ROZPRAWA HABILITACYJNA

Zakład Neurobiologii Naprawczej,
Instytut Medycyny Doświadczalnej i Klinicznej
im. M. Mossakowskiego PAN

Autor:
Dr nauk przyrodniczych Leonora Bużańska

Tytuł pracy:

Neuralne komórki macierzyste z ludzkiej krwi pępowinowej - charakterystyka immunocytochemiczna, fizjologiczna i molekularna

Rozprawa obejmuje cykl publikacji:

1) Bużańska L., Machaj E.K., Zabłocka B., Pojda Z., Domańska-Janik K. Human cord blood-derived cells attain neuronal and glial features in vitro. Journal of Cell Science 2002, 115: 2131-2138.
2) Sun W.*, Bużańska L.*, Domańska-Janik K., Salvi R.J., Stachowiak M.K., "Voltage-sensitive and ligand-gated channels in differentiating neural stem-like cells derived from the nonhematopoietic fraction of human umbilical cord blood" Stem Cells 2005; 23:931-945, * - equally contributing first authors.
3) Bużańska L., Habich A., Jurga M., Sypecka J., Domańska-Janik K., "Human Cord Blood-derived Neural Stem Cell Line – Possible Implementation in Studying Neurotoxicity", Toxicology in Vitro 2005; 19:991-999.
4) Bużańska L., Jurga M., Stachowiak E.K., Stachowiak M.K., Domańska-Janik K. „Neural Stem-like Celi Line Derived from Nonhematopoethic Population of Human Umbilical Cord Blood", Stem Cells and Development 2006;15(3).
5) Bużańska L., Jurga M., Domańska-Janik K. „Neuronal differentiation of Human Umbilical Cord Blood Neural Stem-Like Celi Line" Neurodegenerative Diseases 2006;3:19-26.

Warszawa, czerwiec 2006

PODZIĘKOWANIA

Pani Profesor dr hab. med. Krystynie Domańskiej-Janik
serdecznie dziękuję za inspiracje do mojej pracy
naukowej, a także za pomoc, życzliwość i zrozumienie w
czasie mojej długoletniej pracy w Zespole
kierowanym przez Panią Profesor

Wszystkim współautorom publikacji –
które stanowią istotę niniejszej rozprawy habilitacyjnej –
serdecznie dziękuję
za współpracę oraz ciekawe dyskusje naukowe

Koleżankom i Kolegom
z Zakładu Neurobiologii Naprawczej oraz
Zakładu Neurochemii IMDiK PAN
dziękuję za pomoc i życzliwą atmosferę

KOMENTARZ

Wstęp

Komórki macierzyste (Stem Cells, SC) mają zdolność do samoodnowy przez ciągłe podziały (odpowiednikiem funkcjonalnym jest klonogenność) oraz do różnicowania się w dojrzałe komórki tkanek i narządów. Na określonych etapach rozwoju osobniczego SC wykazują różny stopień ograniczenia potencjału do dalszego różnicowania, począwszy od komorki totipotencjalnej, która może różnicować się we wszystkie pozostałe komórki organizmu (jedynie zapłodniona komórka jajowa i pierwsze komórki potomne), poprzez pluri-, multi- oraz unipotencjalne.

Zarodkowe komórki macierzyste (Embryonic Stem Cells, ESC) mogą namnażać się w hodowli *in vitro* logarytmicznie, w czasie nieograniczonym, zachowując jednocześnie swoją pluripotentność, to jest zdolność do różnicowania we wszystkie(poza płciowymi) komórki organizmu (Thompson i wsp. 1998). Dzięki temu można otrzymać w krótkim czasie dużą ilość komórek do zastosowania w terapii transplantacyjnej. Jednakże linie komórkowe wyprowadzane z ESC wykazują cechy niestabilności genetycznej i epigenetycznej (Humpherys i wsp.2001), co może być powodem ich skłonności do tworzenia guzów w organizmach otrzymujących przeszczep komórkowy.

Te problemy natury biologicznej, a także zastrzeżenia natury etyczno-moralnej związane ze sposobem otrzymywania ESC, skłoniły wielu badaczy do poszukiwania alternatywnych źródeł komórek macierzystych z jednej strony bezpiecznych onkologicznie, z drugiej strony podobnie jak ESC, wykazujących zdolność do ekspansji *in vitro*. Specyficzne tkankowo, somatyczne komórki macierzyste (Somatic Stem Cells, SSC) są drugim źródłem komórek macierzystych stosowanym w terapii transplantacyjnej i podobnie jak ESC wykazują zdolność do strukturalnej i funkcjonalnej integracji z tkanką biorcy przeszczepu, jednakże mają ograniczoną zdolność do podziałów i ekspansji *in vitro*. Początkowo uważano, że SSC mogą różnicować się jedynie w komórki tkanki, z której pochodzą. Badania ostatnich lat wykazały jednak, że SSC są multipotencjalne nie tylko w obrębie jednej tkanki, ale mogą także przekraczać bariery tkankowe i różnicować się w komórki pochodzące z innych listków zarodkowych. Taka zdolność SSC tłumaczona jest zjawiskiem plastyczności (przeprogramowania – transdyferencjacji populacji już ukierunkowanych tkankowo komórek) i/lub transpotencji (wyjściowego braku zaprogramowania - utrzymania autonomicznego, niezróżnicowanego stanu pluripotencjalności części komórek występujących w określonej niszy tkankowej) (Sherley i wsp. 2002). Mechanizmy molekularne tych zjawisk nie są do końca poznane, choć koncepcja „open transcriptosom", która zakłada, że większość genów w

komórkach macierzystych jest utrzymywana w tzw. „stanie otwartym", czyli z aktywacją na niskim poziomie (Liu i Rao 2003) ma coraz więcej zwolenników. Podstawą tej hipotezy było wykrycie, że w niektórych typach somatycznych komórek macierzystych (np. mezenchymalnych) ekspresji podlegają wybrane transkrypty typowe dla różnych listków zarodkowych (Song i Sanchez-Ramos 2003, Tondreau i wsp. 2004). Z drugiej strony istnieje coraz więcej dowodów na to, że w dojrzałych tkankach somatycznych znajdują się pluripotencjalne komórki o charakterze ESC, których podziały w warunkach *in vivo* są skutecznie hamowane przez sygnały plynące z otaczającej niszy tkankowej (Jaing i wsp. 2002, Jurga i wsp. 2006). W sprzyjających warunkach *in vitro*, promujących aktywność proliferacyjną, takie „uśpione" pluripotencjalne komórki macierzyste izolowane z tkanki somatycznej mogłyby podlegać pozytywnej selekcji (Bużańska i wsp. 2006, Kucia i wsp. 2006, Conti i wsp 2005).

Ukierunkowane tkankowo komórki macierzyste (również komórki prekursorowe układu nerwowego) zwykle, w hodowli *in vitro,* po pewnej liczbie podziałów nieodwracalnie przestają się dzielić i spontanicznie różnicują się. Jedną z przyczyn tego zjawiska jest tzw. asymetria podziałów komórek macierzystych pochodzących z tkanek somatycznych (Sommer i Rao 2001). Z pojedynczej komórki tego typu po podziale powstaje jedna komórka macierzysta, która jest kopią komórki matczynej i jedna komórka progenitorowa, czyli komórka macierzysta „ukierunkowana" już w dalszym rozwoju. O podziałach lub dalszej specjalizacji komórki decydują sygnały zarówno pochodzenia wewnątrzkomórkowego jak i z otaczającego ją środowiska (Wurmser i wsp. 2004, Hsieh i Gage 2004, Rao 2004).W niszach mózgu jest to wystarczające do utrzymania stałej puli komórek macierzystych, natomiast *in vitro* prowadzi to do starzenia się i zamierania hodowli komórkowej. Dlatego problemem, wciąż trudnym w przypadku SSC, jest opracowanie metody nieograniczonego namnażania tych komórek *in vitro* i możliwość wyprowadzenia, bez uprzedniego unieśmiertelniania, ustalonych linii komórkowych. Takie linie komórkowe są niezwykle potrzebne zarówno dla dalszych badań podstawowych, jak i dla oczekiwanego zastosowania terapeutycznego.

W publikacji (Bużańska i wsp. 2006), która jest częścią mojej rozprawy habilitacyjnej dokumentujemy wyprowadzenie stabilnej, nietransformowanej linii neuralnych komórek macierzystych z krwi pępowinowej. Tym samym dostarczamy dowodów na to, że możliwe jest w warunkach *in vitro*, wyselekcjonowanie somatycznych komórek macierzystych, które mogą namnażać się w sposób nieograniczony (podobnie jak dzielące się symetrycznie embrionalne komórki macierzyste). Podobną stymulację do podziałów symetrycznych w hodowli *in vitro* uzyskano dla somatycznych komórek macierzystych izolowanych z mózgów

płodowych szczura i człowieka (Conti i wsp.2005). Co więcej, Conti i wsp. zastosowali podobny protokoł doświadczalny do naszego w kontekście zarówno sposobu izolacji komórek zdolnych do podziałów symetrycznych, jak i metody utrzymywania w hodowli komórek proliferujących.

Ludzkie neuralne komórki macierzyste (Human Neural Stem Cells - hNSC) można otrzymywać *in vitro* z hodowli ESC (Li i wsp.1999, Carpenter i wsp. 2003) i z mózgowych tkanek somatycznych (płodowych – Vescovi i wsp. 1999 i dorosłego człowieka – Roy i wsp 2001), ale również ze szpiku kostnego (Woodbury i wsp. 2000, Sanches –Ramos i wsp. 2001, Jiang i wsp. 2002) i skóry człowieka (Toma i wsp. 2001). Praca podjęta przez nasz Zespół i realizowana od 2000 roku we współpracy z Zakładem Hematologii Doświadczalnej Instytutu Onkologii w Warszawie udowodniła, że źródłem neuralnych komórek macierzystych może być również ludzka krew pępowinowa (Bużańska i wsp. 2002). Byliśmy pierwszym zespołem badawczym na świecie, który dostarczył takich dowodów. Zostały one przedstawione na Konferencji ISN/ASN (International Society of Neuroscience/ American Society of Neuroscience) w sierpniu 2001 roku w Buenos Aires (Bużańska i wsp., J Neurochem 2001, 78, suppl 1, p 58). Podobne wyniki rónolegle otrzymał zespół pracujący w Tampie, Floryda; Sanchez-Ramos i wsp. 2001).

Wyniki przedstawione w rozprawie habilitacyjnej dotyczą następujących zagadnień:

- otrzymywanie progenitorów neuralnych z ludzkiej krwi pępowinowej i dowody na ich zdolność do wielokierunkowego różnicowania się w komórki o charakterze neuronów, astrocytów i oligodendrocytów (Bużańska i wp. 2002);
- wyprowadzenie stabilnie ukierunkowanej linii neuralnych komórek macierzystych (Human Umbilical Cord Blood - Neural Stem Cells: **HUCB-NSC**) z progenitorów pochodzących z krwi pępowinowej (Bużańska i wsp. 2006);
- standaryzacja wzrostu i różnicowania HUCB-NSC: opracowanie metod hodowli *in vitro* umożliwiających utrzymywanie HUCB-NSC na różnych eapach zaawansowania rozwojowego (od niezróżnicowanych, poprzez ukierunkowane progenitory neuralne o zawężonym spektrum rozwojowym, do komórek zróżnicowanych) (Bużańska i wsp. 2005, Bużańska i wsp 2006a);
- analiza molekularna mechanizmów leżących u podstaw utrzymania „macierzystości" wyprowadzonej linii HUCB-NSC (Bużańska i wsp. 2006);
- różnicowanie HUCB-NSC w komórki o charakterze funkcjonalnych neuronów: dowody na podstawie badań molekularnych (mikromacierze DNA),

immunocytochemicznych (ekspresja białek) oraz elektrofizjologicznych (metoda „patch clamp") (Bużańska i wsp. 2006, Bużańska i wsp. 2006a, Sun, Bużańska i wsp. 2005).

Otrzymywanie progenitorów neuralnych z ludzkiej krwi pępowinowej

Nasze badania udowodniły, że ludzka krew pępowinowa może być źródłem komórek macierzystych, które pod wpływem odpowiednich warunków środowiska (obecność surowicy i stymulacja neuromorfogenami) mogą różnicować się w komórki o charakterze neuronów, astrocytów i oligodendrocytów (Bużańska i wsp. 2002).

Założeniem wyjściowym do przeprowadzanych doświadczeń było uzyskanie frakcji mononuklearnej krwi pępowinowej zawierającej komórki klonogenne o możliwie najniższym stopniu ograniczenia potencjału do różnicowania. W tym celu przeprowadzono immunodeplecję (metodą sortowania magnetycznego) komórek macierzystych już ukierunkowanych hematopoetycznie (CD34+), a następnie przez 6 tygodni stabilizowano hodowlę w obecności 10% surowicy. Otrzymana jednowarstwowo rosnąca hodowla komórek mononuklearnych była CD34(-), CD45(-), tj. negatywna odpowiednio pod względem powierzchniowych markerów hematopoetycznych i endotelialnych. Dalsza selekcja polegała na zmianie warunków hodowli w kierunku stymulacji do podziałów tej części komórek, która jest wrażliwych na czynnik wzrostowy EGF (Epidermal Growth Factor). Wcześniejsze prace (Roy i wsp.2000) wykazały, że EGF specyficznie stymuluje do podziałów neuralne komórki macierzyste izolowane z OUN. Wybór komórek nieprzylegających do dalszej selekcji i propagacji w hodowli, był również nieprzypadkowy. Założeniem było, że są to komórki niezróżnicowane, wrażliwe na EGF i o dużej zdolności proliferacyjnej. Doprowadziło to do skutecznego wyizolowania frakcji namnażających się komórek o charakterze neuralnych komórek macierzystych. Komórki te wykazywały zdolność do tworzenia klonów, pozytywnych pod względem ekspresji nestyny (zarówno na poziomie białka jak i RNA) – białka typowego dla neuralnych komórek macierzystych. Co wiecej, w obrębie tego samego klonu zidentyfikowano immunocytochemicznie komórki różnicujące się do trzech różnych fenotypów neuralnych (neuronów, astrocytów i oligodendrocytów), co świadczy o multipotencjalnym charakterze izolowanych komórek. Kwas Retinowy (RA) z BDNF (brain derived neurotrophic factor) lub zastosowanie tylko RA, powoduje różnicowanie wyizolowanej frakcji komórek macierzystych krwi pępowinowej w 35% w komórki o charakterze neuronów (35%), astrocytów (30%) i oligodendrocytów (10%). Stymulacja do

różnicowania neuralnego jest jeszcze bardziej skuteczna w obecności czynników typowych dla niszy neurogennej w mózgu, niż sama obecność badanych czynników wzrostowych. Udowodniono to w doświadczeniach, w których komórki macierzyste z krwi pępowinowej znakowano pochodnymi chlorometylowymi dwuoctanu fluoresceiny (Molecular Probs) i hodowano w ko-kulturze z komórkami izolowanymi z kory mózgu szczura. Spowodowało to ok.10% wzrost zdolności do różnicowania zarówno w kierunku neuronalnym jak i w kierunku astrocytalnym.

