~10,000 cells per slide. Cytospin slides are prepared using Shandon-thermo centrifuge (90*g* for 3 min). Slides should put at room temperature for 15 min to dry, and then store them at −20 °C for later immunocytochemistry analysis. Proceed with the rest of cells (**Fig. 4**) to next step.
▲ CRITICAL STEP Use no >10,000 cells per slide. Having too many cells on one slide would form a layer of cells, which would make the localization analysis of markers very difficult.

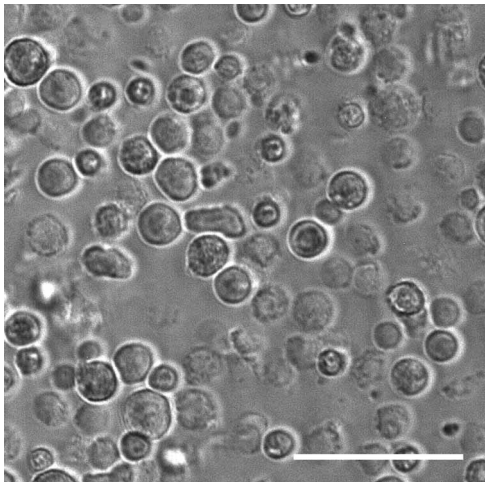

Figure 4 | Embryonic-like stem cells freshly isolated from human umbilical cord blood. Scale bar, 10 μm. Note the relative homogeneity of the population with good sphericity and high nucleus to cytoplasmic ratio, typical of immature stem cell populations.

48| Plate the cells at a concentration ranging from 5 to 10 million CBEs per ml of serum-free harvesting (SF-TPO) medium (see **Table 3**) and incubate at 37 °C at 5% CO_2 for 16–48 h (**Fig. 5a**).
▲ CRITICAL STEP Go to the next step only when CBEs form aggregates (**Fig. 5a**) and the culture is not contaminated with platelets (**Fig. 2**; refer to Step 14).
? TROUBLESHOOTING

49| Change half the medium to serum-free expansion medium (SF-EFS), and incubate the cells at 37 °C in 5% CO_2 for 24 h (see **Table 3**).

50| Again, change half the medium to SF-EFS medium, and incubate cells at 37 °C in 5% CO_2 for 1–7 d (**Fig. 5b**).

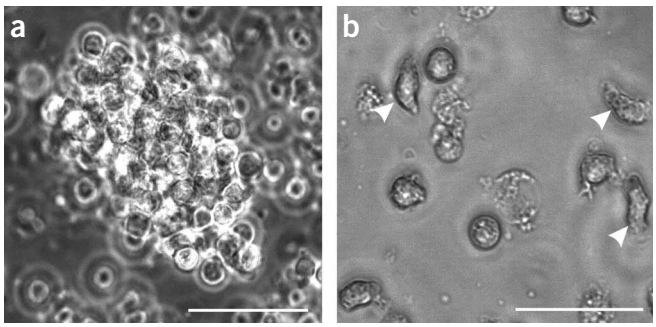

Figure 5 | Harvesting and expansion of CBEs in serum-free media. (**a**) Aggregates of embryonic-like stem cells following the expansion phase (CBEs), formed in SF-TPO medium 16–48 h after isolation from human umbilical cord blood. (**b**) Expected initial shape of the differentiated, collagen-adhered, neural progenitors (arrowheads) after 1–7 d in SF-EFS medium. Scale bars, 50 μm.

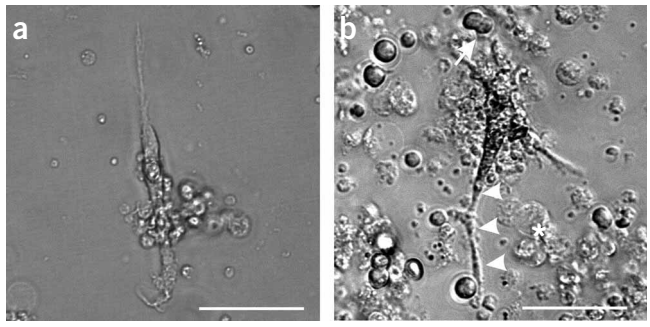

Figure 6 | Differentiation and maturation of CBEs in serum-free media. (**a**) Early stages of neural progenitors developing toward more mature neural cells appear within the first week of differentiation in SF-NEURO/D medium. (**b**) Mature neurons present after 1–2 weeks of maturation in SF-NEURO/M medium (neurite-like process—arrowheads). The maturation process accompanies high apoptosis, particularly of cells that are not able to make the next stage of differentiation (**b**, asterisk). However, the number of proliferating neuroblasts is still detectable though maturation period (**b**, arrow). Scale bars, 50 μm.

