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Embryonic-like stem cells from umbilical cord blood and potential for neural modeling

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Review

Abstract. Stem cells offer the distinct prospect of changing the face of human medicine. However, although they have potential to form different tissues, are still in the early stages of development as therapeutic interventions. The three most used stem cell sources are umbilical cord blood, bone marrow and human embryos. Whilst, cord blood is now used to treat over 70 disorders, at the time of writing this manuscript, not a single disease has been overcome or ameliorated using human embryonic stem cells. Advancing stem cell medicine requires ethically sound and scientifically robust models to develop tomorrow's medicines. Media attention, however, distracts from this reality; it is important to remember that stem cells are a new visitor to the medical world and require more research. Here we describe the utility of human cord blood to develop neural models that are necessary to take stem cells to the next level – into human therapies.

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INTRODUCTION

Stem cells from non-embryonic sources offer a real prospect for clinical intervention in the short term having already shown success in the treatment of over 70 diseases and disorders. At first umbilical cord blood (UCB) and bone marrow (BM) stem cells were used as an intervention in only blood related and immune system related problems. However, their move to assist in the treatment of genetic diseases and application to regenerative medicine has opened new possibilities that had never been imagined, and only before suggested from embryonic stem cells. What slows down the creation of any stem cell therapy is the development of clinical grade protocols to both harvest and process the stem cell source into a form that is suitable for transplant. Determination of what stage a stem cell source should be at for transplant – whether primary stem cells or expanded and/or developed progenitor population and/or fully developed mature cell – is a limiting factor that still requires meticulous research.

Media attention and the ongoing socio-political and religious debates continue to escalate as to which stem cell source should or should not be used for therapies. Whilst this is an important discussion, scientists are ultimately responsible for proving which stem cells can be used for which purposes. Our role therefore, is to prove that adult stem cell sources such as bone marrow, skin and adipose tissue, or umbilical cord blood – our tissue of choice – can be useful to the patients we care for.

EMBRYONIC, ADULT, AND CORD BLOOD STEM CELLS

The debate on choice of adult *versus* embryonic stem cells

Embryonic stem cells (ES) whether from human or animals have three known main limitations: first, the limited amount of tissue producible from one embryo either with or without feeder layers (Klimanskaya et al. 2005, Martin et al. 2005); second, the limitation of producing homogeneous tissue (ES lines grown for more than 80 passages have been shown to acquire mutations (Krtolica 2005); and, third, tissue typing is an immunological reality that when ES cells produce tissue, it will gain Human leukocyte antigens and be recognizable to a host immune system as being foreign, and thus risking rejection (Kofidis et al. 2005, Tajima et al. 2003). The never-ending "breakthroughs" in ES transplantation reported by the media and scientific publications that have aligned themselves rather unfortunately to a particular issue, are generally infrequent and usually sub-primate animal studies that have little 'translatability' to the human setting. The ongoing debate of access to different grades of human embryos, also does nothing to dissuade arguments of tissue typing which can still not be over come and will totally prevent ES cells from being transplanted unless tissue type, according to current scientific evidence (Martin et al. 2005). Nuclear transfer has been suggested to over come this problem, but at time of writing this manuscript only one successful human nuclear transfer embryo has been produced – and at our university (Stojkovic et al. 2005).

Far beyond the general issues of stem cells there are limitations that prevent them being used for the clinic (Korbling and Estrov 2003). Tissue engineering parameters such as biomaterials, growth factor control systems and transplant protocols are also areas that require development to successfully used stem cells outside of the blood and immune systems. The field of tissue engineering is a fast-evolving area with an immense future. A responsibility, however, comes with this area of science which is unparalleled, perhaps since the birth of nuclear atomic science. Hype and media must not be the driving forces to the use of stem cells. Humans must not be used like laboratory animals to be risked in trials of stem cells that have not been rigorously investigated.

Stem cells have been defined as a cell capable of differentiating into one or more cell types as well as maintaining an immature stem cell pool by self-renewal and asymmetric division (Krtolica 2005). Throughout ontogeny, stem cells have historically been grouped into two distinct categories – embryonic or adult stem cells. We would argue that UCB sourced stem cells are a third category, since they possess differences to both ES and traditional adult sourced bone marrow.

Embryonic stem (ES) cells are formed post-fertilization and are termed multipotent by their ability to produce tissues from three germ layers. Strictly speaking ES cells are those which form a human. Embryonic stem cell lines (ES lines) are those which are experimentally isolated from the inner cell mass pre-implantation blastocysts (Atala 2005, Stojkovic et al. 2004). ES lines can only be described as "pluripotent", since no one at the time of writing this has been able to produce every single type of human cell from one ES line.