Wyprowadzenie stabilnie ukierunkowanej linii neuralnych komórek macierzystych (Human Umbilical Cord Blood - Neural Stem Cells: HUCB-NSC) z progenitorów pochodzących z krwi pępowinowej

Stabilna linia komórkowa neuralnych komórek macierzystych wywodzących się z krwi pępowinowej HUCB-NSC (Human Umbilical Cord Blood – Neural Stem Cells) została otrzymana bez uprzedniego unieśmiertelniania komórek (Bużańska i wsp. 2006). Stało się to możliwe, dzięki zastosowaniu metody hodowli *in vitro* przez selekcję komórek niezróżnicowanych (klonogennych, nieprzylegających) i ich propagację – wielokrotne pasażowanie - w obecności mitogennego czynnika wzrostowego EGF, promującego przeżycie komórek ukierunkowanych neuralnie (Roy i wsp. 2000, Singh i wsp. 2004). Ustabilizowanie linii jako neuralnej linii komórek macierzystych nastąpiło po jej przejściowej hodowli w warunkach bez surowicy w obecności czynników wzrostowych EGF, bFGF (basie fibroblast growth factor) i LIF (leukemia inhibitory factor). Umożliwiło to jednoczesną selekcję komórek ukierunkowanych neuralnie i ich stymulację do proliferacji. Badania immunocytochemiczne i molekularne wykazały, że komórki tak wyprowadzonej linii, po zastosowaniu neuromorfogenów (np. dBcAMP - Bużańska i wsp. 2006) lub niektórych czynników wzrostowych (np. CNTF – ciliary neurotrophic factor - Bużańska i wsp. 2006a) mogą się różnicować do fenotypów neuralnych prawie w 100% badanej populacji, co potwierdza neutralny charakter tej linii.

Linia HUCB-NSC utrzymywana jest już ponad cztery lata w ciągłej hodowli, (60-ty pasaż). Wykazano, że komórki HUCB-NSC mają prawidłowy ludzki kariotyp (46xy) i są wysoce klonogenne (wydajność - 10%), co pozwoliło na otrzymanie klonalnych podlini. W celu ustalenia, czy linia komórkowa jest stabilna i czy spełnia cechy linii neuralnych komórek macierzystych, porównywano dane dotyczące kinetyki wzrostu, klonogenności, stabilności kariotypu i potencjału do różnicowania pomiędzy wczesnymi (<10) i późnymi (>25, obecnie

w pasażu 42) pasażami linii podczas prawie trzech lat ciągłej hodowli. Wyniki wykazały brak różnic statystycznych pomiędzy porównywanymi pasażami udowadniając, że linia jest stabilna i spełnia cechy linii neuralnych komórek macierzystych. Stabilność kariotypu, brak zwiększonej ekspresji typowych onkogenów (np. *myc, ras*), inhibicja kontaktowa w warunkach hodowli konfluentnej, a także brak tworzenia guzów nowotworowych po przeszczepie HUCB-NSC do myszy NOD/SCID, pozbawionej odporności immunologicznej, wskazują na to, że nie jest to linia transformowana (Bużańska i wsp. 2006 oraz dane niepublikowane).

Propagacja hodowli komórek HUCB-NSC prowadzona jest w trzech różnych warunkach: 1) bez surowicy w obecności czynników LIF, EGF i bFGF – hodowla niezróżnicowanych, nieprzylegających komórek w formie luźno pływających nieregularnych lub typowych regularnych agregatów określanych jako „neurosfery", 2) w pożywce z niską zawartością surowicy (2%) wzbogaconą ITS (insulin/transferin/selenium), bez dodatkowych czynników wzrostowych – hodowla mieszana: komórek pływających (niezróżnicowanych) i przylegających do podłoża ukierunkowanych progenitorów oraz 3) w obecności 10% surowicy z dodatkiem mitogenów EGF i bFGF – hodowla komórek tylko przylegających, ale ciągle proliferujących zaawansowanych progenitorów neuralnych.

Badaliśmy ekspresję określonych białek typowych dla komórek rozwijającego się i dojrzałego OUN w różnych warunkach hodowli, stosując metodę znakowania immunocytochemicznego. W komórkach niezróżnicowanych HUCB-NSC, hodowanych bez surowicy, wysokiej ekspresji podlegają białka typowe dla neuralnych komórek macierzystych takie jak: nestyna (ok. 90%), GFAP (glial fibrillary acidic protein, ok. 40%) oraz neurofilament NF-200 (ok. 20%), chociaż struktura włóknista, typowa dla tych białek nie była widoczna. Białka typowe dla komórek zaawansowanych w różnicowaniu neuralnym (βtubulina III, MAP2 – microtubul associated protein 2: w kierunku neuronów, S100β w kierunku astrocytów, GalC, O4: w kierunku oligodendrocytów) w tych warunkach nie podlegają ekspresji. W komórkach adherentnych, hodowanych w obecności 2% surowicy zarówno ekspresja ja k i organizacja strukturalna tych białek zmieniła się: dla nestyny i GFAP poziom ekspresji spada odpowiednio do 15% i 23% komórek w populacji, w przypadku NF200 zwiększa się do ok. 30 %, wszystkie te białka występują w formie spolimeryzowanej. Wynik ten tłumaczymy stopniową utratą wczesnych markerów „macierzystych i neuralnych", do których należą nestyna i GFAP, z jednoczesnym zwiększeniem ekspresji białek charakterystycznych dla neuronów takich jak NF-200 w ukierunkowanych progenitorach neuralnych, jakimi są komórki przylegające HUCB-NSC. Równocześnie we frakcji

przylegającej komórek HUCB-NSC hodowanych w pożywce z niską zawartością surowicy, pojawia się ekspresja β tubuliny III, S100β i GalC, co świadczy o stymulacji do różnicowania tej hodowli we wszystkie trzy linie typowe dla OUN. Na uwagę zasługuje pozornie nietypowa ekspresja GFAP – białka charakterystycznego dla zróżnicowanych astrocytów, w niezróżnicowanych, nieadherentnych HUCB-NSC. Badania prowadzone w wielu laboratoriach wykazały, że białko to podlega wysokiej ekspresji również w neuralnychych komórek macierzystych, ale tylko pochodzenia ludzkiego (Doetsch 2003, Conti i wsp. 2005). HUCB-NSC hodowane w wysokim stężeniu surowicy, bez mitogenów, lub w pożywce z małą zawartością surowicy, ale w obecności neuromorfogenów zmieniają fenotyp na bardziej przypominający komórki o charakterze neuronów, astrocytów czy oligodendrocytow, zarówno pod względem morfologii, jak i pod względem ekspresji białek. Świadczy to o znaczącym wpływie warunków hodowli i związanej z tym dostępnością czynników epigenetycznych na różnicowanie HUCB-NSC.

W innej pracy, która nie wchodzi w skład rozprawy habilitacyjnej (Jurga i wsp.2006) wykazaliśmy również wpływ czynnikow genetycznych na zdolność do różnicowania HUCB-NSC. Podobnie jak w przypadku ludzkich transformowanych komórek macierzystych linii DEV (Bużańska i wsp. 2001), podejmowanie decyzji rozwojowej o sposobie różnicowania zależy m.i. od ekspresji czynników transkrypcyjnych typu bHLH. Różnicowanie w kierunku neuronów komórek linii HUCB-NSC hamowane jest obecnością bądź endogennego, bądź dostarczonego drogą transfekcji inhibitora ID1, a rozmieszczenie wewnątrzkomórkowe białka ID1 (jądro lub cytoplazma) może być wskaźnikiem stanu macierzystości komórek HUCB-NSC.

Standaryzacja wzrostu i różnicowania HUCB-NSC

Stabilna linia HUCB-NSC stanowi zarówno łatwo dostępne źródło neuralnych komórek macierzystych, jak również umożliwia standaryzację układów eksperymentalnych do badań *in vitro.* Co więcej, wykazaliśmy (Bużańska i wsp. 2005, Bużańska i wsp. 2006, Jurga i wsp. 2006), że HUCB-NSC mogą być hodowane jako warstwa przylegających do podłoża komórek, lub jako przestrzenne pływające konglomeraty niezróżnicowanych komórek o charakterze neurosfer. „Neurosfery" są to struktury typowe dla hodowli neuralnych komórek macierzystych zarówno somatycznych (izolowanych z mózgu płodu lub mózgu dorosłego osobnika, Svendsen i wsp. 1999), jak i zarodkowych (wyprowadzanych

z blastocysty rozwijającego się zarodka, O'Shea 2002). Zdolność HUCB-NSC do tworzenia „neurosfer" potwierdza neuralny charakter wyprowadzonej przez nas linii komórkowej.

W pracy Bużańska i wsp. 2005, ustaliliśmy optymalne warunki wzrostu i różnicowania HUCB-NSC w długo- i krótkoterminowej hodowli HUCB-NSC. Badania prowadzono w 2-wymiarowej hodowli adherentnej i przestrzennej 3-wymiarowej hodowli reprezentowanej przez neurosfery. W warunkach standaryzowanych hodowli przylegającej oszacowaliśmy tempo proliferacji i przeżywalność komórek oraz ich zdolność do różnicowania. Wykazaliśmy, że podczas różnicowania spontanicznego indukowanego jedynie adhezją komórek do podłoża, w warunkach hodowli 2-wymiarowej, komórki częściej uzyskują fenotyp neuronalny (ok. 30 %) niż astroglialny (astrocyty ok.10%, oligodendrocyty ok.2%). Badaliśmy również proliferację i zdolność do różnicowania komórek HUCB-NSC rosnących w neurosferach. Adhezja neurosfer do podłoża stymuluje spontaniczną migrację i różnicowanie komórek HUCB-NSC. Proces ten może być kontrolowany obecnością czynników wzrostowych (np. LIF/CNTF stymuluje proliferacje komórek w neurosferach), a także składnikami macierzy zewnątrzkomórkowej, (np. fibronektyna hamuje zarówno proliferację jak i migrację komórek) (Bużańska i wsp. 2005).

Procesem różnicowania HUCB-NSC możemy sterować - w warunkach przylegania do podłoża i w obecności neuromorfogenów podejmowane decyzje rozwojowe dotyczące neuronalnego lub astocytalnego, czy oligodendroglialnego fenotypu zależą od rodzaju zastosowanego neuromorfogenu lub czynnika wzrostowego (Bużańska 2006a). Badaliśmy wpływ obecności niektórych czynników wzrostowych i neuromorfogenów (PDGF-AA, PDGF-BB, RA, T3, CNTF, cAMP) w 10-ciu różnych kombinacjach w standaryzowanej hodowli na kierunkowe różnicowanie HUCB-NSC w komórki o charakterze neuronów, astrocytów i oligodendrocytów. Wykazaliśmy, że CNTF promuje różnicowanie w kierunku neuronów (ok.80% komórek βtubulin III pozytywnych), PDGF-BB + RA w kierunku astrocytów (ok.65% komórek S100β pozytywnych), natomiast PDGF-AA + T3 w kierunku oligodendrocytów (ok.12% komórek GalC pozytywnych) (Bużańska i wsp. 2006a oraz dane niepublikowane).

Opracowaliśmy metody umożliwiające utrzymywanie komórek linii HUCB-NSC w hodowli *in vitro,* na różnych etapach rozwoju, odzwierciedlających hierarchię ich wzrostu i różnicowania: od niezróżnicowanych, poprzez progenitory neuralne o zawężonym spektrum rozwojowym, do komórek zróżnicowanych (Bużańska i wsp. 2005, rys. 6). Możliwość izolacji frakcji komórek HUCB-NSC odzwierciedlającej określony etap rozwoju ontogenetycznego neuralnych komórek macierzystych sprawia, że linia HUCB-NSC jest

dobrym modelem badań nad neurotoksycznością rozwojową (Bużańska i wsp. 2005, Bużańska i wsp. 2006a).

Analiza molekularna mechanizmów leżących u podstaw utrzymania „macierzystości" wyprowadzonej linii HUCB-NSC.

Analiza molekularna z zastosowaniem mikromacierzy DNA polegała na badaniu profilu transkrypcyjnego komórek niezróżnicowanych linii HUCB-NSC i różnicowanych pod wpływem dBcAMP (HUCB-NSC Differentiated - HUCB-NSCD) oraz populacji referencyjnej komórek mononuklearnych izolowanych z krwi pępowinowej w taki sam sposób, w jaki izolowano komórki linii HUCB-NSC (frakcja komórek CD 34-, CD 45-) (Bużańska i wsp. 2006).

Porównanie profilu transkrypcyjnego niezróżnicowanych komórek HUCB-NSC i wyjściowych, CD 34(-), komórek mononuklearnych krwi pępowinowej wykazało, że 93% wybranych genów, typowych dla neuralnych komórek macierzystych ludzkich (Wright i wsp. 2002) aktywnych w HUCB-NSC, nie podlega ekspresji w frakcji referencyjnej komórek mononuklearnych (HUCB-MC). Świadczy to o neuralnym charakterze HUCB-NSC.

