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Directed differentiation of umbilical cord blood stem cells into cortical GABAergic neurons

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Umbilical cord blood contains a population of non-hematopoietic multipotent stem cells that are capable of neuronal differentiation *in-vitro*. These cells have shown great potential as a therapeutic tool for central nervous system diseases and disorders. However whether these cells are able to produce neurons with similar developmental and functional characteristics to indigenous neurons within the brain remains poorly investigated. In this study, we used purified umbilical cord blood non-hematopoietic stem cells to produced GABAergic neurons with similar developmental and functional characteristics to cortical GABAergic neurons. We analyzed the expression of transcription factors *MASH1*, *DLX1* and DLX2 throughout the 24 days of a sequential neuronal induction protocol and found that their expression patterns resembled those reported in the developing human cortex. The derived neurons also expressed components of GABAergic neurotransmission including GABA regulatory enzymes, GABA receptor subunits and GABA transporters. Thus we have demonstrated that umbilical cord blood stem cells are capable of producing cortical-like GABAergic neurons *in vitro*. This highlights the potential of umbilical cord blood stem cells as a therapeutic tool for neural injuries and disorders.

Key words: stem cell, umbilical cord blood, cortex, brain, GABA, therapy

INTRODUCTION

The human cortex is composed of two main types of neurons; Pyramidal neurons which are glutamatergic excitatory neurons representing approximately 80% of the cortical neuronal content and interneurons which are GABAergic inhibitory neurons that account for about 20% of the total neuronal cortical content. The excitatory and inhibitory systems, represented by both types of cortical neurons, function in a collaborative and coordinated manner regulating neuronal firing patterns, network activity and synaptic plasticity which contribute to the diverse and complex CNS functions (Foster and Kemp 2006). We have previously shown that directed neuronal differentiation of umbilical cord blood non-hematopoietic stem cells recapitulated key events taking place in human neocortex regulating cortical neurogenesis of glutamatergic neurons (Ali et al. 2012). The ability to additionally model GABAergic neurogenesis would provide a more accurate and relevant model of human cortical neurogenesis.

GABAergic neurons in human central nervous system are derived from two regions; progenitors in the cortex itself which account for around 65% of all the GABAergic neuronal content, and from progenitors in the ganglionic eminences which account for the remaining 35% (Meyer et al. 2000, Letinic et al. 2002, Jones 2009). Expression patterns of certain transcription factors have been shown to control the GABAergic fate specification in human CNS (Letinic et al. 2002). DLX1 and DLX2, which are members of the DLX homeobox family of transcription factors, have been shown to specify the neuronal GABAergic neuronal fate in the human forebrain and repress neuronal glutamatergic and glial oligodendrocytic fates (Letinic et

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al. 2002, Petryniak et al. 2007). Pro-neural b-HLH transcription factors are also involved in regulation of GABAergic differentiation in the forebrain (Poitras et al. 2007). It has been shown that MASH1 expression in progenitor cells of the ventricular (VZ) and sub-ventricular zones (SVZ) promotes a GABAergic fate (Campbell 2005, Mattar et al. 2008, Roybon et al. 2010, Schuurmans et al. 2004). The restricted expression patterns of these transcription factors in human CNS highlights their important regulatory role in regulating GABAergic neurogenesis.

Although advances in understanding neurogenesis have been achieved, the exact mechanisms determining neuronal cortical subtyping are poorly understood in the human CNS as a result of the histological and cellular complexity of the neocortex (Gaspard et al. 2009). Many animal models have been used in attempts to understand these complex molecular mechanisms, nevertheless major molecular, anatomical and physiological differences between animal models and human CNS represent a major limitation for those models and highlight the need of *in vitro* models of human corticogensis (Bystron et al. 2008, Clowry et al. 2010). Such *in vitro* models need to be able to recapitulate main molecular events that govern the specification of neuronal subtypes in the human cortex to allow a more detailed analysis of such complex processes.

