Cell Prolif. 2005, **38**, 245–255

Production of stem cells with embryonic characteristics from human umbilical cord blood

C. P. McGuckin*, N. Forraz*, M.-O. Baradez*, S. Navran†, J. Zhao‡, R. Urban‡, R. Tilton‡ and L. Denner‡

**Stem Cell Therapy Programme, School of Life Sciences, Kingston University, Kingston upon Thames, Surrey, UK, †Synthecon Corporation, Houston, TX, and ‡University of Texas, Medical Branch, Internal Medicine-Endocrinology and Stark Diabetes Center, Galveston, TX, USA*

Received 4 *July* 2005; *revision accepted* 11 *July* 2005

Abstract. When will embryonic stem cells reach the clinic? The answer is simple – not soon! To produce large quantities of homogeneous tissue for transplantation, without feeder layers, and with the appropriate recipient's immunological phenotype, is a significant scientific hindrance, although adult stem (ADS) cells provide an alternative, more ethically acceptable, source. The annual global 100 million human birth rate proposes umbilical cord blood (UCB) as the largest untouched stem cell source, with advantages of naive immune status and relatively unshortened telomere length. Here, we report the world's first reproducible production of cells expressing embryonic stem cell markers, – cord-blood-derived embryonic-like stem cells (CBEs). UCB, after elective birth by Caesarean section, has been separated by sequential immunomagnetic removal of nucleate granulocytes, erythrocytes and haemopoietic myeloid/lymphoid progenitors. After 7 days of high density culture in microflasks, (10^5 cells/ml, IMDM, FCS 10%, thrombopoietin 10 ng/ml, flt3-ligand 50 ng/ml, c-kit ligand 20 ng/ml). CBE colonies formed adherent to the substrata; these were maintained for 6 weeks, then were subcultured and continued for a minimum 13 weeks. CBEs were positive for TRA-1-60, TRA-1-81, SSEA-4, SSEA-3 and Oct-4, but not SSEA-1, indicative of restriction in the human stem cell compartment. The CBEs were also microgravity–bioreactor cultured with hepatocyte growth medium (IMDM, FCS 10%, HGF 20 ng/ml, bFGF 10 ng/ml, EGF 10 ng/ml, c-kit ligand 10 ng/ml). After 4 weeks the cells were found to express characteristic hepatic markers, cytokeratin-18, α-foetoprotein and albumin. Thus, such CBEs are a viable human alternative from embryonic stem cells for stem cell research, without ethical constraint and with potential for clinical applications.

Correspondence: Dr Colin P. McGuckin PhD, Stem Cell Therapy Programme, School of Life Sciences, Kingston University, Penrhyn Road, Kingston upon Thames, Surrey, KT1 2EE, United Kingdom. Tel.: +44 (0)797 1266764; Fax: +44 (0)208 547 7562; E-mail: c_mcguckin@hotmail.com

INTRODUCTION

It is unlikely that embryonic stem cell (ES)-derived treatments will be available for clinical use soon. The prospect of stem cell therapy has heralded much hype and controversy, particularly as a result of development of embyronic stem cell lines. Development of advanced treatments with ES cells is slow because of the scientific reality that it is difficult to produce large quantities of homogeneous tissue/cells for transplantation, particularly bearing in mind that animal feeder layers, on which adult human ES cells tend to rely, might be a contaminant. (Klimanskaya *et al*. 2005; Martin *et al*. 2005). In addition, control of immunological development of ES cells is also a significant problem that will take time to overcome (Tajima *et al*. 2003; Kofidis *et al*. 2005). Adult stem (ADS) cells, however, provide an alternative cell source which is more ethically acceptable and could supply cells for current transplantation. ADS therapies have had successes using bone marrow (BM) stem cells and those derived from umbilical cord blood; for example, treatment of myocardial infarcts with BM-derived stem cells and in haematotherapy using UCB (Stamm *et al*. 2003; Cohen & Nagler 2004; Perin *et al*. 2004).