The reason for this is simple – we do not yet know every type of cell in the human system – the technology for that does not yet exist and precludes the debate that ES are totipotent. Ethical debates and discussions surrounding the use of embryos for research and clinical applications is still extremely passionate and calls for objective education of the public, scientists, physicians and politicians.

Adult stem cells have historically been considered located in adult tissue and "mulipotent", capable of differentiation into one or several lineages. Adult stem cells also organize tissue regeneration and repair upon stress (Korbling and Estrov 2003, Nowlin 2005, Quesenberry et al. 2005).

Umbilical cord blood (UCB) is perhaps one of the most readily available adult stem cell sources. But UCB when harvested are only 9 months old from the point of fertilization and hence could be argued to be different from any population of "adult stem cells" taken from an adult more than – for example – 20 years old. We believe that a new definition of stem cells requires being set – one that realizes that stem cells from different sources may have different potentials and that the term "adult stem cells" may already be out of date.

Whilst bone marrow, skin-related or adipose sourced stem cells may be useful for autologous procedures, umbilical cord blood may have an important role to make in allogeneic intervention, especially when a patient has systemic disease during which their own tissues may not be appropriate to use.

Umbilical cord blood is a useful source of stem cells for clinical applications

Adult stem cells (ADS) are considered immature cell groups useful for tissue repair maintaining tissue homeostasis and repair in a niche-specific controlled microenvironment (Heissig et al. 2005, Jiang et al. 2002, Li and Xie 2005). ADS can, therefore, provide a faster route to clinical intervention than embryonic stem cells, providing a cell source which is both readily accessible and more ethically acceptable to the public.

Although ADS are suggested to be present in multiple tissues (including neural, muscular, hepatic and cardiovascular tissues), blood-derived stem cells remain the most characterized ADS population with clear clinical proof. Bone marrow (BM), mobilized peripheral blood and UCB have provided sufficient quantities of cells to develop clinical translation protocols including development of BM as a cellular therapy for infarcted cardiac tissues and the use of UCB in hematotherapy (Cohen and Nagler 2004, Perin and Silva 2004, Stamm et al. 2003).

UCB further provides distinct advantages over other adult stem cell sources. In terms of ontogeny, UCB cells are at the intermediate point between embryonic/fetal and adult life and have longer telomeres and high proliferation potential. UCB units have also a reduced risk of viral contaminations (cytomegalovirus or Epstein-Barr virus).

Hematological transplantation using UCB further demonstrated the lower incidence of graft-versus-host disease for allogeneic graft (Rocha et al. 2000, Rubinstein et al. 1999). UCB induces better tolerance for human leukocyte antigen (HLA) mismatches when compared to bone marrow, probably due to the immaturity of the immune cells or controlled by UCB dendritic cells and/or natural killer cells (Cohen and Nagler 2004, Liu et al. 2004).

UCB is also a valuable source of stem cells in terms of access and supply. The global population count is in excess of 6 billion and the global birth rate stands at a mammoth 100 million per year. So, UCB is the largest untouched source of stem cells available. Further, the non-invasive nature of its collection confers UCB a distinct advantage over any other source of stem cells.

Potential UCB banking constitutes a powerful route for allogeneic transplants particularly for patients with rare HLA phenotypes (e.g. ethnic minorities) but also siblings and unrelated recipients. These UCB units are already used routinely for hematotherapy mainly for pediatric hematological transplantation. The UK National Blood Service Cord Blood Bank currently stores over 7000 UCB units. All UCB units are HLA-phenotyped, screened for microbiological infections and banked following current Good Manufacture and Practice standards (Watt and Contreras 2005). France hosts two public banks with about 5000 UCB units at clinical grade for transplantation managed by the 'Établissement français du Sang' and the 'Établissement français des Greffes'. In the USA over 40000 UCB units are banked and managed by the National Marrow Donor Program. Further to this, "Bone Marrow Donors Worldwide" registry collected data from 38 cord blood registries from 21 countries and it facilitates direct access to over 200000 UCB units worldwide (from private and public banks; http://www.bmdw.org). Our own Newcastle Cord Blood bank has transplanted cord blood into patients for both hematotherapy and