It has been shown previously that umbilical cord blood contains a distinct population of non-hematopoietic stem cells, reviewed in (Ali and Al-Mulla 2012), that are capable of neuronal differentiation *in vitro* (Domanska-Janik et al. 2006, Ali et al. 2009, Ali and Bahbahani 2010, Janowski et al. 2011). We previously established an *in-vitro* model of human neocortical neurogenesis of glutamatergic neurons by sequential induction protocol of umbilical cord blood non-hematopoietic stem cells (Ali et al. 2012). Being able to model the GABAergic fate commitment using a similar *in vitro* system would provide a more accurate and complete model of human cortical neurogenesis.

As stem cells are being proposed as a potential therapeutic tool for neurological injuries and disorders (Gornicka-Pawlak et al. 2011), it is important to understand the molecular mechanisms that regulate the differentiation of cortical endogenous neurons and see if stem cells can produce neurons with similar characteristics to the ones targeted for therapy.

In this study, we show that purified umbilical cord blood non-hematopoietic multipotent stem cells can differentiate into GABAergic neurons with similar functional and developmental characteristics to endogenous cortical GABAergic neurons. The differentiating cells express certain transcription factors known to be crucially involved in the developmental regulation of GABAergic neurons in human cortex. In addition, the derived neurons expressed proteins associated with GABA synthesis, GABAergic neurotransmission and action potential propagation. Such stem cell *in vitro* system can provide a tool to further analyze the developmental mechanisms regulating the subtyping of human GABAergic cortical neurons which would allow better understanding of the complex molecular regulating systems. This could also aid in developing better stem cells therapeutic interventions for neural injuries and disorders.

METHODS

Umbilical cord blood collection

Umbilical cord blood was collected in the caesarean sections delivery suite of the Maternity Hospital. A negative viral profile and infection status was required. Informed consent from parents was obtained prior to delivery. All the ethical requirements for collection of samples and experimental procedures required by Hospital, University and health authorities have been fulfilled.

Cord blood processing and stem cells purification

Mononuclear cells were separated from whole umbilical cord blood using Ficoll-Paque PREMIUM [GE healthcare] on the same day as cord blood collection. Cord blood diluted with PBS was layered over the Ficoll and centrifuged at 400 g for 30 minutes at room temperature with minimum acceleration and no deceleration to isolate the mononuclear cells fraction (Ali et al. 2009). Targeted stem cells were purified from mononuclear cells fraction using StemSep® Primitive hematopoietic Progenitor cell enrichment kit (Stemcell technologies) following the manufacturer's protocol (Ali et al. 2012).

FACS analysis

The analysis was performed on 100 000 cells of cord blood, mononuclear fraction and purified stem cells.

BD LSRII machine was used and two markers were analyzed SSEA-4 (R & D systems, FAB1435P) and CD45 (BD Pharmingen, 557833). A minimum of 3 trials were performed per each test. Results were analyzed using BD FACSDiva™ 6.0 software.

Culturing and differentiation protocol

Purified cells were cultured initially at a concentration of 1 million cells per ml. Cells were differentiated using our previously published 3-steps neuronal induction (Ali et al. 2012). In the first step, cells were cultured in early commitment media containing 20 ng/ml EGF (Immunotools) and 20 ng/ml bFGF (Immunotools) for 10 days (referred to in text as Day 10). Then cells were switched to differentiation media containing 10 ng/ml EGF (Immunotools), 10 ng/ml brain derived neurotrophic factor (Immunotools) and 1 µM Retinoic acid (Sigma Aldrich) for 7 days (referred to in text as Day 17). Finally cells were transferred into maturation media containing 10 ng/ml EGF (Immunotools), 5 ng/ml Brain Derived Neurotrophic Factor (Immunotools), 10 ng/ml Nerve growth factor (Immunotools) and 1 µM Di-buthyryl cyclic AMP (Sigma Aldrich) for 7 days (referred to in text as Day 24).