Because of volume restriction, single UCB units offer a limited number of cells. However, a growing interest in the scientific community focuses on ways to develop safe and reliable technologies for *ex vivo* expansion, pooling and cryopreservation of UCB-derived stem cells (Querol *et al*. 2000; Ende *et al*. 2001). We have previously reported the importance of characterizing primitive stem cell populations phenotypically, functionally and in terms of proliferation potential (Forraz *et al*. 2002a, 2003; McGuckin *et al*. 2003a,b; McGuckin *et al*. 2004a). This has led us into the development of more stringent and standardized cell separation protocols to identify more and more primitive restricted stem cell groups from UCB. Notably, we have reported on a lineage-restricted group of cells with multipotential differentiational capability, into blood and also into non-haematopoietic cells, including those of neural and hepatic tissues (Forraz *et al*. 2002b, 2004; McGuckin *et al*. 2004b).

From these exciting data, and because of their ontogenic nature, we speculate that, in some part, UCB could be the host of extremely primitive and discrete circulating stem cells, which up to parturition had been embryonic, contributing to embryonic development. Here, we report the world's first reproducible production of cells expressing embryonic stem cell markers (cord blood-derived, embyonic-like stem cells, CBEs, and have developed these for tissue engineering and transplantation, not least for hepatic tissue.

MATERIALS AND METHODS

Umbilical cord blood stem cell processing

Umbilical cord blood units were collected from normal, microbiologically screened and ethics-cleared donors after Caesarean section and were then diluted on collection in phosphate-buffered saline (PBS; Sigma-Aldrich, St Louis, MO, USA) supplemented with 0.6% acid citrate dextrose formula-A acid anticoagulant (Sigma-Aldrich) and bovine serum albumin (0.5% fraction V, Sigma-Aldrich), at pH 7.4. Diluted UCB units were density separated using research grade Ficoll-Paque solution (density: 1.077 g/cm^3, Pharmacia Biotech, Uppsala, Sweden) following the manufacturer's instructions. Primitive lineage-restricted stem cells were purified by sequential immunomagnetic depletion as previously described (Forraz *et al*. 2004; McGuckin *et al*. 2004b).

Growth of cord blood-derived embryonic-like stem cells

UCB lineage-restricted stem cells (10^5 cells/ml) were cultured in tissue culture microflasks (Nunc, Rochester, NY, USA) at 37 °C, 5% CO_2 humidified atmosphere in Iscove's-modified Dulbecco's medium (IMDM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% foetal calf serum (Invitrogen). Cells were stimulated with specific human recombined cytokines (Immunotools, Friesoythe, Germany), thrombopoietin (10 ng/ml), flt3-ligand (50 ng/ml) and c-kit ligand (20 ng/ml). Medium and cytokines were replenished weekly, preserving the formation of adherent CBEs on the microflasks' substrata. When required, developing adherent CBEs were dispersed using × 1 trypsin-EDTA solution (Invitrogen) and were subcultured in new microflasks with the same liquid culture conditions as above at 10^5 cells/ml.

Hepatic 3-dimensional tissue engineering

After 6 weeks of 2-dimensional expansion in microflasks and CBE colony dissociation with trypsin-EDTA, 5×10^5/ml CBEs were harvested and subsequently seeded into a rotating cell culture microbioreactor (RCCS, Synthecon, Houston, TX, USA), specifically custom made to our specifications for microculture and 3-dimensional tissue engineering in a microgravity enhanced environment. In the RCCS, CBEs were grown for 4 weeks in IMDM supplemented with 10% foetal calf serum and human recombinant cytokines (all from Immunotools): hepatocyte growth factor (HGF, 20 ng/ml), basic-fibroblast growth facor (bFGF, 10 ng/ml), epidermal growth factor (EGF, 10 ng/ml) and c-kit ligand (10 ng/ml) to induce hepatic differentiation. Fifty per cent of media were exchanged and cytokines re-added to the RCCS bi-weekly.

Immunophenotyping and fluorescent imaging

Adherent cells were washed with PBS solution. Non-specific Fc receptors were blocked with 2% human γ-globulin in PBS solution (Sigma-Aldrich, 4 °C, 20 min). For intracellular staining, cells were fixed in 4% paraformaldehyde solution (Sigma-Aldrich, 10 min 4 °C) and were permeabilized using 0.1% saponin (Sigma-Aldrich), 0.1% sodium azide (Sigma-Aldrich), 2% foetal calf serum (Invitrogen) in PBS solution (15 min, 4 °C). Throughout immunostaining, excess antibodies were washed away using 0.1% sodium azide, 2% foetal calf serum in PBS solution (with 0.1% saponin when required).