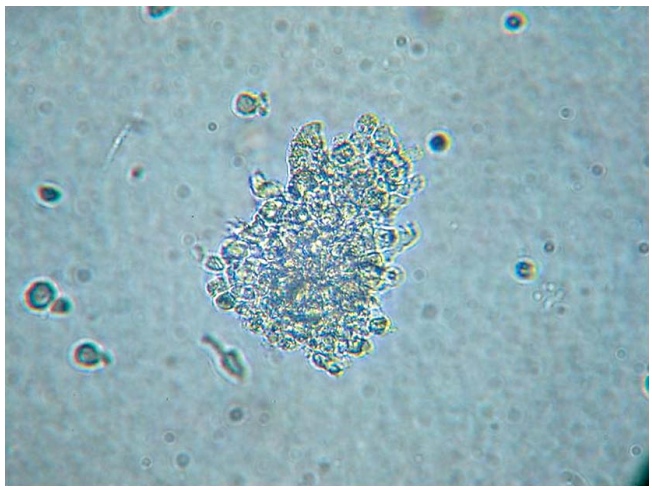

Fig. 1. Cord blood derived embryonic-like stem cells (CBE's). The CBE colony chosen here was photographed at a late stage of growth and just prior to the point of sub-cloning into new cultures. Colonies were generally subcloned by weeks 5–6.

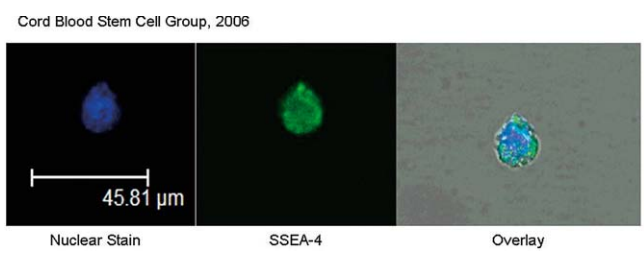

Fig. 2. CBE's express human embryonic stem cell markers. Pictured here is a cell taken from a CBE colony generated from a frozen unit of cord blood from the and stained as example for SSEA-4. Cells from the same colony were similarly positive for other ES markers including Nanog.

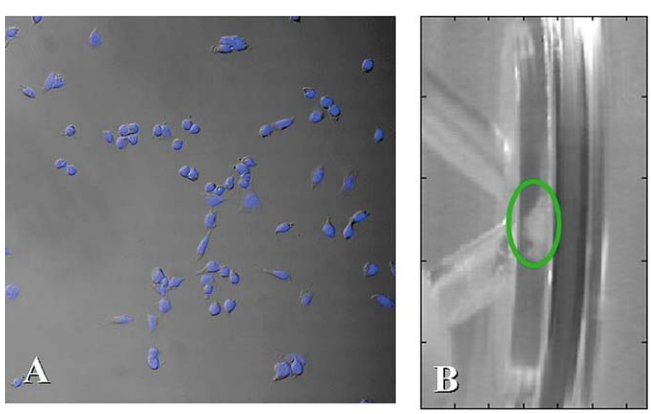

Fig. 3. Differential growth of neural progenitors using both 2- and 3-dimensional modeling systems. (A) Neural progenitor line stained with DAPI nuclear/DNA dye growing in 2-dimensions revealing slowed growth, quick loss of sphericity and accelerated development of maturing spindle shaped neural cells. (B) Neural progenitor construct growing in 3-dimensions inside a micro-bioreactor circulating at low rpm with minimal shear stress and advanced gas exchange capability.