Immunocytochemistry

Cells were fixed with Accustain for 30 minutes. Blocking of nonspecific binding was achieved with 8% fetal calf serum, 8% goat serum and 0.1% tritonX100 in PBS for 60 minutes. Primary antibodies were added after being properly diluted in PBS and incubated overnight. Antibodies used including VGAT (Synaptic biosystems, 131011), GAD1 (Synaptic biosystems, 198011) and β-Tubulin (Abcam, Ab18207). Cells were incubated with appropriate secondary antibody, Alexa 488 (Invitrogen A21121) and Alexa 594 (Invitrogen A11012) for 30 minutes. Cover slips were mounted and cells were visualized using Axio Imager by Zeiss.

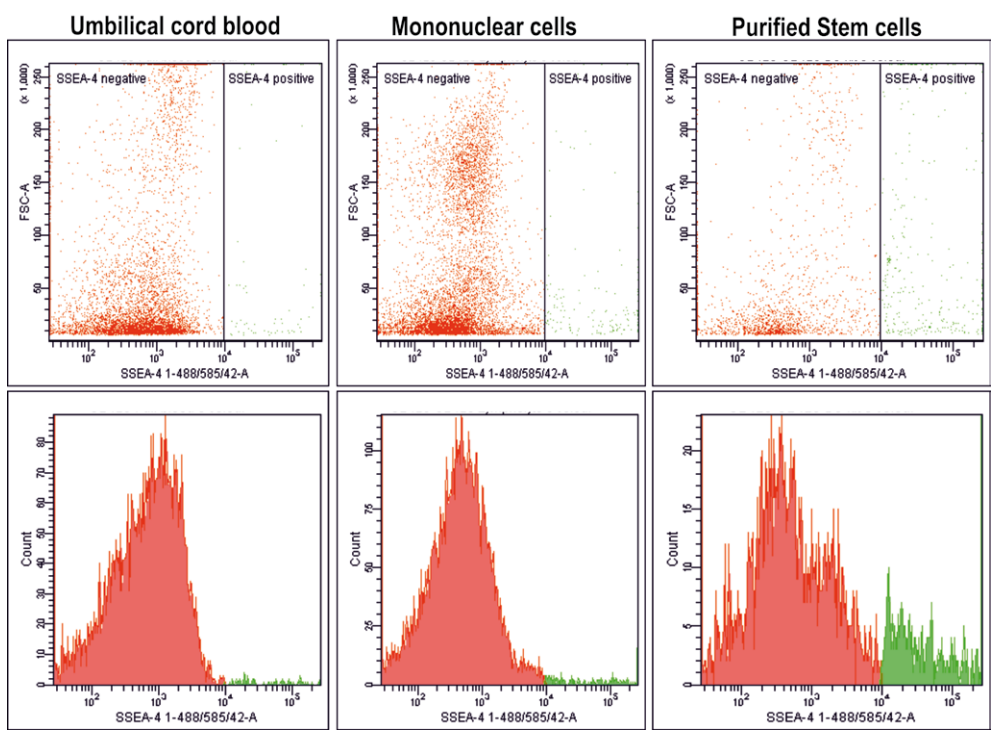

Fig. 1. Purification of non-hematopoietic umbilical cord blood stem cells. FACS analysis of SSEA-4 expression reveals that cells expressing the marker were enriched after the purification process. The upper panel shows the analysis of SSEA-4 expression (X-axis) against the forward scattered beam (Y-axis) in unprocessed samples (Cord blood), after Ficoll treatment (Mononuclear Cells) and after negative depletion (purified stem cells). The lower panel shows a significant increase in signal of SSEA-4 positive cells in the "purified stem cells" sample which reflects the relative number of cells in comparison with samples before the depletion step.

Total RNA isolation and reverse transcription

Total RNA was isolated from the samples using Qiagen RNeasy Plus mini kit following the manufacturer's protocol. For optimal RNA purification DNase digestion step was added. cDNA was produced using script VILO kit (Invitrogen) following manufacturer's protocol.