Primary antibody labelling (45 min, 4 °C) was performed as follows. CBEs were labelled using the 'Embryonic stem cell marker sample' kit (Chemicon, Temecula, CA, USA) including mouse anti-human SSEA-1 (IgM, clone MC-480), SSEA-4 (IgG, clone MC-813-70), TRA-1-60 (IgM, clone TRA-1-60), TRA-1-81 (IgM, clone TRA-1-81), Oct-4 (IgG, clone 9E3) and rat anti-human SSEA-3 (IgM, clone MC-631). CBE-derived differentiated hepatic progenitors were stained with mouse anti-human cytokeratin 18 (IgG, clone CY90, Serotec, Raleigh, NC, USA), α-foetoprotein (IgG, clone F1-6P2A8-P2B9A9, Serotec), albumin (IgG, clone HAS-9, Sigma-Aldrich). Matched isotype control immunolabellings were carried out in all cases.

For secondary labelling (30 min, 4 °C) fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse IgM (Becton Dickinson, San Jose, CA, USA), rabbit anti-mouse IgG (Sigma-Aldrich) or goat anti-rat IgM (Becton Dickinson) were used. When required, nuclear DNA was counterstained by incubating cells at room temperature with a 600 nM 4′, 6-diamidino-2-phenylindole dilactate (Sigma-Aldrich) in PBS for 5 min. Cells were subsequently washed in PBS. Post-staining, cells were mounted on glass slides using anti-fade reagent (Gel Mount Aqueous Medium, Sigma-Aldrich) and they were analysed on a Zeiss laser-scanning confocal microscope.

RESULTS

Cord blood-derived embryonic-like stem cells growing as tight adherent clusters resembling embryoid bodies

Lineage-restricted immunomagnetic-separated stem cells ($0.21 \pm 0.04\%$ mononucleate cells, $n = 10$) had been grown at high density and low volume in tissue culture microflasks. Within 24 h, approximately 70% of the cells adhered to the microflasks' substrata. From six umbilical cord blood units out of 10, adherent cell clusters formed embryoid body-like colonies as early as after 1 week of growth. These embryoid body-like colonies increased in size and number progressively (Fig. 1).

Cord blood-derived embryonic-like stem cells had multistage proliferation patterns

Cell proliferation analysis revealed that CBEs could be expanded for up to 14 weeks in liquid culture. CBEs exhibited a four-stage proliferation pattern: (i) a lag phase from baseline to week 1 with discrete embryoid body-like formation; (ii) an exponential growth phase from week 2 to week 7; (iii) a reduction in proliferation from week 7 to week 9; (iv) a plateau/maintenance phase from week 9 to week 14 (Fig. 2), during which subculture could be carried out for continued *ex vivo* expansion. The maximal expansion peak was reached at week 7 with CBEs having significantly grown from 10^5 cells/ml (baseline) to $3.89 \times 10^7 \pm 4.74 \times 10^6$ cells/ml representing a 389-fold increase from the baseline ($P < 0.001$, $n = 6$). The CBEs demonstrated an interesting capacity for cell number expansion in liquid culture.

Cord blood-derived embryonic-like stem cells subcultured in second generation liquid cultures

For the six umbilical cord blood units which generated CBEs, adherent cell colonies were dissociated at week 6 and re-seeded in second generation liquid cultures. Second-generation CBEs formed embryoid body-like structures with similar morphology to their first-generation progenitor colonies. The second-generation CBEs were grown for up to a further 6 weeks and demonstrated an exponential cell proliferation pattern (Fig. 3). Second-generation CBE populations significantly expanded (168-fold) from the 10^5 cells/ml baseline to $1.68 \times 10^7 \pm 8.84 \times 10^5$ cells ($P < 0.001$, $n = 6$). CBEs retained their exponential proliferation characteristics throughout the first- and second-generation liquid cultures.