Wiadomo obecnie, że za utrzymywanie „macierzystości", czyli zdolności do samoodnowy zarówno komórek somatycznych jak i embrionalnych odpowidzialna jest aktywacja komórkowych szlaków przekazywania sygnału takich jak WNT/βCatenina (Sato i wsp. 2004) oraz LIF/JAK/STAT (Smith i wsp. 1998, Wright i wsp. 2003). W przypadku neuralnych komórek macierzystych wykazano również aktywację szlaku DELTA/NOTCH oraz receptora FGFR1 (D'Amour i Gage, 2003). W naszych badaniach, w niezróżnicowanych komórkach HUCB-NSC wykazaliśmy wzmocnioną aktywność szeregu genów związanych z w/w drogami sygnalowymi. Dla drogi inicjowanej sygnałem liganda Lif są to geny: *lifR* , *lif, jak* oraz *stat.* Aktywacja szlaku WNT koreluje z nadekspresją genów takich jak *wnt, frizzled, lpr, β–catenina, cadheryna i tcf,* natomiast aktywację drogi DELTA/NOTCH reprezentuje zwiększona ekspresja *jagged2, notch3, hey1* oraz *pen-2.* O neuralnym ukierunkowaniu niezróżnicowanej frakcji komórek HUCB-NSC świadczy między innymi wzrost ekspresji genów dla czynników wzrostowych FGF, PDGF, NRG1 oraz ich receptorów: FGFR1, FGFR3, PDGFR oraz ERBB2. Na szczególą rolę w tym procesie receptora FGFR1 wskazuje aż 600-krotne zwiększenie aktywności tego genu w porównaniu z wyjściową frakcją mononulklearną komórek krwi pępowinowej.

Różnicowanie HUCB-NSC w komórki o charakterze funkcjonalnych neuronów.

Analizę molekularną przy użyciu Mikromacierzy DNA zastosowano również do badania ekspresji genów w różnicowanych HUCB-NSC w obecności dBcAMP. W komórkach różnicowanych następuje aktywacja genów związanych z receptorami białek G (*gpr17*), a także wzrost ekspresji genów specyficznych dla neuronów np.: *tau*, *lxn* (lateksyna), *calb2* (kalretynina), dekarboksylaza glutaminianowea (GAD 67), czy też genów kodujących białka zasocjowane z receptorem GABA: GABARAPL3 i GABARAPL1. Analiza immunocytochemiczna potwierdziła obecność białek kodowanych przez te geny. Uwagę należy zwrócić również na wzrost ekspresji genów związanych z przekaźnictwem synaptycznym: *sv2a*(Synaptic vesicle 2a), *synj1*(synaptojanin1), *pclo* (Piccolo) oraz *nptx1*(neuronal pentraxin) (Buzanska i wsp. 2006).

Badania elektofizjologiczne prowadzone metodą „patch clamp" wykazały w komórkach różnicowanych potencjał spoczynkowy wysokości 50mV oraz obecność potencjało-zależnych prądów potasowych (Kir- „inward rectifying potassium current" oraz I_{K+} - „outward recitifying potassium current"). Kir był modyfikowany stymulacją receptorów dopaminergicznych, gabaergicznych, glutamatergicznych i serotoninergicznych. Obecność tych receptorów została potwierdzona immunocytochemicznie. Badania profilu transkrypcyjnego różnicowanych HUCB-NSC przy zastosowaniu mikromacierzy DNA wykazały ekspresję genów dla potencjalo-zależnych kanałów potasowych i sodowych jak również receptorów dla neurotransmiterów takich jak Ach, GABA, 5-HT, DA, glutamina, czy też glicyna. Świadczy to o funkcjonalnym różnicowaniu HUCB-NSC w komórki o charakterze neuronów. Jednakże brak zapisu potencjałów czynnościowych, jak również brak ekspresji niektórych receptorów (np. NMDA) wskazują to, że komórki HUCB-NSC różnicowane w obecności dBcAMP mają cechy funkcjonalnego, ale jeszcze niedojrzałego systemu neuronalnego (Sun,Bużańska i wsp. 2005, Bużańska i wsp. 2006a). Obecnie prowadzone badania, w których HUCB-NSC hodowane są bezpośrednio na podłożu z wbudowanymi elektrodami wskazują na możliwość uzyskania potencjalu czynnościowego w różnicowanych komórkach HUCB-NSC (dane niepublikowane).

Wyniki opisane w komentarzu zawarte są w zbiorze pięciu prac oryginalnych, których jestem pierwszym autorem. Prace te dołączone są do tego komentarza i stanowią jego rozwinięcie dotyczące zastosowanych metod i otrzymanych wyników szczegółowych.

Przedstawione dowody otrzymania neuralnych komórek macierzystych z ludzkiej krwi pępowinowej i wyprowadzenie stabilnej linii komórkowej HUCB-NSC są pionierskie w tej dziedzinie w skali światowej.

Literatura

Bużańska L., Spassky N., Belin M.F., Giangrande A., Guillemot F., Klambt C., Labouesse M., Thomas J.L., Domanska-Janik K., Zalc B. Human medulloblastoma cell line DEV is a potent tool to screen for factors influencing differentiation of neural stem cells. J Neurosci Res 2001; 65:17-23.

Bużańska L., Machaj E. K., Zablocka B., Pojda Z., Berdowska P. and Domańska-Janik K. Human cord blood derived neurons, astrocytes and oligodendrocytes. J. Neurochem. 2001a 78 Suppl. 1, 58.

Bużańska 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 2002, 115: 2131-2138.

Bużańska L., Habich A., Jurga M.., Sypecka J., Domańska-Janik K., "Human Cord Blood-derived Neural Stem Cell Line – Possible Implementation in Studying Neurotoxicity", Toxicology in Vitro 2005; 19:991-999.

Bużańska L., Jurga M., Stachowiak E.K., Stachowiak M.K., Domańska-Janik K. „Neural Stem-like Cell Line Derived from Nonhematopoethic Population of Human Umbilical Cord Blood", Stem Cells and Development 2006, 15(3).

Bużańska L., Jurga M., Domańska-Janik K. „Neuronal differentiation of Human Umbilical Cord Blood Neural Stem-Like Cell Line" Neurodegenerative Dis. 2006a; 3:19-26.

Carpenter M.K., Rosler E., Rao M.S. Characterization and differentiation of human embryonic stem cells. Cloning Stem Cells, 2003; 5(1):79-88.

Conti L, Pollard SM, Gorba T, Reitano E, Toselli M, Biella G, Sun Y, Sanzone S, Ying Q, Cattano E, Smith A: Niche-independent symmetrical self-renewal of mammalian tissue stem cell. PLOS Biol 2005; 3: 1–12.

Doetsch F. A niche for adult neural stem Wells. Cur Opin Genet Dev 2003; 13:543-550

Hsieh J., Gage F. Epigenetic control of neural stem cell fate Cur Opin Genet Dev 2004; 14:461-469.

Humpherys D., Eggan K., Akutsu H., Hochedlinger K., Rideout W.M.3rd, Biniszkiewicz D., Yanagimachi R., Jaenisch R. Epigenetic instability in ES cells and cloned mice. Science. 2001; 293(5527):95-7.

Jiang Y, Jahagirdar Bn, Reinhardt Rl, Schwartz Re, Keene Cd, Oritz-Gonzalez Xr, Reyes M, Lenvik T, Lund M, Du J, Aldrich S, Lisberg A, Low Wc, Largaespada Da, Verfaillie C. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 2002; 418: 41-49.

Jurga M., Makarewicz I, Sarnowska A., Habich A., Kozłowska H., Łukowska B., Bużańska L., Domańska-Janik K. Neurogenic Potential of Human Umbilical Cord Blood-Neural Stem

Cells Depends on Previous Long-Term Culture Conditions. Journal of Neuroscience Research 2006; 83:627–631.

Jurga M., Bużańska L., Habich A. Małecki M., Domańska-Janik K. Function of ID1 protein in human cord blood – derived neural stem-like cells. Journal of Neuroscience Research 2006 (proofs).

Kucia M., Zhang Y.P., Recal R., Wysoczynskil M., Machalinskil M., Majka M., ST Ildstad S.T., Ratajczak J.,CB Shields C.B., Ratajczak M.Z. Cells enriched in markers of neural tissue-committed stem cells reside in the bone marrow and are mobilized into the peripheral blood following stroke. Leukemia (2006) 20, 18–28.

Li M., Pevny L., Lovell-Badge R., Smith A. Generation of purified neural precursors from embryonic stem cells by lineage selection. CurrBiol, 1998; 8: 971-974.

Liu Y., Rao M.S. Transdifferentiation – fact or artifact *J Cell Biochem* 2003
Rao M. Conserved and divergent paths that regulate self-reneval in mouse and human embryonic stem cells Dev Biol 2004; 275:269-286.

O'Shea K.S. Neural differentiation of embryonic stem cells. Ed. T. Zigowa, P.R. Sandberg, J.R. Sanchez-Ramos Neural stem Cells: Methods and Protocols. Methods in Mollecular Biology, vol. 198: 2002, 189: 3-14.

Roy NS, Wang S, Jiang L, Kang J, Benraiss A, Harrison-Restelli C, Fraser RA, Couldwell W.T., Kawaguchi A., Okano H., Nedergaard M., Goldman S.A. In vitro neurogenesis by progenitor cells isolated from the adult human hippocampus. Nat Med. 2000; 6(3):271-7.

Sanchez-Ramos, J., Song, S., Cardozo-Pelaez, F., Hazzi, C., Stedeford, T., Willing, A., Freeman, T. B., Saporta, S., Jansen, W., Patel, N. et al. Adult bone marrow stromal cells differentiate into neural cells in vitro. Exp. Neurol. 2000; 164,247 -256.

Sanchez-Ramos J.R., Song S., Kamath S.G., Zigova T., Willing A., Cardozo-Pelaez F., Stedeford T., Chopp M., Sanberg P.R. Expression of neural markers in human umbilical cord blood. Exp Neurol 2001;171:109-15.

Sherley J.L. Asymmetric cell kinetics genes: the key to expansion of adult stem cells in culture. Stem cells 2002; 20: 561-572.

Singh S.K., Ian D Clarke I.D.,Hide T., Dirks P.D. Cancer stem cells in nervous system tumors. Oncogene (2004) 23, 7267–7273

Sommer N., Rao M. Neural stem cells and regulation of cell number. Progress in Neurobiology 2002; 66, 1-18.

Song S., Sanchez-Ramos J. Brain as the sea of marrow. Exp. Neurology 2003; 184:54-60.

Svendsen, C. N., Caldwell, M. A. and Ostenfeld, T. Human neural stem cells: isolation, expansion and transplantation. Brain Pathol. 1999; 9, 499-513.

Sun W.*, Bużańska L.*, Domańska-Janik K., Salvi R.J., Stachowiak M.K., "Voltage-sensitive and ligand-gated channels in differentiating neural stem-like cells derived from the nonhematopoietic fraction of human umbilical cord blood" Stem Cells 2005; 23:931-945, * - equally contributing first authors.

Thomson J. A, Itskovitz-Eldor J., Shapiro S.S, Michelle A. Waknitz M.A., Swiergiel J.J, Marshall V.S., Jones J.M., Embryonic Stem Cell Lines Derived from Human Blastocysts Science1998; 282, 1145.

Toma J.G., Akhavan M., Fernandes K.J., Barnabe-Heider F., Sadikot A., Kaplan D.R., Miller F.D. Isolation of multipotent adult stem cells from the dermis of mammalian skin, Nat Cell Biol. 2001 3(9):778-84.

Tondreau T, L Lagneaux, M Dejeneffe, M Massy, C Mortier, A Delforge and D Bron. Bone marrow derived mesenchymal stem cells already express specific neural proteins before any differentiation. Differentiation 2004 72:319–326.

Vescovi A.L, Parati E.A, Gritti A., Poulin P., Ferrario M., Wanke E., Frolichsthal Schoeller P., Cova L., Arcellana-Panlilio M., Colombo A., Galli R. Isolation and cloning of multipotential stem cells from the embryonic human CNS and establishment of transplantable human neural stem cell lines by epigenetic stimulation. Exp Neurol 1999156:71-83.

Woodbury, D., Schwarz, E. J., Prockop, D. J. and Black, I. B. Adult rat and human bone marrow stromal cells differentiate into neurons. J. Neurosci. Res. 2000 61,364 -370.

Wright LS, J Li, MA Caldwell, K Wallace, JA Johnson and CN Svendsen. (2003). Gene expression in human neural stem cells: effects of leukemia inhibitory factor. J Neurochem 86:179–195.

Wurmser A.E., Palmer T.D., Gage F.H. Cellular interactions in the stem cell niche Science 2004; 304: 1253-1254.

PUBLIKACJA 1

Human cord blood-derived cells attain neuronal and glial features in vitro

L. Bużańska[1], E. K. Machaj[2], B. Zabłocka[1], Z. Pojda[2] and K. Domańska-Janik[1,*]

[1]Medical Research Centre, Polish Academy of Sciences, 5 Pawinskiego St. 02-106 Warsaw, Poland
[2]Institute of Oncology, 5 Roentgena St. 02-781 Warsaw, Poland
*Author for correspondence (e-mail: kd-j@cmdik.pan.pl)

Accepted 20 February 2002
Journal of Cell Science 115, 2131-2138 (2002) © *The Company of Biologists Ltd*

Summary

Neural stem cells are clonogenic, self-renewing cells with the potential to differentiate into brain-specific cell lines. Our study demonstrates that a neural-stem-cell-like subpopulation can be selected and expanded in vitro by the use of human umbilical cord blood cells, which are a relatively easily available starting material. Through a combination of antigen-driven magnetic cell sorting and subfractionation according to cell surface adhesive properties, we have isolated a clonogenic fraction devoid of hematopoietic or angiogenetic properties but with relatively high self-renewal potency. The resulting clones express nestin, a neurofilament protein that is one of the most specific markers of multipotent neural stem cells. In the presence of selected growth factors or in the rat brain co-culture system, the progeny of these cells can be oriented towards the three main neural phenotypes: neurons, astroglia and oligodendroglia. The cells show high commitment (about 30% and 40% of the population) to neuronal and astrocytic fate, respectively. Interestingly, upon differentiation, the neural-type precursor cells of cord blood origin also give rise to a relatively high proportion of oligodendrocytes – 11% of the total population of differentiating cells.