! CAUTION During this incubation, change half culture medium every other day. If many floating cells are observed upon microscopical evaluation of the cell culture flask (inverted microscope), centrifuge the old medium, resuspend the pellet in a fresh medium and place it back in culture well with fresh medium.

▲ CRITICAL STEP Within this step, at least some of free-floating single or aggregated CBEs should begin to adhere spontaneously and change their morphology (see **Fig. 5b**).

Neural differentiation of lineage-negative cells ● TIMING ~2–3 weeks

51| Put sterile glass slide inserts in the wells of a 24-well plate, one slide per well.

52| Coat the slides with collagen (1 mg ml^{-1} in 0.25% acetic acid) and incubate at 37 °C in 5% CO_2 for 3 h to dry the collagen (see REAGENT SETUP).

! CAUTION Washing the coated wells after the drying period with fresh medium might be required because collagen is initially too acidic and this can affect cell viability.

53| On the basis of the concentration in the cell suspension, plate half a million cells in each well and then add 500 μl of fresh neural differentiation medium (SF-NEURO/D) to each well (see **Table 4**).

54| Incubate at 37 °C at 5% CO_2. Change half the SF-NEURO/D medium every other day for 1 week (**Fig. 6a**).

▲ CRITICAL STEP Go to the next step only when CBEs are already attached and differentiated toward unipolar neuroblasts (see **Fig. 6a**).

? TROUBLESHOOTING

55| After 1 week, change half the medium to neural maturation media (SF-NEURO/M) in each well (see **Table 4**).

56| Change half the medium every third day for 1–2 weeks to obtain mature multiprocessed neural phenotypes (see **Fig. 6b**).

57| Do immunocytochemistry using Abs against adequate neural markers for differentiated cells and

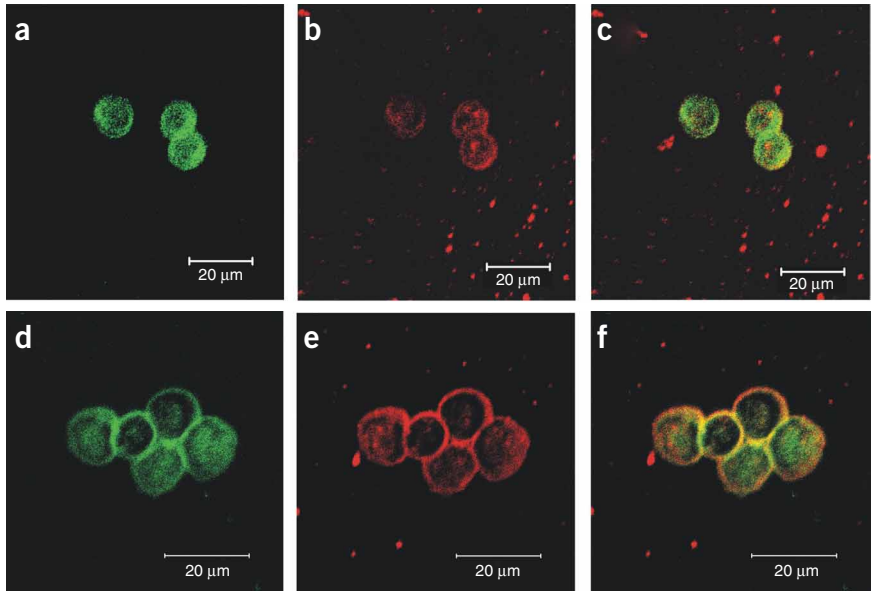

Figure 7 | Intracellular localization of markers of pluripotency. Fluorescent microscope staining confirmation of cord blood embryonic-like stem cells, expressing, as example in this case, Oct4 (**a**) and Sox2 (**b**); (**c**) is a composite overlay of (**a**) and (**b**) and Ki67 (**d**), with additional Nanog (**e**); (**f**) is a composite overlay of (**d**) and (**e**).

embryonic/pluripotency markers for the undifferentiated cells on cytospin slides (see **Table 2** for used and suggested markers) as reported previously[1,5] (**Fig. 7**).

▲ CRITICAL STEP At this stage, you should have obtained the neural cells. Although some of the cells may still be of the neural progenitor variety rather than terminally differentiated cells, this provides more flexibility in the final use and analysis of the cells.

● TIMING

The collection, processing and negative depletion procedures can be performed in ~6 h. The entire procedure from umbilical cord blood collection to obtaining cells with multiprocessed neural phenotypes can be performed in ~3–4 weeks.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 5**.

TABLE 5 | Troubleshooting table.