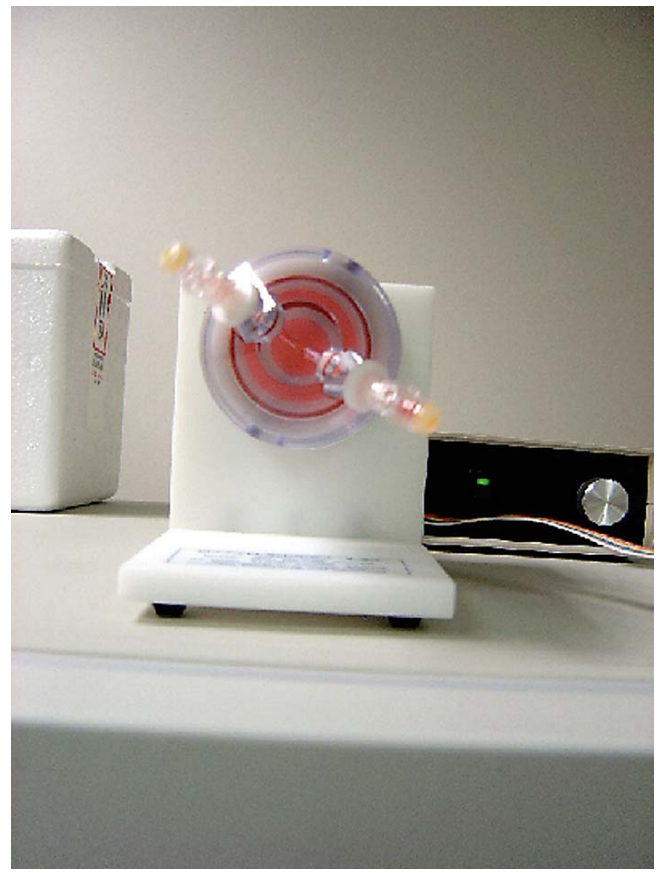

Fig. 4. Micro-bioreactor. Pictured – a micro-bioreactor system from the Synthecon Corporation set for 1 ml liquid (sealed) capacity. Gas exchange takes place to the rear wall of the liquid air interface.

immunotherapy successfully, including multiple cord blood units into single patients.

UCB adult stem cells are therefore an exciting therapeutic prospect and with the advent of international cord blood banking provide an increasing resource of stem cells useful for the treatment and the research of global disease.

Clinical uses of umbilical cord blood

Historically, transplanters quantified and predicted hematological grafts success using CD34 (a cell surface sialomucin) as the main identifying marker for stem/progenitor cells. However, one of the drawbacks to using UCB clinically was the limited number of cells available from a single cord. The number stem/progenitor cells present in an average UCB units was believed to represent only 5% of the optimal dose

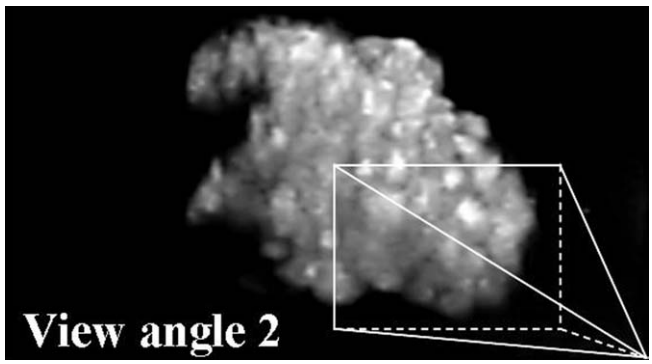

Fig. 5. Three-dimensional neural progenitor construct created by microgravity-aided bioreactor cell culture. The construct pictured in Fig. 3B was removed, stained with nuclear/DNA DAPI dye and imaged on a standard multi-laser confocal microscope revealing true 3-dimensional growth developing at a controlled rate, allowing gentle adherence of the cells to each other.

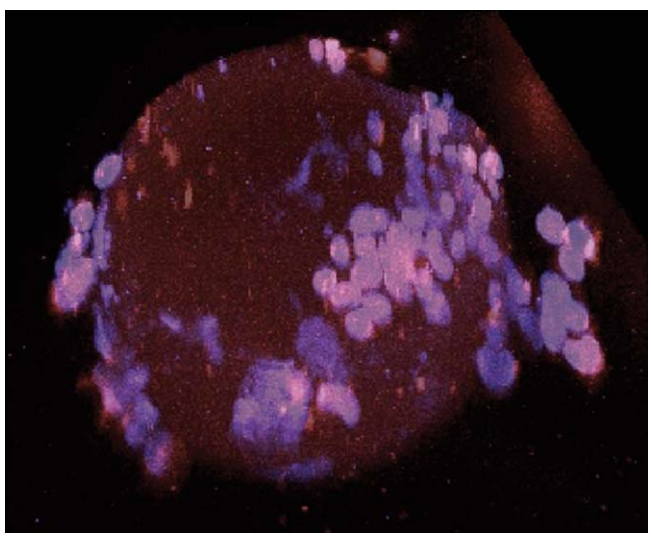

Fig. 6. Microbioreactor support system for neural cultures moving towards mature cells. A collagen bead (slight orange glow) is pictured with neural cells gently adhering to the collagen surface. Upon adherence the cells begin to migrate across the surface of the bead connecting with neighbors.

required for adult hematopoietic transplantation (2–4 × 10^6/kg) (Gilmore et al. 2000). Such a limitation focused research to developing safe and reliable technologies for *ex vivo* expansion, pooling and cryopreservation of UCB stem cells (Ende et al. 2001, Querol et al. 2000). Using multiparametric phenotyping, we demonstrated that UCB contained a complex succession of heterogeneous stem and progenitor cell groups up- and down-regulating a range of surface antigen including CD133, CD34, CD38, CD7, CD90 etc., as they differentiated (Forraz et al. 2002a, McGuckin et al. 2003a). High definition laser-scanning confocal microscopy imaging enabled us to better understand cell adhesion molecules' role in stem cell development, maintenance and differentiation, and 3-dimensional interactions with surrounding cells and extracellular matrix components (McGuckin et al. 2003b).