Key words: Human stem cells, Cord blood, Neural differentiation, Neural progenitors, Transdifferentiation

Introduction

Long-standing dogma states that neural stem cells with the ability to differentiate into neurons, astrocytes and oligodendrocytes are derived embryologically from neuroepithelial progenitors. However, several studies from different laboratories have recently reported that human or rodent mesenchymal bone marrow (BM) cells can be directed in vivo and in vitro into a neuronal or astrocytic fate (Azizi et al., 1998; Kopen et al., 1999; Sanchez-Ramos et al., 2000; Woodbury et al., 2000). Vice versa, neural stem cells isolated from the adult brain can develop blood cell elements (Bjornson et al., 1999) and give rise to all germ cell layers (Clarke et al., 2000) owing to the surprising versatility of their differentiation program. Moreover, rodent BM cells can invade the brain of previously irradiated or genetically myeloid-ablated recipients and give rise not only to mesenchyma-derived brain macrophages or microglia but also to astroglia and neurons, which were previously thought to belong exclusively to the neuroepithelial lineage (Eglitis and Mezey, 1997; Brazelton et al., 2000; Mezey et al., 2000). The above data together with the recently reported ability of stem cells residing in various organs to omit tissue-restricted specification (Alison et al., 2000; Krause et al., 2001; Zuk et al., 2001) suggest that classical barriers of cell differentiation can be broken down under certain permissive conditions, rendering the dogmatic 'point of no return' of cellular lineage inaccurate. These results also indicated a remarkable plasticity in tissue-specific stem cells and encouraged us to look for a source of precursors that could be committed to neural fate in tissues other than neuroectodermal.

We selected umbilical cord blood (CB) cells for our study. These cells are easily available and preserved, and they could potentially serve as a routine starting material for isolation and expansion of cells for allogenic as well as authologous transplantations. The preliminary results of this study have already been presented (Bużańska et al., 2001a; Machaj et al., 2001). Here, using the method of CB cell subfractionation and their subsequent culturing in the presence of defined media and growth factors, we were able to generate a self-renewing, clonogenic cell population with neural-type precursor characteristics.

Materials and Methods

Collection, isolation and propagation of CB cells in vitro

Cord blood was collected after obtaining the approval of a local Ethical Committee and the mother's informed consent. Blood was collected after delivery of the placenta by puncturing umbilical cord veins (>40 ml/sample). Cell numbers, viability and blood sterility were evaluated, and blood storage time did not exceed 12 hours. The mononuclear cell fraction was isolated on a Ficoll/Hypaque gradient, and the cells that bound to the immunomagnetic beads coated with anti-CD34 antibody were eliminated by immunomagnetic sorting (MilteneyiBiotek anti-CD34 Isolation Kit). After washing, the remaining cells were resuspended in Iscove's modified Dulbecco's (IMDM, Gibco) medium supplemented with 10% fetal calf serum (FCS, Gibco) at a final concentration of 10^6 cells/ml. Plastic-adherent cells were cultured for 3 weeks in IMDM plus 10% FCS at 37°C, 5% CO_2, in a fully humidified atmosphere and with 50% of the media being changed every week. Before reaching the monolayer phase, cells were trypsinised and re-cultured in similar conditions for the next 3 weeks. At the beginning and the end of the culturing period, the cells were analysed by flow cytometry.

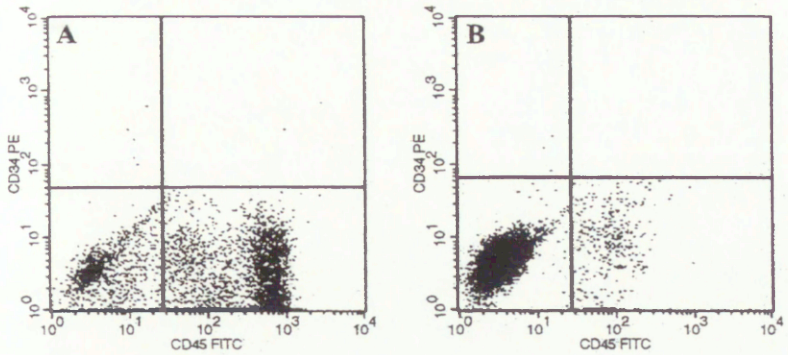

Fig. 1. Flow cytometry of fresh $CD34^+$-immunodepleted cells (A) and cells following 6 weeks of selection in vitro (B).
(A) Immunomagnetic depletion of the initial mononuclear fraction resulted in approximately 100% elimination of $CD34^-$ cells, whereas up to 80% of cells expressed CD45 antigen.
(B) Following 6 weeks of culture of $CD34^-$-depleted cells in conditions described in the Materials and Methods, $CD45^-$ cells were reduced to almost negligible levels (less than 4% of the whole cell population).

Flow cytometry analysis

A Becton-Dickinson FACS Scan and commercial antibodies (HPCA-2 anti-CD34 phycoerithrine-conjugated and anti-CD45 fluoresceine-conjugated) were used for FACS examination.

Cell culture expansion and clone formation

For cell culture expansion, trypsin-removed cells were plated in plastic 25 cm^2 culture flasks at a density of 5×10^4 cells/cm^2 in DMEM (Gibco) supplemented with 10% FCS, EGF (epidermal growth factor, Sigma) at 10 ng/ml and antibiotic-antimycotic solution (AAS, Sigma, 1:100). The cells were grown for 7 days to obtain a monolayer. Some confluent cultures were re-seeded after trypsinisation, whereas some were kept for 5 days longer in order to obtain free floating, non-adherent cells. Both kinds of cell, when transferred to separate flasks or multi-well plates at a density of about 10 cells/cm^2 in the presence of EGF, started to grow clones within the next 7 days in culture. The clones were observed to grown in size during the following 14 days. As the cells proliferated, some of them detached from the plastic and remained floating in suspension; however, they stayed viable and could give rise to new clones. After reseeding, these cells can be maintained as an adherent, undifferentiated, clonogenic population in the presence of EGF and FCS during the six, already tested, passages.

In additional experiments, these cells were cultured in the commercially available clonal cell culture system (Methocult H 4330, Stem Cell Technologies), which support the growth of both erythroid and myeloid precursors. The cells were analysed for the possible appearance of erythropoietic (BFU-E), granulo/macrophagopoietic (GM-CFC) and mixed (CFU-GEMM) colonies after 14 days of culture at 37°C in 5% CO_2 in a fully humidified atmosphere.

Differentiation of nestin-expressing cells derived from cord blood

In culture media

Clones that had been grown for 14 days in the conditions described above were treated directly with Neurobasal Media supplemented with 10% FCS and 0.5 μM all-trans-retinoic acid (RA, Sigma) for the following 4 days.

In separate experiments, clone-growing cells were collected by trypsinisation and plated on poly-L-lysine 24-well tissue culture plates at a density 5×10^4 cells/cm^2. The media used for promoting cellular differentiation was as follow: (1) Neurobasal Medium (Gibco) supplemented with 10% FCS (Gibco); (2) Neurobasal Medium supplemented with 10% FCS and 0.5 μM RA; (3) Neurobasal Medium supplemented with 10% FCS, 0.5 μM RA and BDNF (Sigma) at a concentration of 10 ng/ml.

In each case, cells were incubated at 37°C in 5% CO_2 in a fully humidified atmosphere for 4 days and fixed for immunocytochemical detection of neural-specific antigens.

In the presence of the cortical primary culture

Mixed primary cultures were prepared from the brain cortex of 18-19 day-old rat embryos (Wistar) under sterile conditions. Dissected tissue was placed in Ca^{2+}- and Mg^{2+}-free HBSS (Gibco), dispersed mechanically (10-12 pipette strokes) and then enzymatically by a 15 minute incubation in 0.2% trypsin (Gibco). After centrifugation at 200 *g* for 3 minutes the pellets were resuspended in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% FCS (Gibco) under antibiotic-antimycotic protection (AAS, Sigma 1:100). After triturating, the debris was removed by filtration through Millipore cell strainers (45 μm in diameter). Viable cells were plated at a density of 5×10^4 cells/cm^2 on poly-L-lysine 24-well tissue culture plates in 500 μl DMEM supplemented with 10% FCS and AAS (1:100). The cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C and allowed to grow for 7 days before CB-derived cells were added. Undifferentiated cells from nestin-positive clones were collected by trypsinisation and prelabelled with green 'cell tracker' (5-chloromethyl-fluorescein-diacetate, Molecular Probes Inc), according to the manufacturer's recommendation. CB-derived cells were seeded on the monolayer of rat brain cells at a density of 5×10^4 cells/cm^2. Cells were allowed to grow in such co-culture conditions for between 4 and 8 days and were then fixed for immunocytochemistry.

Western blotting

The cultures were harvested in PBS, counted and lysed in the Laemmli (Laemmli, 1970) gel loading buffer in the proportion of 3.4×10^5 cells per 100 μl. The equal-volume samples were separated by SDS-PAGE on a 10% polyacrylamide gel and transferred onto Hybon-C-Extra. Immunodetection was performed using the monoclonal anti-β-tubulin III (Sigma), polyclonal anti-GFAP (DAKO) and polyclonal anti-PLP/DM-20 (gift from J.-M. Matthieu). The immunoblots were incubated with horseradish-peroxidase-conjugated secondary antibodies, anti-rabbit for GFAP and PLP/DM-20 antigens and anti-mouse for β-tubulin III detections, then developed by ECL (Amersham).

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde diluted in PBS for 20 minutes, then washed with PBS and blocked in PBS containing 50% sheep serum and 10% FCS (60 minutes). First antibodies were applied overnight at 4°C. Anti-human nestin, a rabbit polyclonal antibody (gift of U. Lendahl, Karolinska Institute, Stockholm) was applied at a concentration 1:1000, according to Grigelioniene et al. (Grigelioniene et al., 1996). The three following primary antibodies, mouse monoclonal TUJ1 (Easter et al., 1993) directed against the β-tubulin isoform III (gift of A. Frankfurter, University of Virginia, Charlottesville, VA), mouse monoclonal anti-MAP-2 (microtubule

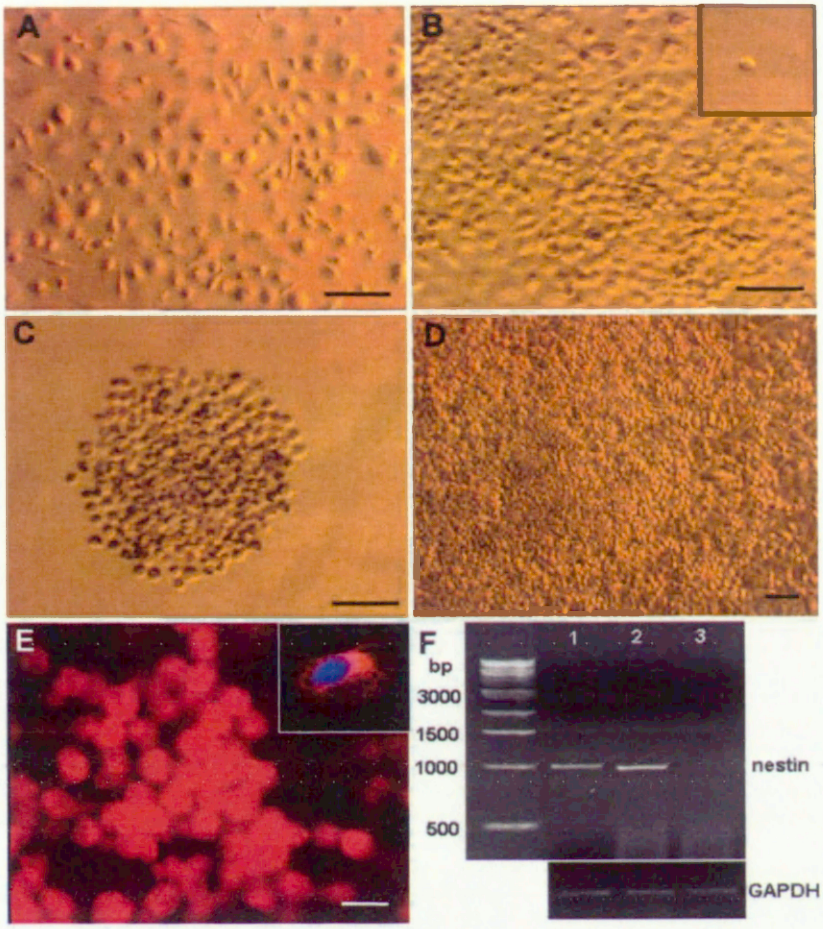

Fig. 2. Nestin-positive clones obtained from the selected human cord blood subpopulation. Phase-contrast images of CB-derived cells. (A) The $CD34^+$-depleted cells that were characterised by FACS in Fig. 1B. (B) Cells from A after being re-seeded and expanded in DMEM/10% FCS and 10 ng/ml EGF to form a monolayer of homogenous, round, proliferating cells. An example of a typical, single, clonogenic cell from this culture is shown in the insert. (C) A clone growing for 14 days in the presence of EGF, after low-density (10 cells/cm^2) suspension of cells from culture presented in B. (D) The same clone as in C after another 7 days in culture displays a 10-fold increase in the number of cells as quantified by cell counting. (E) Immunocytochemical staining with the anti-human nestin polyclonal antibody shows that the majority of cells are immunoreactive in growing the clone. The insert shows a higher magnification (40×) of a nestin-positive cell with a Hoechst 33258-stained nucleus, showing a typical filamentous pattern of immunostaining. (F) RT-PCR analysis of a *nestin* gene expression in cells growing in clones (lines 1 and 2) contrasted with an almost complete lack of signal in mRNA sample extracted from CB-derived (not EGF expanded) cells growing in a monolayer as shown in A. Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in parallel samples served as a semi-quantitative control for RT-PCR products. Bars: A, 50 µm; B-D, 100 µm; E, 20 µm.