Cord blood-derived embryonic-like stem cells expression of human embryonic stem cell-specific markers

CBE colonies were tested for human embryonic stem cell-specific antigens. CBE colonies were found to express stage-specific embryonic antigens, SSEA-3 and SSEA-4, respectively. CBE colonies were, however, not immunoreactive for stage-specific embryonic antigen-1 (SSEA-1). The cell colonies expressed embryonic extracellular matrix components Tra 1-60 and Tra 1-81. Finally, CBEs also expressed embryonic stem cell transcription factor Oct-4 (Fig. 4). Taken together, these data suggest that CBEs represent a primitive cell group with an embryonic stem cell phenotype.

Application of cord blood-derived embryonic-like stem cells in 3-dimensional tissue bio-engineering of hepatic progenitors

Six-week-expanded CBEs had been dissociated into single cells and then injected into a 3-dimensional rotating cell culture (RCCS) microbioreactor. In the RCCS, CBEs were stimulated

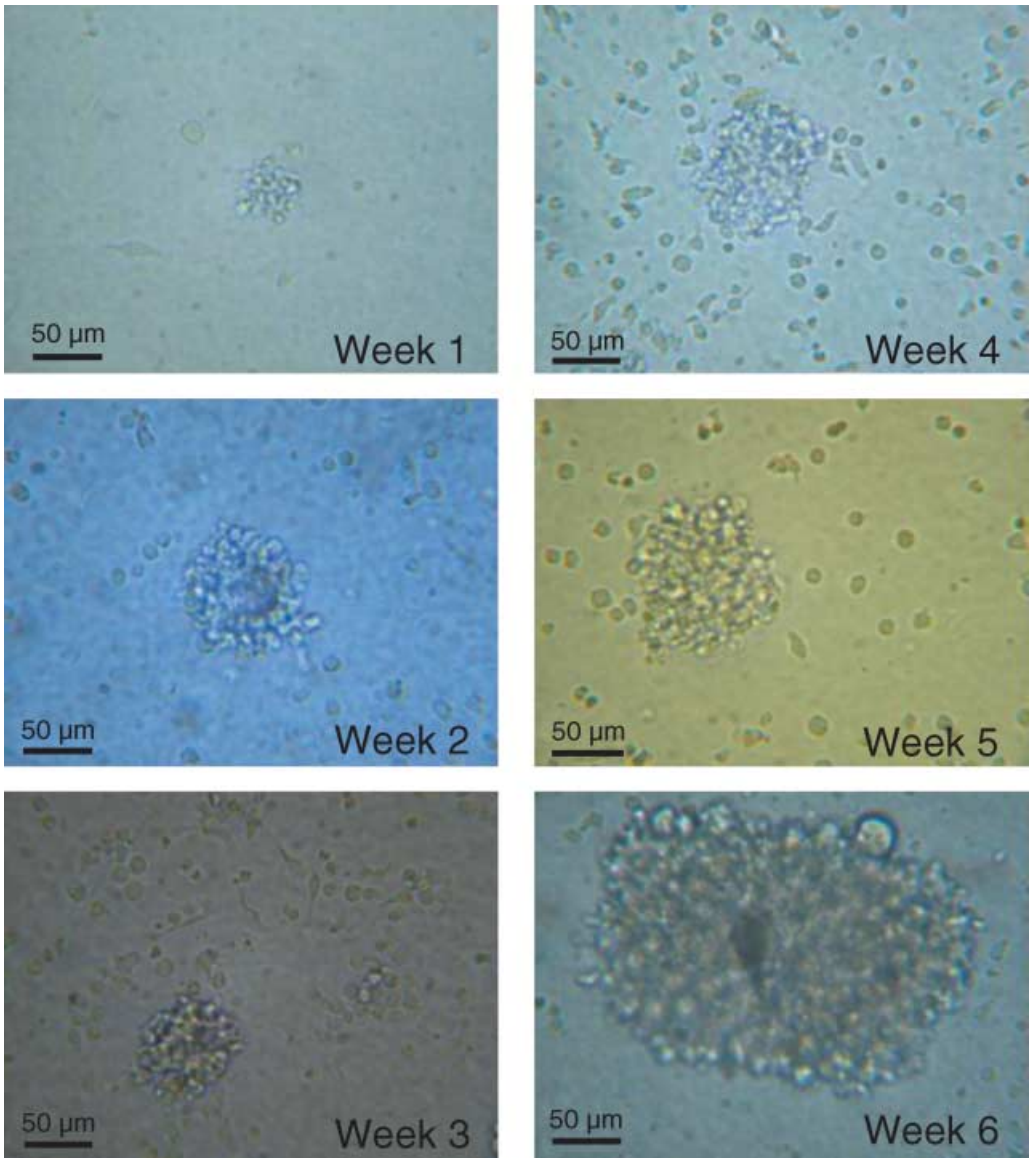

Figure 1. Cord blood-derived embryonic-like stem cell morphology. CBEs were identified as early as week 1 of liquid culture as tight clustered colonies which progressively increased in size and number.

with a range of differentiative hepatic cues for 4 weeks; cells were counted weekly (Fig. 5). The rotating microbioreactor supported cell proliferation in 3-dimensions for at least 2 weeks (respective single cell expansion counts: baseline = 5×10^5; week 1 = 5.2×10^5; week 2 = 5.1×10^5). Following