Quantitative polymerase chain reaction (PCR)

For Q-PCR reactions, we used Profiler™ PCR arrays purchased from Sabiosciences [PAHS-060E and PAHS-036E] in addition to conventional reactions. Primers sets were obtained from the PrimerBank website (http://pga.mgh.harvard.edu/primerbank) (Wang and Seed 2003, Spandidos et al. 2008) and purchased from Sigma, *GAPDH* 5-TGTTGCCATCAATGACCCCTT-3 and 5-CTCCACGACGTACTCAGCG-3, *DLX1* 5-CCATGCCAGAAAGTCTCAACA-3 and 5-GGCCCAAACTCCATAAACACC-3, *DLX2* 5-GCCTCAACAACGTCCCTTACT-3 and 5-TCACTATCCGAATTTCAGGCTCA-3, *OLIG1* 5-AAAAGACCAGTTAGGCGGTG-3 and 5-AAGAGCGAAAACTCTCTGCG-3, *S100B* 5-CCACCAATATTCTGGAAGGG-3 and 5-GTGGCAGGCAGTAGTAACCA-3, *DLG4* 5-GGACCAGATCCTGTCGGTCA-3 and 5-CCTCGAATCGGCTGTACTCTT-3, *MASH1* 5-CGCGGCCAACAAGAAGATG-3 and 5-CGACGAGTAGGATGAGACCG-3. Q-PCR reactions were carried out using RT2 SYBR® Green/ROX™ qPCR master mix (Sabiosciences, PA-012-8) and ABI7900HT machine according to a two steps cycling program published previously (Ali et al. 2012). PCR results for each gene were normalized to *GAPDH*. A minimum of triplicates for each reaction has been performed.

Statistical analysis

For two groups of data, the student's paired *t*-test was used to obtain probability (*P*) values. For three or more groups of univariate data, single-factor analysis

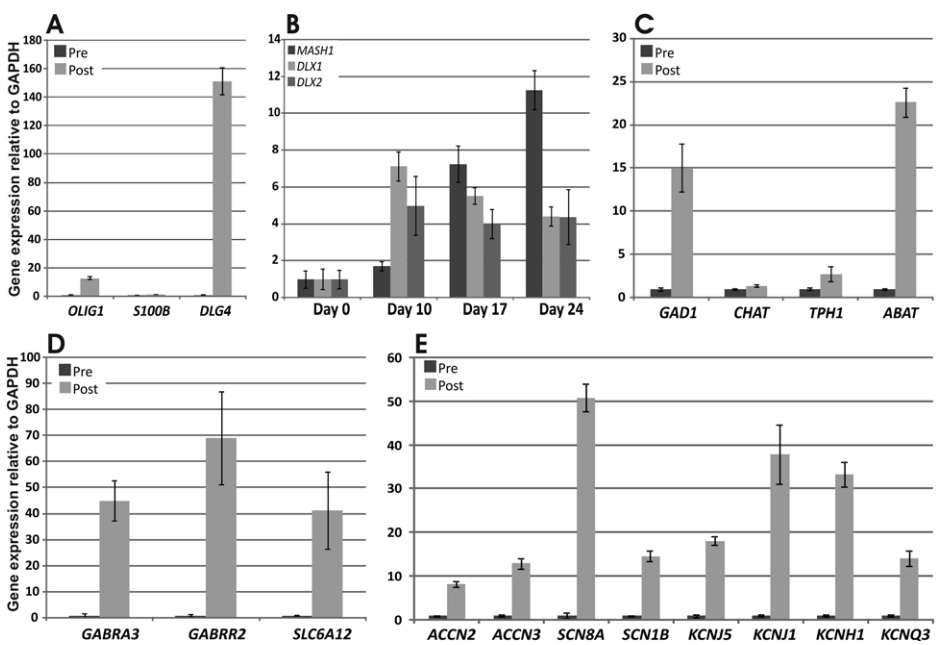

Fig. 2. Q-PCR expression analysis reveals an induction profile of GABAergic-specific markers during the differentiation process. (A) Analysis of neuronal (*DLG4)*, astrocytic (*S100B*) and oligodendrocytic (*OLIG1*) markers between undifferentiated cells (Pre or Day 0) and cells after neuronal maturation (post or Day 24). Expression profiles of (B) transcription factors *MASH1*, *DLX1* and *DLX2* in samples isolated from undifferentiated cells (day 0), cells after neuronal commitment (day10), cells after neuronal induction (day 17) and cells after neuronal maturation (day 24), (C) genes encoding enzymes involved in neurotransmitter synthesis and regulation, (D) genes encoding GABA receptors and one transporter and (E) genes encoding subunits of ion channels involved in action potential generation and propagation.