associated protein 2, Sigma) and rabbit polyclonal anti-GFAP (glial fibrillary acidic protein, purchased from Dakopatts), were diluted 1:2000, 1:100 and 1:200, respectively, in PBS/gelatine containing 0.2% Triton X-100. The mouse monoclonal anti-GalC (galactosylceramide) antibody (Ranchst et al., 1982), a culture supernatant obtained from R-mAb hybridoma cells (gift of B. Zalc, INSERM U-495, Paris) was used at a dilution of 1:50 in DMEM with 10% FCS. Secondary antibodies, anti-mouse IgG FITC for MAP2 (Sigma), anti-mouse IgG2a-TxR for TUJ1, anti-mouse IgG3-TxR for GalC or anti-rabbit IgG-TxR for GFAP (all from Pharmingen), were

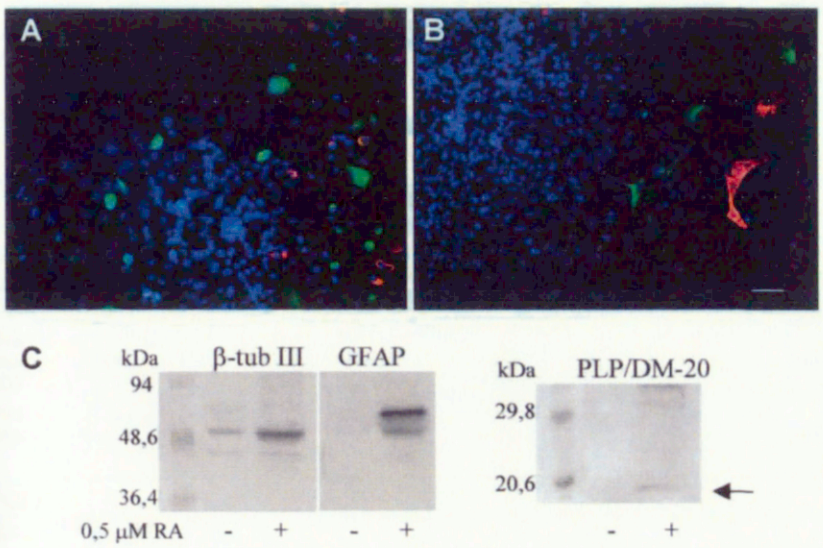

Fig. 3. Expression of neural marker proteins by the clone-growing cells after 4 days of treatment with retinoic acid. (A,B) Double immunostaining for the presence of β-tubulin III (FITC-conjugated secondary antibody) and GFAP or GalC (Texas-Red-conjugated secondary antibodies). Cells derived from the same clone can express neuronal and astroglial (A) or neuronal and oligodendroglial (B) markers. All cell nuclei were additionally stained with Hoechst 33258. Bar, 50 µM. (C) Western blots of the untreated (RA−) controls and the RA-treated (RA+) differentiated cells probed with anti-β-tubulin III (neuronal marker), anti-GFAP (astrocytic marker) and anti-PLP/DM-20 (oligodendrocytic marker). The position of the PLP splicing variant, DM-20, is marked by arrow.

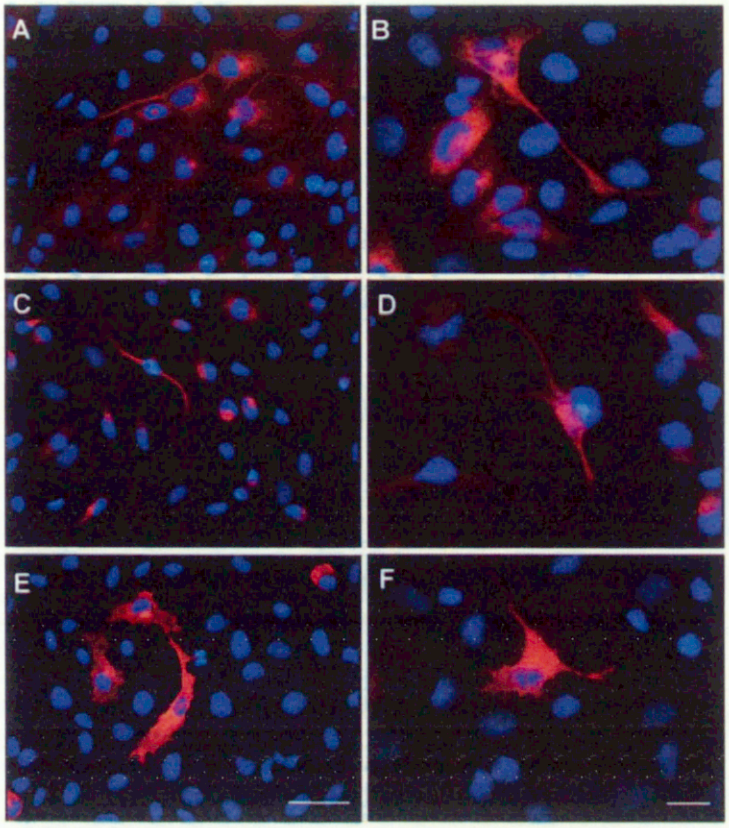

Fig. 4. Cell-type-specific immunostaining for neurons (A,B), astrocytes (C,D) and oligodendrocytes (E,F) in CB-derived cells growing in differentiation-promoting media. (A,B) Cells expressing class III β-tubulin recognised by the TUJ1 antibody and displaying neuron-like morphology with long axonal projections. The filamentous feature of cytoplasmic structures that are immunoreactive with the TUJ1 antibody and corresponding to neuronal microtubules are clearly visible under higher magnification (B). (C,D) Cells immunostained with the anti-GFAP antibody. Some of the cells are round and relatively small, whereas others contain long projections with immunoreactive filamentous structures that are visible in the cytoplasm (D). (E,F) GalC-immunoreactive cells expressing galactosylceramides and displaying morphology characteristic of oligodendrocytes, with flat cell body and short or long branched projections. Smaller, round immunoreactive cells are also occasionally present (see the right upper corner). Bars: A,C,E, 50 μm; B,D,F, 20 μm.

diluted 1:100 in the same solution as the first antibody and applied for 1 hour at room temperature. As a control for immunocytochemistry (in order to exclude non-specific background staining), first antibodies were omitted during the procedure. To visualize the nuclei, the cultures were then incubated with 5 μM Hoechst 33258 (Sigma) (20 minutes at room temperature) before being mounted in Fluoromount-G (Southern Biotechnology Associate Inc., USA) either directly on the bottom of 24-well plates or on glass slides of poly-L-lysine-coated cover slips.

Microscopy and quantification

The live growing cells or prefixed immunocytochemically labelled cultures were observed either in the phase contrast or in the UV light under fluorescence microscopes using Axiovert 25 or Axioscope 2 (Carl Zeiss), respectively. Images were captured by the Videotronic CCD-4230 camera coupled with the microscope and processed using the computer-based programmable image analyser KS300 (Carl Zeiss).

The formation of clones by mitogen-expanded cells, which were selected from three independent cord blood preparations, was followed for at least four weeks for three or more randomly chosen clones. Differentiation towards a particular cell phenotype was quantified as a percentage of the total number of CB-derived cells growing in defined conditions. Cells from three culture plates (at least 600 cells each time) were counted in parallel for every cord blood preparation using the computer-assisted image analysis system described above.

PCR

Total RNA was isolated from cells using TRIzol Reagent (Life Technologies) and quantified spectrophotometrically. Then 5 μg samples were reverse transcribed using Superscript II and oligo $(dT)_{12\text{-}18}$ primers (Gibco). Each sample was amplified in duplicate, with and without reverse transcriptase, to control the amplification of genomic DNA.

An equal volume of each sample was amplified by PCR using the following primers: for the human *nestin* gene 5′-GAGGACCAGGACTCTCTATC-3′ and 5′-AGCGAGGAGGATG-AGCTCGG-3′ and for the *GAPDH* gene 5′-CATGTGGGCCATGA-GGTCCACCAC-3′ and 5′-TGAAGGTCGGAGTCAACGGATTTG-GT-3′. Following 30 cycles of amplification (1 minute at 94°C, 1 minute at 58°C and 1 minute at 72°C using the MJ Research Thermal Cycler PTC-100), the PCR products were resolved on a 1% agarose gel. The appearance of 998 bp nestin bands was photographed under UV light.

Results

Clonogenic potential of a selected CB-derived cells subpopulation

CB-derived cells, which were negatively selected for hematopoietic (CD34) and endothelial (CD45) cell surface markers during 6 weeks of culture in the conditions given in Material and Methods (Fig. 1A,B), when treated with mitogens such as epidermal growth factor (EGF) display relatively high potency to expand (Fig. 2B). Then, after planting at a low-cell dispersion (approximately 10 cells per cm^2), they grow clones. The clone-forming cells express nestin (Fig. 2E,F), a protein that was previously shown to be a reliable marker for central nervous system (CNS) stem and progenitor cells (Lendahl et

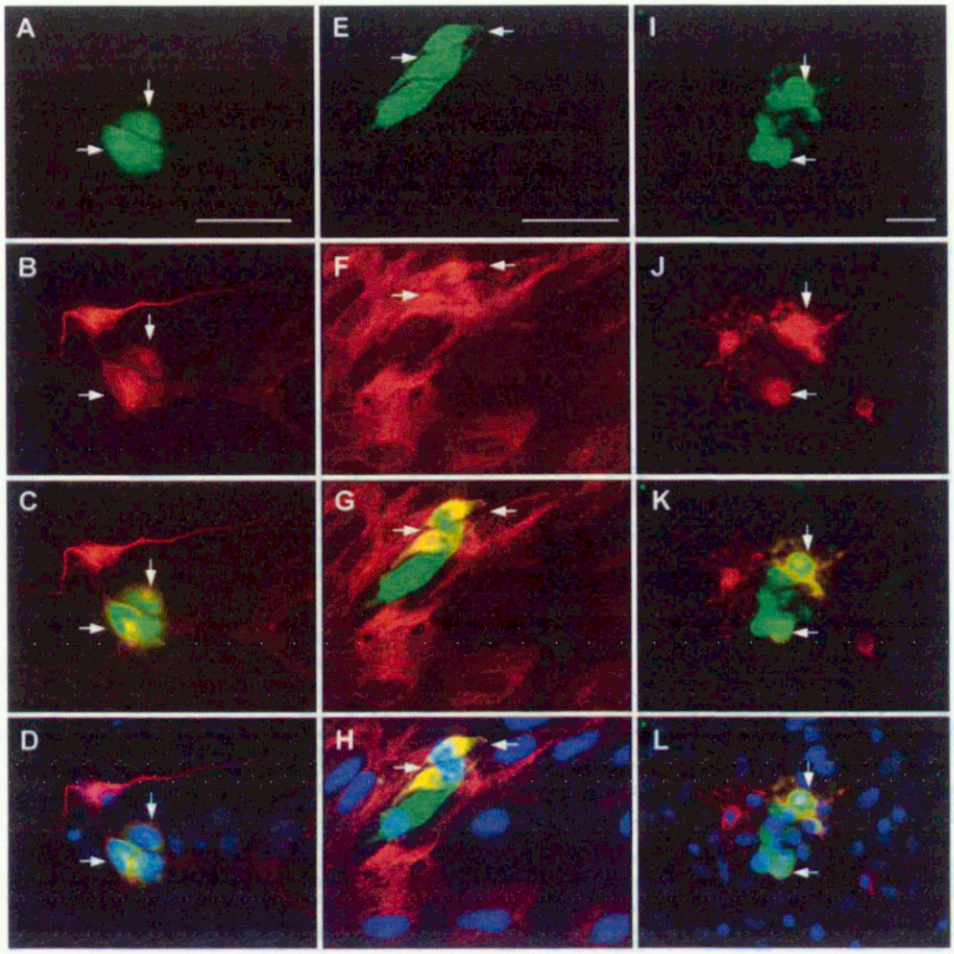

Fig. 5. Neural differentiation of CB-derived cells after their plating on the monolayer of rat primary cortical culture. The CB-derived cells (coloured green by the 'cell tracer' in A,E,I and by Texas Red after phenotype-specific immunoreactions in B,F,J) can be detected in the vicinity of cells originating from rat cortex (only red). Both stains were detected simultaneously (C,G,K) and together with nuclei of all cells forming rat primary cortical monolayer revealed by the use of Hoechst 33258 staining (D,H,L). Colocalisation of red and green labelling in C,G,K and D,H,L appears yellow after overlaying these two images. (B,C,D) Neuron-specific immunostaining for type III β-tubulin with TUJ1 antibody. (F,G,H) Astrocyte-specific immunostaining with anti-GFAP antibody. (J,K,L) Oligodendrocyte-specific immunoreaction with galactosylceramide recognised by the GalC antibody. Arrows indicate immunopositive CB-derived cells. Scale bars for A to H shown in A and E correspond to 50 μm. The scale bar for I to L shown in I corresponds to 20 μm.

al., 1990). Cell clones multiply at the rate of about 10 times the cell number per week (Fig. 2C,D), and after reseeding in the presence of EGF, they can grow further to form a monolayer of adherent, undifferentiated cells or, depending on the planting density, can re-establish new clones as observed up to six passages. Moreover, these new clones were totally unable to produce any hematopoietic colonies in standardised in vitro tests provided by Stem Cell Technologies (see Materials and Methods).