this, initial analysis led to the result that single cell expansion counts diminished at week 3 (4.7×10^5) and week 4 (2.1×10^5). However, differential interference microscopy observations at week 4, indicated that the tissue bio-engineered cells had formed distinct self-aggregating 3-dimensional clusters in the RCCS microbioreactor.

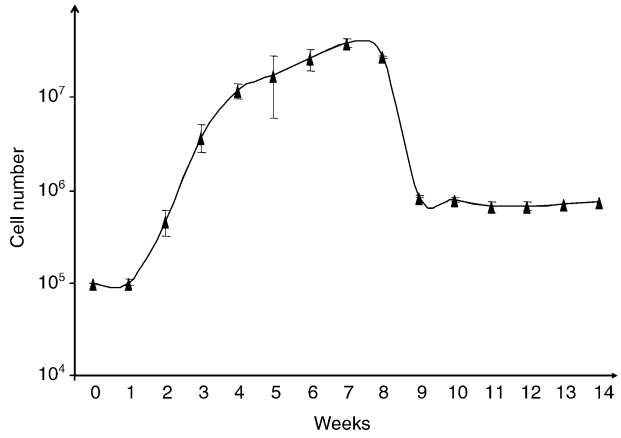

Figure 2. Cord blood-derived embryonic-like stem cells' proliferation profile. CBEs exhibited a four-stage proliferation pattern: (i) a lag phase (baseline to week 1); (ii) exponential phase (week 2 to week 7); (iii) reduction in proliferation (week 7 to week 9); (iv) plateau/maintenance phase (week 9 to week 14). CBEs could be expanded 389-fold from baseline at week 7.

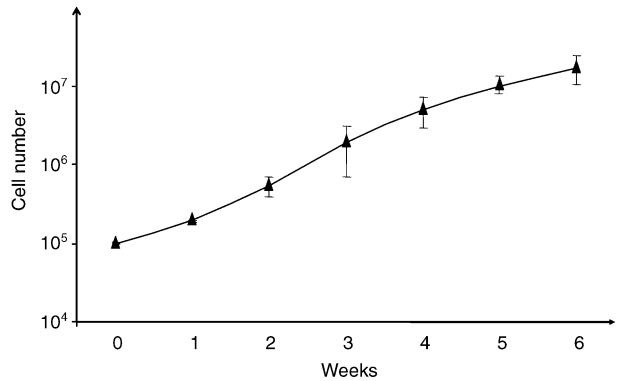

Figure 3. Cord blood-derived embryonic-like stem cells' production of second generation colonies. First-generation CBEs were dissociated and subcultured yielding 168-fold expansion from baseline for 6 weeks.

Cell clusters collected from the microbioreactor were further immunophenotyped for hepatic progenitor cell markers and were histologically examined using high-definition laser-scanning confocal microscope. These 3-dimensional tissue bio-engineered cell clusters were observed to express hepatic-specific antigens, including cytokeratin 18, α-foetoprotein and albumin (Fig. 5). This demonstrated the potential of CBEs for 3-dimensional tissue bio-engineering applications, particularly for hepatic progenitor cell production.