By integrating our data together, we moved away from traditional positive cell isolation methods targeting progenitor cell groups (CD34+ or CD133+ cells) which are largely committed towards hematopoiesis. This led us to progressively refine our stem cell separation strategy by removing hematopoietic mature and progenitor cells in order to yield pre-hematopoietic stem cells from UCB (Forraz et al. 2004). Interestingly, this immature lineage-restricted stem cell group proved to bear multipotential for differentiation into blood and also non hematopoietic tissues including neural and hepatic (Forraz et al. 2002b, 2003, 2004, McGuckin et al. 2004b).

Potential for harvesting embryonic-like stem cells from umbilical cord blood

We proved the cells exist in UCB that have multi-tissue differentiation capability. Further, since UCB is ontogenically young, we hypothesized that discrete circulating immature stem cell populations may remain from the embryonic and fetal development stages.

To prove this we optimized a series of stringent and rapid cell separation methods whereby sequential immunomagnetic removal of mature cells in UCB combined to standardized subculture protocols reproducibly revealed immature stem cell groups with astonishing embryonic characteristics: We called these cord blood derived embryonic-like stem cells (CBE's), since they were found to reproducibly express markers contiguous with embryonic stem cells. To do this the UCB units were collected post-birth from caesarean sections donors and submitted to centrifugal density separation to remove most erythrocytes, granulocytes and platelets. UCB mononucleated cell fraction was further depleted of nucleated red cells, myeloid and lymphoid progenitors, and leukocytes at various stages of differentiation by sequential immunomagnetic cell separation targeting Glycophorin-A, CD33, CD7, and CD45 surface antigen (Forraz et al. 2004, McGuckin et al. 2004b, 2005). Post-selection, lineage-restricted cells represented a highly rare and specific cell group

from UCB, as little as 0.16 ± 0.02% of starting mononucleated cell fraction (n=21).

Lineage-restricted stem cells were then grown at high concentrations in low volume (promoting cell-cell interaction and endogenous growth factor release) in micro tissue flasks with Iscove's-modified Dulbecco's medium supplemented with human recombined thrombopoietin, flt3-ligand, c-kit ligand (McGuckin et al. 2005).

For every 10 UCB units submitted to this protocol six yielded CBE's with similar growth patterns consisting of a majority of cells (70%) adhering to the microflasks' matrix and forming tight organized colonies which appeared to grow in a similar colony formation as that seen in embryonic stem cell colonies after only one week (Fig. 1). However, we would also point out that these cells were not at all like embryos growing in culture, and were not encapsulated in any way. The cells cannot therefore, be at all confused with blastocyst or later stages of the embryo.

We confirmed the "embryonic" similarity of CBE's nature by testing immunoreactivity against a selection of human embryonic stem (ES) cell markers. CBE's were notably positive for stage specific embryonic antigen-3 and -4 (SSEA-3 and SSEA-4) which are sialoproteins expressed by human ES cells but negative for SSEA-1, which confirmed their undifferentiated phenotype (Thomson et al. 1998). CBE's were further immunoreactive for tumor rejection antigen TRA 1-60 and TRA 1-81, ES cell-specific extracellular matrix sialylated keratin sulphate proteoglycans, which most likely contributed to the colony – like nature of their clustered organization (Hoffman and Carpenter 2005). CBEs were also positive for the pluripotency transcription factor Oct-4 involved in differentiation inhibition and ES cell self-renewal (Matin et al. 2004).

CBE's demonstrated high potential for cellular expansion in liquid culture. Following a short lag phase, CBE colonies could be significantly propagated for 14 weeks and over, with a 389 maximal fold expansion at week 7. Exponential CBE growth occurred between week 2 and week 7. Following a slowing down in proliferation, CBE's underwent a maintenance phase (week 9 to week 14) which coincided with near-confluence/overcrowding on the substrata and colonies stopping releasing non adherent single cells in the supernatant.