Differentiation of nestin-positive CB-derived cells into neural-specific cell phenotypes

Differentiation of nestin-positive cells was achieved either by direct treatment of growing clones with differentiation-promoting media (Fig. 3A,B) or by plating clone-forming cells onto poly-L-lysine-coated coverslips in the presence of neurobasal/10%FCS medium. Cell differentiation was supported by addition of retinoic acid (RA) alone (Fig. 3A,B,C) or in combination with brain-derived neurotrophic factor (BDNF) (Fig. 4) as recommended previously by Sanchez-Ramos et al. (Sanchez-Ramos et al., 2000). Under these conditions the CB-derived cells start to differentiate along the three major CNS lines, which can be identified by their immunochemical properties. Cell-type-specific antigens were recognised by a TUJ1 monoclonal antibody directed against a neuron-specific class of III β-tubulin (Fig. 3A,B, Fig. 4A,B), by GFAP polyclonal antibody against an astrocyte-specific fibrillary acidic protein (Fig. 3A, Fig. 4C,D) and by a GalC monoclonal antibody against the oligodendrocyte-specific galactosylceramide (Fig. 3B, Fig. 4E,F) (for details see the Materials and Methods). Moreover, as is shown in Fig. 3A,B, cells belonging to the same clone can express neuronal/astrocytic or neuronal/oligodendrocytic markers, confirming directly their dual differentiation potential. The appearance of the neural marker proteins upon CB cell differentiation was additionally proved by western blotting (Fig. 3C). A low level expression of the neuronal marker β-tubulin III can be found even in the initial, non-differentiated clonogenic cultures, whereas two other, astrocytic (GFAP) and oligodendrocytic (PLP/DM-20) markers, are detected only after growing the cells in differentiation-promoting conditions. For western blots, we have used, instead of a classic oligodendrocyte immunomarker, GalC, a proteolipid protein (PLP) and its splicing variant DM-20 expression. An early appearance of DM-20, which is known to overtake expression of PLP as well as GalC in the oligodendrocyte lineage, is clearly visible on the blot (arrow).

The degree of differentiation of CB-derived cells depends on supplementation of the medium (Fig. 6). Spontaneous

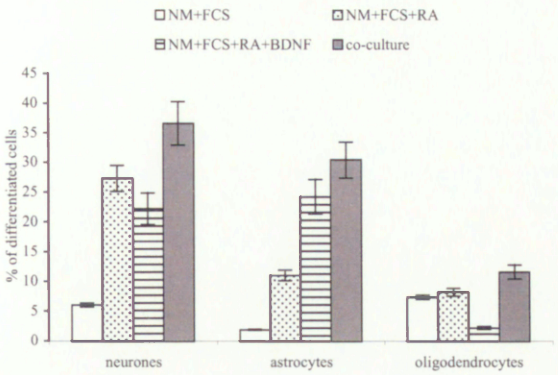

Fig. 6. Extent of CB-derived neural cell differentiation. Quantification of the extent of neuronal, astrocytic and oligodendrocytic differentiation in cultures grown for 4 days in differentiation-promoting conditions. White bars: neurobasal medium (NM) supplemented with 10% FCS; dotted bars: NM with 10% FCS plus 0.5 μM of RA; striped bars: NM, 10% FCS, 0.5 μM RA with addition of 10 ng/ml BDNF; grey bars: cells after seeding on monolayer of rat primary cortical culture grown for 4 days in DMEM supplemented with 10% FCS. Note that promotion of differentiation was best in co-culture (grey bars). In pure, CB-derived cell systems, the presence of RA was decisive for neuronal differentiation, whereas additional supplementation with BDNF promoted mainly astrocytes, with a significant retention of oligodendrocytes. A subpopulation of CB-derived cells growing in a monolayer before clone formation was found to be negative for all investigated antigens. The results are expressed as the mean±s.d. of cell number from nine independent cultures (three parallel experiments from three separate cord blood preparations).

differentiation after plating of the clone-growing cells on poly-L-lysine substratum in 10%-FCS-supplemented neurobasal medium was minimal for neurons and astrocytes (less than 5% of the whole cell population). Addition of RA into the medium promotes differentiation of neurons and, to a lesser extent, astrocytes. Supplementation of the medium with BDNF does not increase the number of neurons in comparison with RA alone, whereas it significantly promotes the development of astrocytes and suppresses that of oligodendrocytes. This may indicate that at this stage of differentiation of CB-derived cells (4 days after poly-L-lysine plating), neurons are not able to produce BDNF at a concentration that is optimal for its physiological paracrine effect on the neighbouring cells.

Differentiation by reseeding of nestin-expressing CB-derived cells on a monolayer of rat brain primary culture

A similar or even higher differentiating effect was achieved after plating CB-derived cells on cover slips with already growing rat primary cortical culture in the presence of 10% FCS in DMEM medium. The phenotype-specific markers for neurons, astrocytes and oligodendrocytes (red in Fig. 5B,F,J) co-stained numerous CB-derived cells that were pre-labelled with 5-chloromethyl-fluorescein-diacetate (green in Fig. 5A,E,I). The close vicinity of rat-brain-differentiated cells appears to promote CB-derived cell differentiation. It seems that under these conditions the CB-derived neural precursors get an optimal paracrine neurotrophic support that promotes

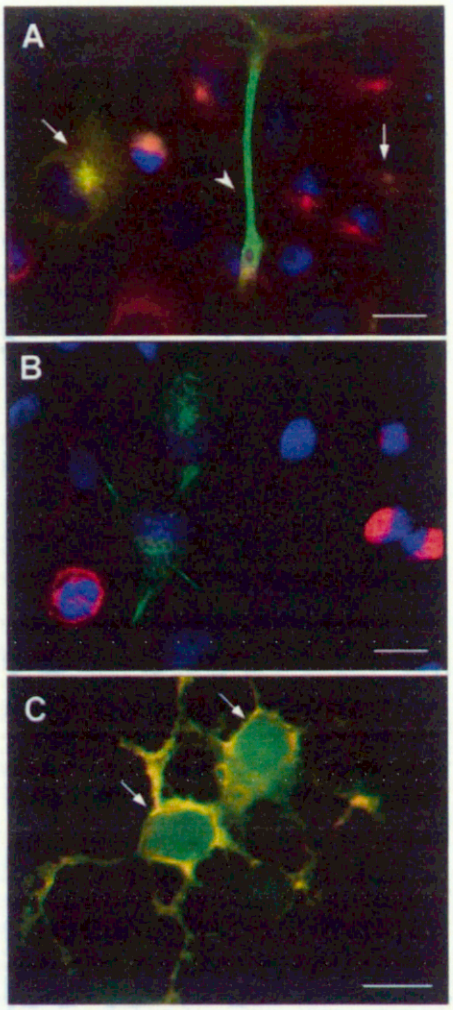

Fig. 7. Examples of phenotypic diversity among a progeny of CB-derived neural precursors induced to differentiate in various culture conditions. (A) Overlaying images of expression of two neuron-specific proteins: class III β-tubulin (immunostained by a Texas-Red-conjugated secondary antibody) and MAP2 (immunostained green by afluorescein-conjugated secondary antibody). The TUJ1-positive structures, which are also immunoreactive with the anti-MAP2 antibody, appeared yellow when detected simultaneously (arrows). Some of these cells display characteristic neuron-like morphology (arrowhead). Cell nuclei are blue contrastained by Hoechst 33258. (B) Distinct distribution of neuron- and astrocyte-specific proteins is indicated by MAP2 (green) and GFAP (red) immunostaining, respectively. Cell nuclei are blue-stained by Hoechst 33258. Very fine, green, anti-MAP2 immunoreactive cytoskeletal filamentous structures can be seen in the cytoplasm of neuron-like cells. (C) CB-derived cells prelabelled green by 'cell tracer' reveal the typical morphology of matured oligodendrocytes with long, branched projections expressing GalC-immunoreactive galactosylceramides. In the figure, fragments of green-traced cells positive for GalC (recognised by Texas-Red-conjugated secondary antibody) appear yellow owing to the overlaying of these two colours when detected simultaneously.

the appearance of all three types of neural progeny. After 4 days in co-culture, almost 40% of cells that were previously

marked by green 'cell tracker' differentiate into neurons, and for the other cells types 30% differentiate into astrocytes and 11% into oligodendrocytes (Fig. 6).

Examples of phenotypic diversity among differentiating cells

Fig. 7 presents the characteristic future of cells belonging to all three types of neural lineage. Some cells display typical neuron-like morphology, with long neurite projections. These cells express, in addition to TUJ1-labelled β-tubulin III, MAP-2 protein, which is characteristic of later steps of neuronal development. Overlaying images of cells double-labelled for these two neuronal proteins are shown in Fig. 7A. In Fig. 7B, distinct populations of anti-GFAP-reactive astrocytes (red) and anti-MAP2-stained neurons (green-labelled cytoskeletal structures) are shown to grow in proximity. Occasionally cells co-expressing both markers (GFAP and MAP-2) were observed; however this was seen in less than 2% of all the GFAP-labelled cells. Cells showing a typical morphology of matured, myelin-forming oligodendrocytes, with irregular, branched projections that stain with an anti-GalC antibody (owing to overlaying with the green 'cell tracker' they are yellow in Fig. 7C) were often found in differentiating CB-derived cell cultures (Figs 4 and 5).

Discussion

The CB-derived cells show a relatively high commitment to neuronal and astrocytic fate; a level similar to that previously observed with foetus-derived neural stem cells (Carpenter et al., 1999; Svendsen et al., 1999). Occasionally, in differentiating cultures a low level (about 2%) of cells co-expressing GFAP and MAP2 (being astrocytic and neuronal markers, respectively) was observed. This observation seems to match a recent finding that newborn neurons appearing in subventricular zone (Barres, 1999), as well as those differentiating from foetal stem cells in vitro (Rosser et al., 1997), co-express GFAP and probably originate from a certain type of common, neuro-astroglia progenitor.

Interestingly, neural cells obtained from CB show a relatively high spontaneous differentiation into oligodendrocytes, which amounts to about 11% of the cells in co-culturing conditions, a higher percentage than previously reported for CNS stem cells (Palm et al., 2000; Zhang et al., 2000).

Attempts are under way to test the responses of CB-derived cells to defined trophic or genetic signals, which are known to be effective in promoting differentiation of oligodendrocytes and neurons in vitro (Cameron et al., 1998; Josephson et al., 1998; Bużańska et al., 2001b) and to recruit them to the damaged brain in vivo (Fricker et al., 1999; Bjorklund and Lindvall, 2000; Rosser et al., 2000). Our data utilising a co-culture system as an alternative to in vivo injection studies indicate that brain tissue itself can provide optimal trophic support for neural progenitor cell differentiation.

The other challenge is to elucidate further the origin of the CB-derived neural precursors described here. We have already shown that this selected cell population, which is able to differentiate towards neural phenotypes, is practically devoid of cells expressing CD34 and CD45 antigens (Fig. 1B), which are characteristic of angiogenic or blood-forming stem cells (Kim et al., 1999). In this respect, their antigenic properties are similar to those described in the foetal human CNS stem cell subpopulation (Uchida et al., 2000). In contrast, the neural precursors examined for this study originate from a plastic adherent mononuclear fraction, which may suggest a mesenchymal origin. In spite of this, at the final stage of in vitro propagation and selection, which directly precedes nestin-expressing clone formation, these cells are totally unable to produce any hematopoietic colonies in vitro. This result corresponds with the antigenic properties estimated by FACS analysis in this paper (Fig. 1B). A similar fraction of plastic-adherent mouse bone marrow stromal cells was reported to transdifferentiate into a neural lineage by Kopen et al. (Kopen et al., 1999). As we have already shown, the CB-derived precursor cells can produce nestin-expressing clones that are able to differentiate toward neuronal/astrocytic or neuronal/oligodendrocytic phenotypes (Fig. 3A,B), thus displaying a bipotentiality. The question of whether these clones can differentiate simultaneously into all three types of neural progeny, a rigorous demand of a neural stem cells, must be answered in further experiments. It is also conceivable that CB-derived neural cells may originate from even more 'primitive' pluripotent stem cells residing in cord blood and resembling those discovered recently in mouse bone marrow (Krause et al., 2001). These ancestor cells can differentiate in vivo toward a variety of cell types, including epithelial cells of the lung, gastrointestinal tract, liver, brain and skin. This striking potential for transdifferentiation of adult stem cells from various tissues into a neural fate as well as into cells of others organs (Kopen et al., 1999; Peterson et al., 1999) is a matter of increasing interest and discussion (Morrison, 2001). Thus, it will be scientifically and practically important to understand by which mechanisms the cells from cord blood give rise to developmentally unrelated CNS tissue and to further purify and characterise these cells.

In conclusion, this study has provided evidence, to our knowledge for the first time*, that each of the three cell types of human brain – neurons, astrocytes and oligodendrocytes – can be propagated in vitro from CB cells. These results raise the possibility that cord blood may provide an efficient source of cells differentiating into the neural lineage, with a potential to be employed in the therapy of human CNS diseases.

We thank Anne Rosser for helpful suggestions, U. Lendahl and B. Zalc for their generous gifts of antiserea and Jan Albrecht for careful reading, discussion and editorial assistance. The work was supported by the grant No. 6P05A04920 from the State Committee for Scientific Research and partially by the grant No. PBZ 29/12 from the Polish State Committee for Scientific Research and Ministry of Health.

References

Alison, M. R., Poulsom, R., Jeffery, R., Dhillon, A. P., Quaglia, A., Jacob, J., Novelli, M., Prentice, G., Williamson, J. and Wright, N. A. (2000). Hepatocytes from non-hepatic adult stem cells. *Nature* **406**, 257.

Azizi, S. A., Stokes, D. A., Augelli, B. J., DiGirolando, C. M. and Prockop, D. J. (1998). Engraftment and migration of human bone marrow stromal cells implanted in the brains of albino rats-similarities to astrocyte grafts. *Proc. Natl. Acad. Sci. USA* **95**, 3908-3913.

*During revision of this manuscript the paper of Sanchez-Ramos et al. (Sanchez-Ramos et al., 2001) appeared and described the phenomenon of CB-derived cells differentiating into neurons and astrocytes.

Barres, B. A. (1999). A new role for glia: generation of neurons! *Cell* **97**, 667-670.

Björklund, A. and Lindvall, O. (2000). Cell replacement therapies for central nervous system disorders. *Nat. Neurosci.* **3**, 537-544.

Bjornson, C. R. R., Rietze, R. L., Reynolds, B. A., Magli, M. C. and Vescovi, A. L. (1999). Turning brain into blood: a hematopoietic fate adopted by adult neural stem cells in vivo. *Science* **283**, 534-537.