DISCUSSION

Here, we report the first in the world reproducible production of untransformed adherent human stem cell populations with embryonic stem cell phenotype, from primary umbilical cord blood

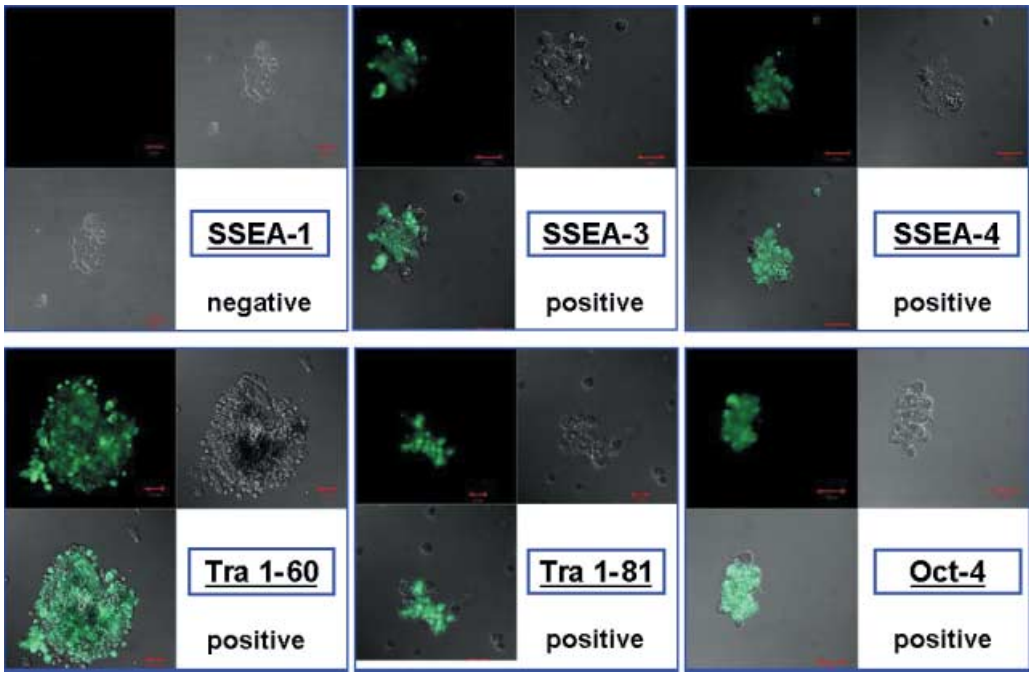

Figure 4. Cord blood-derived embryonic-like stem cells expression of embryonic stem cell-specific markers. CBEs expressed embryonic sialoproteins SSEA-3, SSEA-4, extracellulamatix components Tra 1-60, Tra 1-81, and the pluripotency transcription factor Oct-4 (scale bar 20 μm).

units. Cord blood-derived embryonic-like stem cells were successfully cultured from 6 out of 10 UCB units tested. The non-100% result would most likely be because of the scarcity of potential circulating primitive stem cells in UCB units. We speculate that purity of the initial stem cell source may be critical in CBE production. We have previously characterized, in depth, a highly lineage-restricted stem cell group originating in the UCB (Forraz *et al*. 2004; McGuckin *et al*. 2004b) from which CBE colonies were derived. Those cells represented a very discrete cell population, and we hypothesize, that the UCB might host rare multipotent cells still present after embryonic and foetal development. Data presented here strongly support such a hypothesis as CBEs have formed embryoid-body like colonies, which were immunoreactive for primitive human embryonic stem cell markers.

CBEs were found to express stage-specific embryonic antigen-3 and -4, which are sialoproteins characteristic of human ES cells, but did not express stage-specific embryonic antigen-1, a non-human protein. This phenotype (SSEA-3+, SSEA-4+, SSEA-1–) has previously been reported as specific for human undifferentiated ES cells. Murine ES cells, for example, have the opposite phenotypic profile (Thomson *et al*. 1998). Further to this, CBE colonies also organized into and produced ES cell-specific extracellular matrix sialylated keratin sulphate proteoglycans; they expressed tumour rejection antigens TRA 1-60 and TRA 1-81 (Hoffman & Carpenter 2005). Finally, CBEs also expressed the transcription factor Oct-4 which has been shown to control ES cell pluripotency by suppressing their differentiation and promoting self-renewal (Gerrard *et al*. 2005; Matin *et al*. 2004).