of variation (ANOVA) was used to obtain *P* values. Results with *P* values of less than 0.05 were considered statistically significant.

RESULTS

Effective enrichment of non-hematopoietic umbilical cord blood stem cells

FACS analysis of SSEA-4 expression revealed that cells expressing SSEA-4 were enriched by 7.62 fold (SD ± 1.43, n=6, P<0.005) after negative depletion as compared to unprocessed cord blood (Fig.1). The cells expressing SSEA-4 were negative for the major hematopoietic marker CD45 (Appendix Figure 1).

Efficient neuronal fate commitment

Quantitative PCR (Q-PCR) analysis showed highly significant increase in expression of the neuronal gene *DLG4* between undifferentiated cells (Pre) and cells after neuronal differentiation (Post) (n=6, P<0.005) while expression of oligodendrocytic marker *OLIG1* was less significant in comparison to *DLG4* (n=6, P<0.05) and for the astrocytic marker *S100B*, there were no significant increase in expression levels between samples before and after differentiation (Fig. 2A).

Up regulation of key cortical GABAergic transcription factors in differentiating cells

Q-PCR results showed that the expression of transcription factors *MASH1*, *DLX1* and *DLX2* increased significantly throughout the differentiation process of cord blood non-hematopoietic stem cells *in vitro*. *MASH1* expression increased gradually from (Day 0) reaching its highest level after neuronal maturation (Day 24) (n=6, P<0.005) (Fig. 2B). *DLX1* and *DLX2* expression showed similar pattern to each other as they increased significantly after neuronal commitment (Day 10) and stayed roughly at the same level after neuronal differentiation and maturation (Day 17 and Day 24 respectively) (n=6, P<0.005, for *DLX1* and *DLX2*) (Fig. 2B).

Differentiated cells expressed specific GABAergic markers

Q-PCR results showed the expression of genes encoding enzymes involved in the biosynthesis and