Steps	Problem	Reason/solution
14, 32	Contamination of cell suspension with platelets	Centrifuge the cells slowly (400*g*/10 min), discard the supernatant with platelets and collect the pellet. Repeat if necessary
40	Existence of magnetic beads with negative fraction cells	Reapply cells to magnetic particle concentrator to remove all magnetic beads along with positive cells
46	Total number of cells is less than one million	Use "U" shape 96-well plate to enhance the cells' survival and aggregate formation
52	Low level of cell adhesion to plate surface	Decrease the volume of media (minimum 300 μl) to enhance cell attachment to the plate surface

ANTICIPATED RESULTS

Our recent study indicated that umbilical cord blood contains various stem cells at different stages of their development[3]. Such cell fractions (e.g., CD133+; CBEs) might be isolated separately from one cord blood unit, expanded and committed toward different lineages: endothelial[1] and neural[14], respectively.

We found that CBEs could be differentiated very effectively toward neuronal phenotypes *in vitro*. Soon after isolation, CBEs are very small (diameter of 2–3 μm) and resemble embryonic[13] or very small embryonic-like[2] phenotypes reported previously by other research groups. CBEs express at this stage markers characteristic for pluripotent human embryonic stem cells (e.g., surface markers: SSEA-3/4 and transcription factors: Oct4A and Sox2). During the stage of *in vitro* expansion (in the presence of EGF and bFGF), some of the cells start to differentiate spontaneously; they lose expression of embryonic markers (mentioned earlier) and they increase in size (diameter up to 20 μm). These cells are in majority of neural progenitors expressing markers of proliferating neural stem cells (Nestin, GFAP and Ki67, respectively). During the expansion stage, most of the undifferentiated cells remain in the aggregates formed within a few hours after isolation. Therefore, we consider such aggregates as an artificial stem cell niche able to prevent the stem cells from spontaneous differentiation[14].

This protocol uses 2D culture conditions for the differentiation of CBEs into neural cells. Culture media, which promote neuronal differentiation, are supplemented with RA, BDNF and dBcAMP, and they are introduced sequentially. It is worth noting that proper sequential addition of neuromorphogenes is critical to the success of this protocol. We found, for example, that introducing cAMP and BDNF too early (when the cells

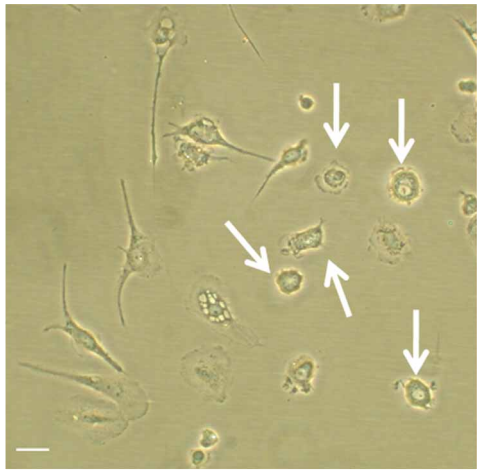

Figure 8 | Example of later stage neural phenotype from culture. The proportion of ovoid cells (highlighted by white arrows) reduces from previous cultures and epithelioid cells start to proliferate, acquiring a more defined neuroglial morphology with longer membrane projections and neurite-like extensions. Adherent cells which are more spherical can also at this stage spontaneously cluster into compact neurospheres. Scale bar, 10 μm.

do not express Nestin yet) may trigger apoptosis. Development of neurons *in vitro* from CBEs reflects the morphological changes of the neural stem cells observed *in vivo*. Round-shaped neural stem cells (Nestin+) (**Fig. 5**) give rise to bipolar proliferating neuroblasts (**Fig. 6a**), and subsequently they generate mature neuronal phenotypes (**Figs. 6b** and **8**) with one main, axon-like protrusion and many dendritic-like small processes. These stages may be additionally characterized by expression of specific markers (see **Table 2**).

We found that cord blood-derived lineage-negative stem cells differentiate significantly faster when they grow in 3D bioscaffolds. Such cells can be further co-cultured on biodegradable scaffolds to produce artificial neural tissue supported with microvessels[1]. We found that the optimal time for implantation of the cells on 3D scaffolds is the end of the expansion phase (stage 2). At this stage, CBEs are still maintained in culture as floating aggregates which are easy to transplant, and most of the cells are already neural stem cells. CBEs accompanying other stem cell types present in cord blood are very useful in tissue engineering studies where more then one cell type is desired. Stem cells can potentially be obtained from only one cord blood unit for future autologous transplantation. Developed in our laboratory, this technique of separation of pluripotent and more committed stem cells from a single cord blood unit will allow in future the creation of artificial brain tissue constructs for regenerative medicine.