CBEs can be expanded *in vitro* under the influence of the TPOFLK cytokine combination which we have previously shown to play a role in cell self-renewal and in maintenance of the

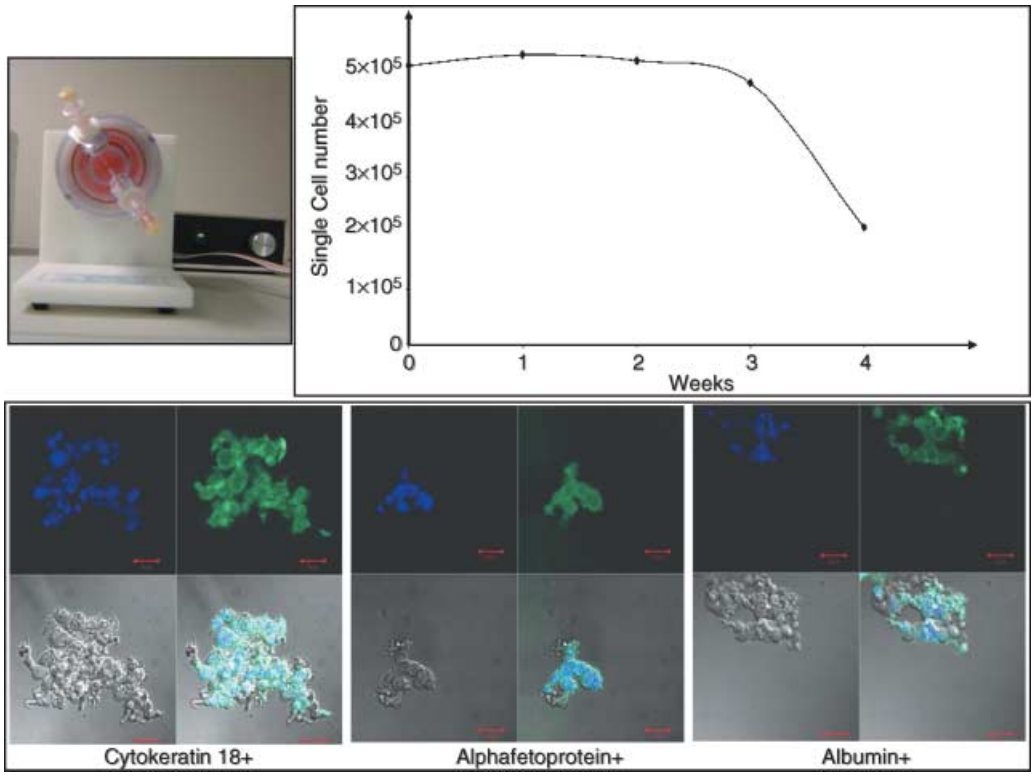

Figure 5. Cord blood-derived embryonic-like stem cells in 3-dimensional hepatic tissue bio-engineering. CBEs were dispersed and grown in a Synthecon microgravity simulating rotating cell culture system microbioreactor (top left quadrant). In this 3-dimensional shear stress-free environment cell growth was maintained until week 3. The observed decrease in single cell numbers was explained by the formation of 3-dimensional hepatic aggregates (top right quadrant). Phenotypic analysis confirmed that CBEs had successfully differentiated into hepatic progenitors (bottom quadrant, scale bar 20 μm).

undifferentiated phenotype (Forraz *et al*. 2004; McGuckin *et al*. 2004a). First generation CBEs grew exponentially for 8 weeks. This was followed by maintenance of a homogeneous CBE colony group for up to 14 weeks. Decrease in proliferation coincided with CBEs not releasing non-adherent single cells in the supernatant. This may be as a result of (i) the cells self-limiting their growth, (ii) requiring more 'embryonic-like' stimuli to further propagate, or (iii) limitation of the geography in the microflask, preventing further free enlargement of colonies. Ongoing experiments test for CBE expansion using a novel feeder-free system (Klimanskaya *et al*. 2005; Wang *et al*. 2005). Sub-cultured second generation CBEs' 6-week exponentional growth further demonstrated the tremendous potential of CBEs for the continued establishment of UCB-derived stem cell lines with embryonic properties.