The finding of these CBE's suggests they: (i) have the ability to self-limit their growth *via* cell-cell contact or release of soluble growth inhibitors, (ii) require sustained "embryonic-like" stimuli for further propagation, or (iii) be inhibited by the *in vitro* surface geography of the microflask, preventing further free enlargement of the colony. CBE's are currently being tested in novel feeder-free system specific for embryonic stem cells (Klimanskaya et al. 2005, Wang et al. 2005). However, during this plateau phase CBE's could be dissociated with trypsin and replated into second generation liquid cultures where they expanded exponentially for over six weeks. CBE's could also be frozen down and thawed without affecting their *in vitro* proliferation potential.

Following publication of this protocol, much resistance was received from several "embryonic stem cell research advocates", who suggested the technique would be unlikely to be useful to the existing cord blood stored in worldwide banks. This unnecessary criticism was made irrelevant early in 2006 when we repeated the work on frozen cord blood units, demonstrating, for example, positivity for stem cell markers including Nanog and SSEA-4 (Fig. 2).

Whilst we do not say that these cells are identical to embryonic stem cells, we would also point out that we don't want them to be. Unlike embryonic stem cell lines, CBE's come from a wide range of tissue types and have, therefore, more potential for matching to a potential transplant recipient.

CORD BLOOD STEM CELLS FOR NEURAL MODELING

Neural cell modeling for regenerative medicine

We and other researchers have the ability of UCB to derive neural cells *in vitro* (McGuckin et al. 2004a). In the United States of America (Bicknese et al. 2002, Sanchez-Ramos et al. 2001) and also in Poland with our collaborator Professor Domanska (Buzanska et al. 2002, 2006a,b, Jurga et al. 2006a,b). Our methods developing CBE's may now enable preliminary expansion of the primitive CBE stem cells prior to differentiation into neural progenitors and differentiated tissues. We are therefore developing new techniques to investigate optimization of neural cell growth *in vitro*. In 2004 we published protocols for the production of a wide range of neural phenotypes *in vitro*. However, one of the problems highlighted was that when neural cells grow on a flat surface their growth rate slows

down (Fig. 3A). Further, since up to only half the potential surface area of the cell is then available to the growth medium, the cells may potentially not respond to mediators in the same way as expected *in vivo*.

Application of stem cells for neural cell development

Since stem cells are not just useful for transplantation but can also be applied to understanding the development of disease and for drug analysis. We have now moved to investigate 3-dimensional systems of growth. By partnering with the NASA spin-out company Synthecon, we have developed a micro-bioreactor protocol which allows 3-dimensional groups of cells to be formed (Fig. 4). Figure 3A shows a neural cell population growing in a standard 2D flat plate system. Here growth is slow and response to mediators limited. When seeded into the Synthecon 3D circulating bioreactor system, a 3D group of the same cells can be formed, which is more representative of the *in vivo* system (Fig. 5). The bioreactor system mimics simulated microgravity and allows the developing tissue construct to engage in cell to cell communication. Potentially such models are useful to understand the connection breakdowns and drug interactions in disorders such as Alzheimer's disease and Parkinson's disease. Models can also be grown on supporting scaffolds and bead systems to allow investigation of the early stages of neural progenitor growth in the fetal environment (Fig. 6).

CONCLUSIONS

We are developing reproducible and robust protocols to advance our understanding of the utility and application of adult stem cells, not least from umbilical cord blood. Given that the cell group that can be produced from cord blood is increasing year by year, the ability to use these cells for tissue engineering research and potentially for transplant is advancing rapidly. For example we have worked with scientists at the BioE Corporation, USA, to characterize a cell line named "multi-lineage progenitor cells" (MLPC™). These cells, which are cord blood-derived, are karyotypically normal by spectral genomic typing and have shown to date, to form over 12 cell tissue groups, not least, hepato-pancreatic progenitors, neuronal and glial, endothelial, osteoblasts and myocytes. This validates that one clonal cell type from cord blood is multipotential. The multiphenotypic production of neural cells in our lab also demonstrates the utility of cord blood for research in neuroscience.

In summary, cord blood, has a long history of successful transplant for hematological and immune system diseases and now with the multipotentiality confirmed in vitro to produce tissues from all three human germ layers, ectodermal, endodermal and mesodermal, demonstrates that it has viability for the regenerative medicine arena.

Given that human cord blood is already the world's largest source of stem cells, we must continue to develop it for the treatment of patients with serious disease worldwide.

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