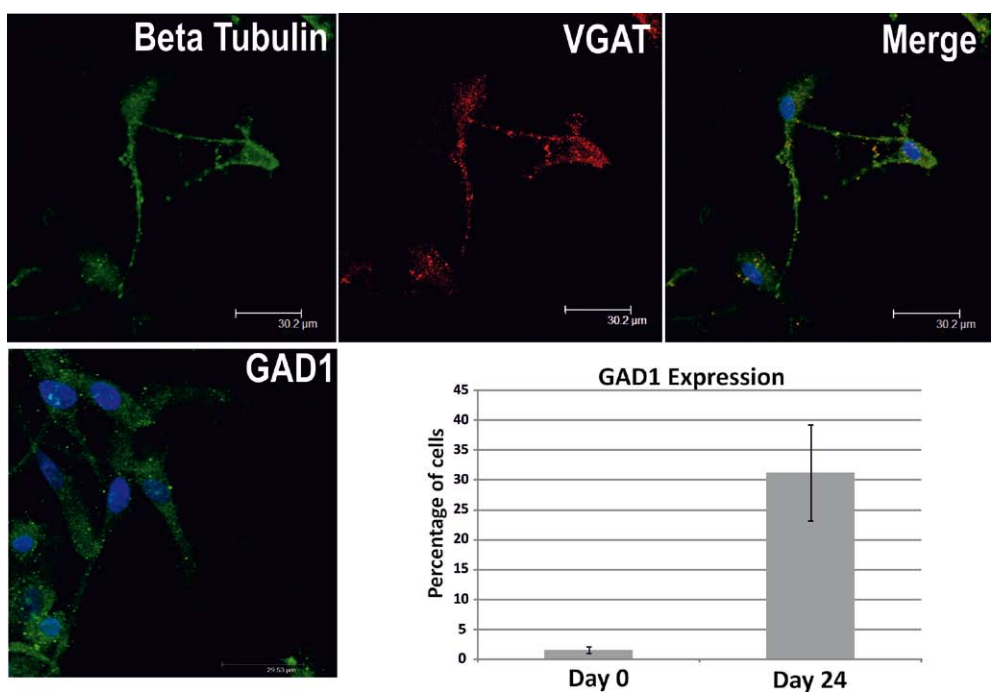

Fig. 3. Immunocytochemistry analysis of GABAergic markers in cells post differeniation (Day 24). Upper panel shows that the cells expressed β-Tubulin, a neuronal structural protein, and the GABA trasporter VGAT. Lower panel shows the porportion of cells expressing GAD1 post-neuronal differentation (Day 24) in comparison with pre-differentation (Day 0).

regulation of GABA increased significantly after the differentiation process (Post) including *GAD1* and *ABAT* (n=6, P<0.005, for both genes) (Fig. 3C). However no similar statistically significant changes were observed in expression levels of *CHAT* and *TPH1*, which encode enzymes involved in the synthesis of Acetylcholine and Serotonin respectively (Fig. 3C).

Q-PCR results also showed similar results for genes encoding subunits of GABA receptors (*GABRA3* and *GABRR2*) and the GABA transporter (*SLC6A12*). In these three cases expression levels increased significantly after the differentiation protocol (Post) (n=6, P<0.005, for all genes) (Fig. 2D).

Immunocytochemistry results showed that the cells after neuronal maturation (Day 24 or Post) adapted a neuronal morphology and expressed neuronal specific protein β-Tubulin (Fig. 3). The cells also expressed the Vesicular GABA Transporter (VGAT) and it appeared as punctuated staining concentrated in the cell's terminals (Fig. 3). Only 1.65% ± 0.34 expressed GAD1 before differentiation (Day 0) while 31.2% ± 8.03 (n=3, P<0.005) of cells expressed GAD1 post differentiation (Day 24) (Fig. 3).

Elevated expression of genes involved in action potential propagation after differentiation

Q-PCR analysis of umbilical cord blood stem cells between (Day 0 or Pre) and (Day 24 or Post) showed a significant increase in ion channel genes expression levels (Fig. 2E). Cells at day 24 also showed significant increase in expression levels of sodium channels genes including amiloride-sensitive channels *ACCN2* and *ACCN3* (P<0.005, n=3, for both genes), and voltage-gated channels *SCN1B* and *SCN8A* (P<0.005, n=3, for both genes) (Fig. 2E).

Q-PCR also showed significant increase in expression levels of potassium channels genes included inward rectifier channels *KCNJ1* and *KCNJ5* (P<0.005, n=3, for both genes) and voltage-gated channels *KCNH1* and *KCNQ3* (P<0.005, n=3, for both genes) (Fig. 2E).

DISCUSSION

In this study we showed that neuronal differentiation of umbilical cord blood non-hematopoietic stem cells *in vitro* produced GABAergic neurons with similar developmental and potential functional characteristics to human indigenous cortical GABAergic neurons.

Effective purification of the umbilical cord blood non-hematopoietic stem cells

To purify the targeted stem cell population we used a negative depletion strategy to select out the hematopoietic cells in order to purify the non- hematopoietic stem cells (Ali et al. 2012). As a marker of the targeted population, we used SSEA-4, which has been widely used to characterize and select embryonic stem cells (Thomson et al. 1998, Mitalipova et al. 2003, Carpenter et al. 2004) and that is also expressed by umbilical cord blood non-hematopoietic multipotent stem cells (Kucia et al. 2007, Ali et al. 2012). Cells expressing SSEA-4 increased by 7.62 folds (SD ± 1.43, n=6, P<0.005) (Fig. 1) after the purification step which suggest successful enrichment and purification of the targeted population. The non-hematopoietic identity of the population was confirmed by the negative expression of the major hematopoietic marker CD45 (Appendix Figure 1).