Brazelton, T. R., Rossi, F. M. V., Keshet, G. I. and Blau, H. M. (2000). From marrow to brain: expression of neuronal phenotypes in adult mice. *Science* **290**, 1775-1779.

Bużańska, L., Machaj, E. K., Zablocka, B., Pojda, Z., Berdowska, P. and Domańska-Janik, K. (2001a). Human cord blood derived neurons, astrocytes and oligodendrocytes. *J. Neurochem.* **78 Suppl. 1**, 58.

Bużańska, L., Spassky, N., Belin, M. F., Giangrande, A., Guillemot, F., Klambt, C., Labouesse, M., Thomas, J. L., Domanska-Janik, K. and Zalc, B. (2001b). The human medulloblastoma cell line DEV is a potent tool to screen for factors influencing differentiation of neural stem cells. *J. Neurosci. Res.* **65**, 17-23.

Cameron, H. A., Hazel, T. G. and McKay, R. D. (1998). Regulation of neurogenesis by growth factors and neurotransmitters. *J. Neurobiol.* **36**, 287-306.

Carpenter, M. K., Cui, X., Hu, Z., Jackson, J., Sherman, S., Seiger, A. and Wahlberg, L. U. (1999). In vitro expansion of a multipotent population of human neural progenitor cells. *Exp. Neurol.* **158**, 265-278.

Clarke, D. L., Johansson, C. B., Wilbertz, J., Veress, B., Nilsson, E., Karlstrom, H., Lendhal, U. and Frisen, J. (2000). Generalised potential of adult neural stem cells. *Science* **288**, 1660-1663.

Easter, S. S., Jr, Ross, L. S. and Frankfurter, A. (1993). Initial tract formation in the mouse brain. *J. Neurosci.* **13**, 285-299.

Eglitis, M. A. and Mezey, E. (1997). Hematopoietic cells differentiate into both microglia and macroglia in the brains of adult mice. *Proc. Natl. Acad. Sci. USA* **94**, 4080-4085.

Fricker, R. A., Carpenter, M. K., Winkler, C., Greco, C., Gates, M. A. and Björklund, A. (1999). Site-specific migration and neuronal differentiation of human neural progenitor cells after transplantation in the adult rat brain. *J. Neurosci.* **15**, 5990-6005.

Grigelioniene, G., Blennow, M., Tõrõk, C., Fried, G., Dahlin, I., Lendahl, U. and Lagercrantz, H. (1996). Cerebrospinal fluid of newborn infants contains a deglycosylated form of the intermediate filament nestin. *Pediatr. Res.* **40**, 809-814.

Josephson, R., Muller, T., Pickel, J., Okabe, S., Reynolds, K., Turner, P. A., Zimmer, A. and McKay, R. D. (1998). POU transcription factors control expression of CNS stem cell-specific genes. *Development* **125**, 3087-3100.

Kim, D. K., Fujiki, Y., Fukushima, T., Ema, H., Shibuya, A. and Nakauchi, H. (1999). Comparison of hematopoietic activities of human bone marrow and umbilical cord blood CD34 positive and negative cells. *Stem Cells* **17**, 286-294.

Kopen, G. C., Prockop, D. J. and Phinney, D. G. (1999). Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. *Proc. Natl. Acad. Sci. USA* **96**, 10711-10716.

Krause, D. S., Theise, N. D., Collector, M. I., Henegariu, O., Hwang, S., Gardner, R., Neutzel, S. and Sharkis, S. J. (2001). Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. *Cell* **105**, 369-377.

Laemmli, U. K. (1970). Structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lendahl, U., Zimmerman, L. B. and McKay, R. D. G. (1990). CNS stem cells express a new class of intermediate filament protein. *Cell* **60**, 585-595.

Machaj, E. K., Buzanska, L., Gajkowska, A., Oldak, T., Zablocka, B., Berdowska, P., Domanska-Janik, K. and Pojda, Z. (2001). Cord blood derived stem cells differentiates in vitro into neurons, astrocytes and oligodendrocytes. *Exp. Hematology* **29 Suppl. 1**, 4.

Mezey, E., Chandross, K. J., Harta, G., Maki, R. A. and McKercher, S. R. (2000). Turning blood into brain: cells bearing neuronal antigens generated in vivo from bone marrow. *Science* **290**, 1779-1782.

Morrison, S. J. (2001). Stem cell potential: Can anything make anything? *Curr. Biol.* **11**, R7-R9.

Palm, K., Salin-Nordstrom, T., Levesque, M. F. and Neuman, T. (2000). Foetal and adult human CNS stem cells have similar molecular characteristics and developmental potential. *Brain Res. Mol. Brain Res.* **78**, 192-195.

Peterson, B. E., Bowen, W. C., Patrene, K. D., Mars, W. M., Sullivan, A. K., Murase, N., Boggs, S. S., Greenberger, J. S. and Goff, J. P. (1999). Bone marrow as a potential source of hepatic oval cells. *Science* **284**. 1168-1170.

Ranchst, B., Clapshaw, P. A., Price, J., Noble, M. and Seifert, W. (1982). Development of oligodendrocytes and Schwann cells studied with a monoclonal antibody against galactocerebroside. *Proc. Natl. Acad. Sci. USA* **79**, 2709-2713.

Rosser, A. E., Tyers, P., TerBorg, M., Dunnett, S. B. and Svendsen, C. N. (1997). Co-expression of MAP-2 and GFAP in cells developing from rat EGF responsive precursor cells. *Brain Res. Dev. Brain Res.* **98**, 291-295.

Rosser, A. E., Tyers, P. and Dunnet, S. B. (2000). The morphological development of neurons derived from EGF- and FGF-2-driven human CNS precursors depends on their site of integration in the neonatal rat brain. *Eur. J. Neurosci.* **12**, 2405-2413.

Sanchez-Ramos, J., Song, S., Cardozo-Pelaez, F., Hazzi, C., Stedeford, T., Willing, A., Freeman, T. B., Saporta, S., Jansen, W., Patel, N. et al. (2000). Adult bone marrow stromal cells differentiate into neural cells in vitro. *Exp. Neurol.* **164**, 247-256.

Sanchez-Ramos, J., Song, S., Kamath, S. G., Zigova, T., Willing, A., Cardozo-Pelaez, F., Stedeford, T., Chopp, M. and Sanberg, P. R. (2001). Expression of neural markers in human umbilical cord blood. *Exp Neurol* **171**, 109-115.

Svendsen, C. N., Caldwell, M. A. and Ostenfeld, T. (1999). Human neural stem cells: isolation, expansion and transplantation. *Brain Pathol.* **9**. 499-513.

Uchida, N., Buck, D. W., He, D., Reitsma, M. J., Masek, M., Phan, T. V., Tsukamoto, A. S., Gage, F. H. and Weissman, I. L. (2000). Direct isolation of human central nervous system stem cells. *Proc. Natl. Acad. Sci USA* **57**, 14720-14725.

Woodbury, D., Schwarz, E. J., Prockop, D. J. and Black, I. B. (2000). Adult rat and human bone marrow stromal cells differentiate into neurons. *J. Neurosci. Res.* **61**, 364-370.

Zhang, S.-C., Ge, B. and Duncan, I. D. (2000). Tracing human oligodendroglial development in vitro. *J. Neurosci. Res.* **59**. 421-429.

Zuk, P. A., Zhu, M., Mizuno, H., Huang, J., Futrell, J. W., Katz, A. J., Benhaim, P., Lorenz, H. P. and Hedrick, M. H. (2001). Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng.* **7**, 211-228.

PUBLIKACJA 2

STEM CELLS®
Original Article

Voltage-Sensitive and Ligand-Gated Channels in Differentiating Neural Stem–Like Cells Derived from the Nonhematopoietic Fraction of Human Umbilical Cord Blood

WEI SUN,[a] LEONORA BUZANSKA,[b,d] KRYSTYNA DOMANSKA-JANIK,[d] RICHARD J. SALVI,[a] MICHAL K. STACHOWIAK[c]

[a]Hearing Research Lab, [b]Molecular and Structural Neurobiology and Gene Therapy Lab, and
[c]Department of Pathology and Anatomical Sciences, SUNY University at Buffalo, Buffalo, New York, USA;
[d]NeuroRepair Department, Medical Research Center, Warsaw, Poland

Key Words. Neural stem cells • Human umbilical cord blood • Neurotransmitter receptors • Patch clamp
Inward rectifying potassium current • Outward rectifying potassium current

ABSTRACT

Fetal cells with the characteristics of neural stem cells (NSCs) can be derived from the nonhematopoietic fraction of human umbilical cord blood (HUCB), expanded as a nonimmortalized cell line (HUCB-NSC), and further differentiated into neuron-like cells (HUCB-NSCD); however, the functional and neuronal properties of these cells are poorly understood. To address this issue, we used whole-cell patch-clamp recordings, gene microarrays, and immunocytochemistry to identify voltage-gated channels and ligand-gated receptors on HUCB-NSCs and HUCB-NSCDs. Gene microarray analysis identified genes for voltage-dependent potassium and sodium channels and the neurotransmitter receptors acetylcholine (ACh), γ-aminobutyric acid (GABA), glutamate, glycine, 5-hydroxytryptamine (5-HT), and dopamine (DA). Several of these genes (GABA-A, glycine and glutamate receptors, voltage-gated potassium channels, and voltage-gated sodium type XII alpha channels) were not expressed in the HUCB mononuclear fraction (HUCB-MC), which served as a starting cell population for HUCB-NSC. HUCB-NSCD acquired neuronal phenotypes and displayed an inward rectifying potassium current (Kir) and an outward rectifying potassium current (I_{K+}). Kir was present on most HUCB-NSCs and HUCB-NSCDs, whereas I_{K+} was present only on HUCB-NSCDs. Many HUCB-NSCDs were immunopositive for glutamate, glycine, nicotinic ACh, DA, 5-HT, and GABA receptors. Kainic acid (KA), a non–N-methyl-D-asparate (NMDA) glutamate-receptor agonist, induced an inward current in some HUCB-NSCDs. KA, glycine, DA, ACh, GABA, and 5-HT partially blocked Kir through their respective receptors. These results suggest that HUCB-NSCs differentiate toward neuron-like cells, with functional voltage- and ligand-gated channels identified in other neuronal systems. STEM CELLS *2005;23:931–945*

INTRODUCTION

Neuronal and glial populations in the developing brain are generated from multipotent neural stem cells (NSCs) located predominantly in the subventricular zone and hippocampus [1]. Recent studies have demonstrated that a small number of NSCs provide a source of new neurons in the olfactory bulb, hippocampus, cortex, and basal ganglia [2, 3]. NSCs have been cultured in vitro in the form of neurospheres and used to investigate the molecular mechanisms of lineage determination and mechanisms of neuronal and glial differentiation. Transplantation of NSCs into brain or spinal cord could potentially be used to replace damaged neurons and glial cells and thus treat a wide range of neurodegenera-

Correspondence: Michal K. Stachowiak, Ph.D., Department of Pathology and Anatomical Sciences, 206A Farber Hall, SUNY University at Buffalo, Buffalo, New York 14214, USA. Telephone: 716-829-3540; Fax: 716-829-2911; e-mail: mks4@buffalo.edu Received November 12, 2004; accepted for publication March 15, 2005. ©AlphaMed Press 1066-5099/2005/$12.00/0 doi: 10.1634/stemcells.2004-0316

tive disorders and central nervous system (CNS) injuries [4–6]. Experiments in rodents and primates show that cultured NSCs or their neuronal-committed progeny from fetal rat or human brain can survive, mature, and develop axonal connections after transplantation into a damaged brain, thereby providing both structural and functional replacements for damaged neurons [7, 8]. Mouse embryonic NSCs transplanted into animal models of Parkinson's differentiate into dopamine (DA)–containing neurons with their characteristic electrophysiological properties [4]. Moreover, intravenous or intrathecal injections of adult neural precursor cells into animal models of multiple sclerosis promote multifocal remyelination and functional recovery [9, 10].

Although the use of embryonic, fetal, or adult brain-derived NSCs holds great therapeutic potential, their limited supply and evoked immunogenicity in case of allografts limit their usefulness for human therapy. An alternative approach has been to use animal and human bone marrow stromal cells, which have been shown to integrate into rat brain tissue [11, 12] and even to differentiate into astrocytes and tyrosine hydroxylase (TH)–expressing neurons that synthesize DA when grafted into the Parkinsonian mouse [13]. Moreover, in patients receiving therapeutic bone marrow transplants, some transplanted cells migrate into the brain and display fusion-independent neuronal phenotype [14–16]. This suggests that nonembryonic stem cells from sources other than the nervous system could be used for neuron-replacement therapy [17].

Human umbilical cord blood (HUCB) mononuclear fraction contains stem cells belonging to hematopoietic and nonhematopoietic lineages, which may represent a source of multipotent NSCs with relatively low antigenicity [12, 18–22]. Indeed, when transplanted into the brain, the mononuclear fraction of HUCB was shown to survive in brain tissue [23, 24] and reduce motor and neurological deficits [25]. However, due to limited implantation of these cells into brain tissue, the mechanisms underlying the therapeutic effect of these transplanted cord blood cells are still under discussion [26].

Cord blood contains a relatively well-defined population of $CD34^+$ stem cells committed to a hematopoietic lineage. Although it is possible that such cells transdifferentiate into other types of stem cells [27], HUCBs may also contain nonhematopoietic stem cells from the fetus with wider potential, which can give rise to NSCs. Using the $CD34^-/CD45^-$ mononuclear fraction of HUCB as starting material, Buzanska et al. [22] obtained human, multipotent, neural stem–like cells. From these cells, a clonogenic nonimmortalized cell line (HUCB-NSC) with relatively high self-renewal potency and expressing several NSC markers including nestin and glial fibrillary acidic protein (GFAP) was further developed [28]. HUCB-NSCs can differentiate in vitro and give rise to all three brain cell types. In the presence of the neuromorphogen/retinoic acid, 40% of cells attained a neuronal phenotype expressing β-tubulin III and MAP-2, 30% developed astrocyte phenotypes expressing GFAP and S100β, whereas 11% of cells developed oligodendroglial phenotypes expressing galactosylceramide (GalC) [22, 28]. These results suggest that HUCB might be expanded in vitro to produce a population of human fetal NSCs that could be useful clinically. For therapeutic application, HUCB cells must not only develop morphological characteristics of neurons but also voltage- and ligand-gated ion channels that would allow them to function within a neural network and respond to neurotransmitters released from neighboring neurons. To begin to address these issues, we used the whole-cell patch-clamp technique to characterize the electrophysiological properties and ligand-gated receptors on HUCB–differentiated NSCs (NSCDs) and HUCB-NSCs, and gene microarray analysis and immunocytochemistry were used to confirm and further characterize HUCB-NSCD, HUCB-NSC, and HUCB mononuclear cells (HUCB-MCs).