By establishing CBE colonies, we have confirmed the presence of stem cells with ES cell characteristics in UCB. We have shown that these cells of primitive phenotype can be maintained undifferentiated for over 13 weeks. Other reports have also supported this theory concerning the existence of circulating embryonic stem-cell like, adult somatic cell ancestors, during foetal development (Jiang *et al*. 2002; Korbling & Estrov 2003). Jiang *et al*. (2002) notably have shown that multipotent progenitors can be isolated from post-natal murine bone marrow, muscle and brain.

Conventional cell culture methods generally employ 2-dimensional culture systems to propagate cells *in vitro*. Although this approach is useful to understand key biological features like cell cycle parameters, growth factor stimulation, proliferation rates and signal transduction events, large-scale clinical grade *ex-vivo* tissue and organ expansion generation calls for 3-dimensional tissue bio-engineering. The emerging challenge for tissue bio-engineers is to successfully integrate cells, matrices or scaffolds, nutrients and mechanical forces in a 3-dimensional culture system to generate functioning tissues and organs for regenerative medicine. A key finding in this has been our ability to expand, maintain and differentiate CBEs into potential hepatic progenitor cells in 3-dimensions, with and without supporting bio-scaffolds (synthetic scaffold data – separate manuscript, in preparation), using the rotating cell culture systems microbioreactor. The RCCS microbioreactor has previously been reported to support differentiation of human ES cells by complementing chemical cues efficiently (Gerecht-Nir *et al*. 2004). This NASA-derived ground-breaking technology has enabled CBEs to be grown in this 3-dimensional microgravity-simulating environment, optimizing nutrition and gas exchange, endogeneous extracellular matrix production and cellular aggregation, for 360° tissue bio-engineering. Ongoing studies are assessing terminal differentiation and functionality of CBE-derived hepatic progenitor cells (endodermal origin). Further, although we have already produced successful tissue culture of cells from all three germ layers in a 2-D culture environment, the suitability of the RCCS microbioreactor for differentiation of CBEs into ectodermal and mesodermal tissues is currently being additionally tested.

Given the current human birth rate, and with the global population in excess of 6 billion people, the use of ADS from UCB provides perhaps the most readily accessible and under-utilized stem cell source. Cord blood cells also provide distinct immunological advantages, primarily because of the immunologically protected nature of the foetal environment. Ontogenically, because of the relatively untapped length of these cells' telomeres, successful UCB-based haematotherapy protocols have already been explored (Dickinson *et al*. 2002; Rocha *et al*. 2004). In developed areas of the world, including Europe and North America, the number of cord blood banks is increasing, providing the prospect of being able to select an ADS unit with an appropriate immunological phenotype for individual patients (Proctor *et al*. 2001; Warwick & Armitage 2004). These unique primitive cord blood-derived embryonic-like stem cells) offer multiple advantages over truly adult stem cells and over embryonic stem cells, and are a viable and ethically sound alternative to them for tissue bio-engineering applications. Clearly, they provide a faster translation of stem cell use to the clinic and to robust cellular therapeutic protocols. They also provide, for the first time, a useful third phenotypic origin for scientific comparison against adult stem cells and those of embryoinic origin.

ACKNOWLEDGEMENTS

Dr McGuckin and Dr Forraz were funded by the British Department of Trade and Industry, UK-Texas-Biosciences programme and are grateful to Dr Parton, Dr Darnborough and Lord Sainsbury for their continuous support of our Adult Stem Cell programme. The continued support of Her Majesty's British Consulate staff of the Foreign and Commonwealth Office – Houston, and in particular Dr Akrawi, are noted. Urban, Tilton, Zhao and Denner are supported by the McCoy Foundation, San Marcos, TX and the Clayton Foundation, Houston, TX, USA.

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