The cortical GABAergic neuronal identity

Our neuronal *in vitro* differentiation system of umbilical cord blood non-hematopoietic stem cells showed that the differentiating cells recapitulated major molecular events regulating the GABAergic cortical differentiation. *MASH1*, a member of the basic helix-loop-helix (bHLH) family of transcription factors, plays a critical role in GABAergic fate specification in the cortex (Parras et al. 2002, Jo et al. 2007, Oishi et al. 2009). It is expressed by a distinct population of cortical progenitors cells in ventricle zone (VZ) and sub ventricle zone (SVZ) as well as post-mitotic differentiated GABAergic neurons arising from progenitors within the cortex but not in post-mitotic GABAergic neurons arising from the ganglionic eminences (Letinic et al. 2002, Parras et al. 2004). *DLX1* and *DLX2* are members of DLX family of homeodomain transcription factors. They are necessary for differentiation of progenitor cells in the cortex into GABAergic neurons (Panganiban and Rubenstein 2002). They also specify the neuronal fate and repress the oligodendroglial fate in the forebrain (Petryniak et al. 2007). Letinic and coauthors (2002) showed that the majority (65%) of GABAergic cortical neurons in the human cortex express *MASH1*, *DLX1* and *DLX2*.

In our *in vitro* system, freshly purified stem cells (Day 0 or Pre) showed the lowest expression of *MASH1*, *DLX1* and *DLX2* (Fig. 2B) and this represent the undif-

ferentiated and multipotent status of the stem cells which we showed previously to express markers normally expressed by undifferentiated cells including *OCT4*, *SOX2* and *NANOG* (Habich, et al. 2006, McGuckin et al. 2008, Ali et al. 2009, 2012,). Following the induction with bFGF and EGF (Day 10) the expression levels of *MASH*1, *DLX1* and *DLX2* were elevated which correspond to the GABAergic cortical progenitors. After neuronal differentiation and maturation (Day 17 and 24), the expression of *MASH1* elevated significantly and *DLX1* and *DLX2* expression levels stayed within the same range. Cells at this stage adapted clear neuronal morphology and expressed specific neuronal markers including *DLG4* and β-Tubulin (Fig. 2A, 3).

Our differentiation protocol demonstrated GABAergic specificity as the differentiated cells expressed *GAD1* and *ABAT* (Fig. 2C, 3), the enzymes responsible for GABA synthesis and regulation respectively. On the other hand the differentiated cells did not express either *TPH1* or *CHAT* which are the enzymes involved in the synthesis of neurotransmitters serotonin and acetylcholine respectively (Fig. 2C). Moreover, the differentiated cells expressed components responsible for GABAergic neurotransmission including GABA receptor subunits and transporters (Fig. 2D, 3) which supports their GABAergic neuronal functional identity. In addition, the cells also expressed subunits of ion channels involved in neuronal action potential generation and propagation (Fig. 2E) which may suggest active neuronal functional status.

In vitro model of cortical neurogenesis: Completing the model

We have shown previously that the sequential induction of umbilical cord blood purified stem cells recapitulated key events taking place in human neocortex regulating cortical neurogenesis of glutamatergic neurons (Bayatti et al. 2008, Ali et al. 2012). And here we have shown that umbilical cord blood stem cells are also capable of modeling cortical neurogenesis of GABAergic neurons. The elevated expression of *MASH1* which reached its highest level in the cells after neuronal maturation (Day 24) shows that our stem cell population is even more relevant to a model of cortical development than to a model of GABAergic neurogenesis in the ganglionic eminences. As showed by Letinic and colleagues (2002), *MASH1* is only expressed in post-mitotic GABAergic neurons arising from progenitors in the cortex and not ganglionic eminences.