ACKNOWLEDGMENTS Our work and the development of these protocols have been supported by: (i) the National Health Service Obstetrics and Gynaecology Directorate, Newcastle Hospitals Trust; (ii) Fondation Jerome LeJeune, Paris; (iii) Novus Sanguis adult stem cells charity, Paris; (iv) The Kuwaiti Government sponsorship of H.A.; and (v) The Blood Transfusion Service of Llubjana, Slovenia sponsorship of M.S.

Published online at http://www.natureprotocols.com
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions

1. McGuckin, C.P. *et al.* Production of stem cells with embryonic characteristics from human umbilical cord blood. *Cell Prolif.* **38**, 245–255 (2005).
2. Kucia, M. *et al.* Morphological and molecular characterization of novel population of CXCR4+ SSEA-4+ Oct-4+ very small embryonic-like cells purified from human cord blood: preliminary report. *Leukemia* **21**, 297–303 (2007).
3. McGuckin, C.P. & Forraz, N. Potential for access to embryonic-like cells from human umbilical cord blood. *Cell Prolif.* **41** (suppl. 1): 31–40 (2008).
4. Ende, M. & Ende, N. Hematopoietic transplantation by means of fetal (cord) blood. A new method. *Va. Med. Mon.* **99**, 276–280 (1972).
5. Buzańska, L., Machaj, E.K., Zabłocka, B., Pojda, Z. & Domańska-Janik, K. Human cord blood-derived cells attain neuronal and glial features *in vitro*. *J. Cell Sci.* **115**, 2131–2138 (2002).
6. Jurga, M. *et al.* Neurogenic potential of human umbilical cord blood: neural-like stem cells depend on previous long-term culture conditions. *J. Neurosci. Res.* **83**, 627–637 (2006).
7. Chen, N. *et al.* Trophic factor induction of human umbilical cord blood cells *in vitro* and *in vivo*. *J. Neural. Eng.* **4**, 130–145 (2007).
8. Walczak, P. *et al.* Long-term cultured human umbilical cord neural-like cells transplanted into the striatum of NOD SCID mice. *Brain Res. Bull.* **74**, 155–163 (2007).
9. Kang, K.S. *et al.* A 37-year-old spinal cord-injured female patient, transplanted of multipotent stem cells from human UC blood, with improved sensory perception and mobility, both functionally and morphologically: a case study. *Cytotherapy* **7**, 368–373 (2005).
10. Willing, A.E. *et al.* Intravenous versus intrastriatal cord blood administration in a rodent model of stroke. *J. Neurosci. Res.* **73**, 296–307 (2003).
11. McGuckin, C. *et al.* Embryonic-like stem cells from umbilical cord blood and potential for neural modeling. *Acta Neurobiol. Exp. (Wars)* **66**, 321–329 (2006).
12. Sanberg, P.R. *et al.* Umbilical cord blood-derived stem cells and brain repair. *Ann. NY Acad. Sci.* **1049**, 67–83 (2005).
13. Pan, G. & Thomson, J.A. Nanog and transcriptional networks in embryonic stem cell pluripotency. *Cell Res.* **17**, 42–49 (2007).
14. Habich, A. *et al.* Early appearance of stem/progenitor cells with neural-like characteristics in human cord blood mononuclear fraction cultured *in vitro*. *Exp. Hematol.* **34**, 914–925 (2006).
15. McGuckin, C.P., Forraz, N., Pettengell, R. & Thompson, A. Thrombopoietin, flt3-ligand and c-kit-ligand modulate HOX gene expression in expanding cord blood CD133 cells. *Cell Prolif.* **37**, 295–306 (2004).
16. Imreh, M.P. *et al. In vitro* culture conditions favoring selection of chromosomal abnormalities in human ES cells. *J. Cell. Biochem.* **99**, 508–516 (2006).
17. Buzańska, L., Jurga, M., Stachowiak, E.K., Stachowiak, M.K. & Domańska-Janik, K. Neural stem-like cell line derived from a nonhematopoietic population of human umbilical cord blood. *Stem Cells Dev.* **15**, 391–406 (2006).
18. McGuckin, C.P., Basford, C., Hanger, K., Habibollah, S. & Forraz, N. Cord blood revelations: the importance of being a first born girl, big, on time and to a young mother. *Early Hum. Dev.* **83**, 733–741 (2007).
19. Jurga, M., Buzanska, L., Małecki, M., Habich, A. & Domańska-Janik, K. Function of ID1 protein in human cord blood-derived neural stem-like cells. *J. Neurosci. Res.* **84**, 993–1002 (2006).