We have shown here that 31.2 ± 8.03% of the cells after neuronal maturation (Day 24) expressed GAD1, an enzyme that defines GABAergic neurons. In our previous paper, we found that around 60% of the cells after differentiation expressed TBR1, a marker of post-mitotic glutamatergic neurons (Ali et al. 2012). The co-existence of both types of the cortical neurons in the same culture which was exposed to the same culturing conditions over the 24 days duration suggests that the neuronal fate specification (Glutamatergic *versus* GABAergic) was determined by molecular mechanisms independent from culturing conditions.

The ability of umbilical cord blood stem cells to model cortical neurogenensis provide us with a very promising tool to study the molecular developmental mechanisms regulating human corticogenesis. Improving our understanding of cortical development will also help in the development of therapeutic approaches aimed toward treating injuries and disorders affecting the human cortex in particular and CNS in general.

Umbilical cord blood stem cells are being used in a number of clinical trials targeting the development of cell based therapies for neural injuries and disorders including Traumatic Brain Injury, Cerebral Palsy and Spinal cord injuries, reviewed in (Ali and Al-Mulla 2012). However, the ability of transplanted cells to produced neurons with similar developmental characteristics remains poorly investigated. Our findings provide a proof of concept the umbilical cord blood stem cells can actually produce neurons with similar developmental and potentially functional characteristics to cortical indigenous neurons. However further studies need to be directed towards the functional integration of cord blood derived neurons into the lesion sites which important to restore the function lost for injury or disease.

CONCLUSIONS

Umbilical cord blood can be viewed as a promising source of stem cells for research and clinical applications. We showed that these cells can produce GABAergic neurons *in-vitro* with similar developmental and potentially functional characteristics to endogenous cortical neurons. Pre-clinical trials using umbilical cord blood on animal models of neural injuries and disorders showed promising results. Clinical trials are still at its

early stages but results obtained so far demonstrated high potential and hope toward developing effective therapies for various CNS injuries and disorders.

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APPENDIX

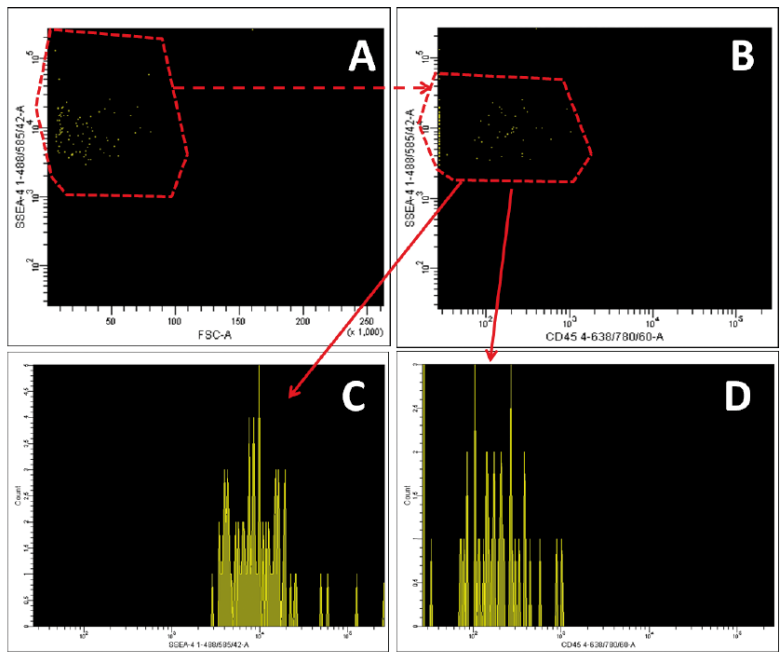

Fig. 1. FACS analysis on SSEA-4 positive cells (gated) showed that this subset of cells is negative for CD45. SSEA-4 positive cells were gated and then analyzed. Forward scattered (FSC-A) analysis showed that SSEA-4 positive cells are relatively small homogenous population of cells (A). The same population was negative for major hematopoietic marker CD45 (B) which is also demonstrated by histograms (C and D) where (C) indicates positivity for SSEA-4 and (D) negativity for CD45.