Materials and Methods

Cell Culture

The nonimmortalized HUCB-NSC line, immuno-negative for CD34 and CD45 hematopoietic cell markers [22], was derived from the MC fraction as described previously [22, 28]. The cell line was established through sequential in vitro passaging and further selection in a culture of nonadherent proliferating cells (unpublished data). The HUCB-NSC line was expanded as a culture of only nonadherent and nondifferentiated cells in serum-free medium that consisted of Dulbecco's modified Eagle's medium (DMEM)/F12, antibiotic-antimycotic solution (AAS) (diluted ×100; Sigma, St. Louis, http://www.sigmaaldrich.com), supplemented with B27 (1:50; Invitrogen, Grand Island, NY, http://www.invitrogen.com), and mitogens (epidermal growth factor [EGF] [10 ng/ml], basic fibroblast growth factor [bFGF] [10 ng/ml], and leukemia inhibitory factor [10 ng/ml]; Sigma). Alternatively HUCB-NSCs were expanded in a culture containing adherent as well as floating, rounded cells using DMEM/F12, supplemented with 2% fetal bovine serum (FBS), insulin-transferrin-selenium (ITS) (1:100; Invitrogen), and AAS (diluted ×100; Sigma) (unpublished data). In the present study, HUCB-NSCs were cultured in DMEM/F12, 2% FBS, supplemented with ITS and AAS. Nonadherent cells, which were undifferentiated, are hereafter referred to as HUCB-NSCs. To induce differentiation, nonadherent HUCB-NSCs were collected and plated on glass coated with poly-L-lysine plus laminin (0.5–1 $\mu g/cm^2$) in 12-well plates at a density of 10^4 cells per cm^2. Attached cells were treated with dBcAMP/CPT (300 μM; Sigma) and placed in an incubator (5% CO_2, 37°C) for 1 day to 4 weeks.

Gene Microarray Analysis

Total RNA was isolated using Trizol Reagent (Invitrogen) from approximately 4 million cells for three experimental groups: (a) control HUCB-MCs, (b) nonattached HUCB-NSCs, and (c)

attached HUCB-NSCDs differentiated for 4 weeks on poly-L-lysine and laminin-coated coverslips and treated with dBcAMP/CPT (300 μM; Sigma). cRNA samples were hybridized to Affymetrix HG-U133 Set A and B Gene Chips with probes for approximately 33,000 human genes (Gene Core Laboratory, Roswell Park Cancer Institute, Buffalo, NY, http://www.roswellpark.org). Streptavidin-phycoerytrin–stained gene chips were scanned with a GeneChip System confocal scanner (Agilent, Affymetrix Inc., Santa Clara, CA, http://www.affymetrix.com) at 3-μm resolution. Fluorescence intensity for each gene was normalized to the average fluorescence intensity of the entire chip. Relative abundance of each gene was assessed according to its p value, calculated according to the statistical algorithms provided by Affymetrix Microarray Suite (Affymetrix Inc.). Present (P), marginal (M), and absent (A) calls for gene expression were defined as $p < .05$, $.05 < p \leq .06$, and $p > .06$, respectively. Mean values from two separate experimental runs were calculated. GeneChip absolute analysis and comparisons among the three experimental conditions were performed using Data Mining Tool (Affymetrix Inc.). Data were transferred to Excel files for later analysis. Genes of interest in this study were genes associated with voltage-gated and ligand-gated ion channels. Identification of relevant genes in the data set was found by searching the Affymetrix data set for key words such as *receptors*, *neural*, and *neuronal*. Results of all remaining genes will be published elsewhere.

Immunocytochemistry

HUCB-NSCDs, attached to poly-L-lysine/laminin–covered glass and treated with dBcAMP/CPT (300 μM), were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate (pH 7.2) for 20 minutes at room temperature. Cultures were washed three times with 0.1 M phosphate-buffered saline (PBS), blocked in 5% normal goat serum for 1–2 hours. Samples were incubated with primary antibody (Ab) overnight at 4°C. For cytoskeletal staining, cells were additionally permeabilized in PBS plus 0.1%–0.25% Triton X-100 for 20 minutes. Primary antibodies against cytoskeletal proteins were monoclonal anti–β-tubulin III (1:300; Sigma), monoclonal neurofilament NF-200 (1:400; Chemicon, Temecula, CA, http://www.chemicon.com), and monoclonal anti–light chain of neurofilament NF-70 (1:100; Chemicon). The antibodies used for neurotransmitter receptors were anti-glutamate receptor 2 Ab (polyclonal, 1:100; Chemicon), anti–5-HT1C (5-HT1CR, 1:500, polyclonal Ab; Santa Cruz Biotechnology, Santa Cruz, CA, http://www.scbt.com), anti–γ-aminobutyric acid (GABA)–A receptor (GABA-AR, beta chain 1:100, monoclonal; Research Diagnostic Inc., Flanders, NJ, http://www.researchd.com), glycine receptor Ab (GlyR, 1:100, polyclonal; Chemicon), acetylcholine (ACh) nicotinic receptor beta subunit Ab (1:100, monoclonal; Transduction Laboratories, http://www.bdbiosciences.com), and anti–DA D2 receptor polyclonal Ab (D2; 1:100; Chemicon). An Ab for the neurotransmitter GABA was also evaluated (anti-GABA, 1:200, monoclonal; Chemicon). In some experiments, cell nuclei were stained with ToPro-1 (Molecular Probes, Eugene, OR, http://probes.invitrogen.com) at a final concentration of 2 μM. After rinsing in PBS, cells were incubated with secondary Ab (goat anti-mouse immunoglobulin G [IgG] Alexa-555, goat anti-rabbit IgG Alexa 488, 1:300 [Molecular Probes]; goat anti-mouse IgG FITC, 1:300 [Sigma]; or goat anti-rabbit Cy3, 1:1000 [Jackson ImmunoResearch, West Grove, PA, http://www.jacksonimmuno.com]) for 1 hour at room temperature. Double immunostaining of neurotransmitter receptors and cytoskeletal proteins was performed sequentially. Cells were incubated first with primary anti-receptor Ab overnight at 4°C, followed by 30-minute incubation with secondary Ab at 37°C. Cells were subsequently permeabilized for 20 minutes in 0.25% Triton X-100 and incubated with primary Ab against cytoskeletal proteins for 2 hours at room temperature and with secondary Ab at 37°C for 30 minutes. As a negative control, the primary Ab was omitted, with other steps remaining the same. Images of cells mounted on Fluoromont G (SouthernBiotech, Birmingham, AL, http://www.southernbiotech.com) were taken using a fluorescence microscope (Axioskop; Carl Zeiss, Jena, Germany, http://www.zeiss.com) or confocal Bio-Rad MRC microscope (Bio-Rad Laboratories, Hercules, CA, http://www.bio-rad.com), processed with Adobe PhotoShop (Adobe Systems, San Jose, CA, http://www.adobe.com), and printed on a dye sublimation printer (Kodak 8670; Eastman Kodak Company, Rochester, NY, http://www.kodak.com).

Whole-Cell Patch-Clamp Recording

Our procedures for making whole-cell, patch-clamp recordings have been described in detail previously [29–31]. Before recording from HUCB-NSC or HUCB-NSC-NSCD, the culture medium was replaced with Hanks' balanced salt solution (HBSS), which contained (in mM) NaCl 137, Na_2HPO_4 0.2, KCl 5.4, KH_2PO_4 0.4, $MgSO_2$ 0.8, $CaCl_2$ 1.3, glucose 5.6, and HEPES 10. The recording pipette was filled with a solution containing (in mM) KCl 120, KF 20, NaCl 2, $MgCl_2$ 2, EGTA 10, and HEPES 10; pH was buffered to 7.3 with NaOH, and osmolarity was adjusted with sucrose to 290 mOsm. The pipette resistance in the bath solution was typically 2–6 MΩ. The whole-cell recording configuration was established on the soma of HUCB-NSC or HUCB-NSCD (1–3 GΩ), and recordings were made at 22°C. In voltage-clamp configuration, series resistance and series compensation were applied as reported in our previous publications. Current and voltage signals were amplified using a patch-clamp amplifier (Multiclamp 700A), digitized (DigiData 1200; Axon Instruments, Union City, CA, http://www.axon.com), and analyzed by pCLAMP (version 8.01; Axon Instruments). Kainic acid (KA), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), DA, glycine, strychnine, 4-aminopridine (4-AP), tetraethylammonium (TEA), $CdCl_2$, $BaCl_2$, and CsCl were all purchased from Sigma. The solutions were made in HBSS and applied alone or mixed

together through a puffer electrode connected to a DAD-12 super fusion system (ALA Instruments, Westbury, NY, http://www.alascience.com). Electrophysiological results were evaluated using Student's *t*-test (Sigma Stat, SPSS, version 2) as indicated in the Results section.

Results

Expression of Neural Markers

In our previous study, HUCB-NSCs were shown to express several structural or cytoskeletal proteins characteristic of cells of neural lineage while maintained in DMEM, 10% FBS (Invitrogen), and 10 ng/ml EGF [22]. The same characteristics were observed in the present study when HUCB-NSCs were maintained in 2% FBS medium, without growth factors, but supplemented with ITS (1:100; Sigma). HUCB-NSC cultures proliferated at a high rate and consisted of two cell populations. The first population was represented by round, floating cell aggregates consisting of cells of approximately 8–10 μm in diameter with relatively large nuclei and scant cytoplasm (Fig. 1A, arrowhead; Fig. 1B). These cells expressed nestin and GFAP but did not express β-tubulin III or other markers of advanced neuronal (MAP2) or astroglial (S100β) differentiation (data not shown). The second population consisted of flattened cells that attached to the plastic wells (Fig. 1A, arrow; Fig. 1C). These cells expressed early markers of neuronal differentiation, including NF-200, NF-70, and β-tubulin III (not shown). When HUCB-NSCs were plated on polylysine/laminin-coated glass plates and maintained in medium containing dBcAMP/CPT for 1–24 days, they readily attached to the substratum and developed long, neurite-like processes after 5 or more days in culture (Figs. 1D–1F). These HUCB-NSCDs were immunopositive for β-tubulin III (80%) (Fig. 1D), neurofilament NF-200 (Fig. 1E), and NF-70 (Fig. 1F), a late, structural, neural marker. Relatively few cells (<19%) with astrocyte-like morphology and GFAP immunoreactivity were observed.

Genes Associated with Ion Channels and Neurotransmitters

Microarray RNA analysis was applied to (a) the starting population of HUCB-MCs from which our neural progenitors had been selected and expanded [22], (b) floating HUCB-NSCs, and (c) HUCB-NSCDs attached to poly-L-lysine/laminin–coated coverslips and differentiated for 4 weeks in dBcAMP/CPT. Expression of approximately 33,000 genes was analyzed using Affymetrix HG-U133 Set A and B Gene Chips. Receptor and ion channel genes important for neuronal function, the focus of the present study, are listed in Table 1. Their relative expression in HUCB-MCs, HUCB-NSCs, and HUCB-NSCDs was evaluated using statistical algorithms provided by Affymetrix Microarray Suite as described above. The results with the remaining genes will be reported elsewhere.

As shown in Table 1, 14 neurotransmitter receptors genes and 7 ion channel genes considered important for neural development and function were expressed (present or marginal calls for expression; see Materials and Methods) in HUCB-NSCDs. The neurotransmitter receptor genes expressed in HUCB-NSCDs consisted of three cholinergic receptor subtypes, two dopaminergic receptor subtypes, three GABAergic receptors subtypes, two glutamate receptor subtypes, one glycinergic subtype, and three serotonergic subtypes. None of the three GABAergic receptor subtypes was expressed in

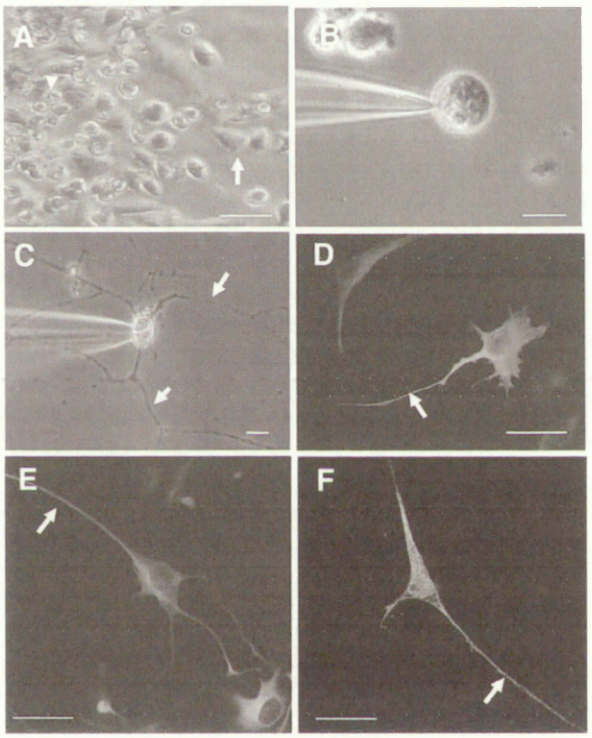

Figure 1. (A): Cultures of HUCB-NSCs maintained in 2% FBS medium contained flattened cells that attached to plastic dish (arrow) and round, floating aggregate-forming cells with relatively large nuclei and scant cytoplasm (arrowhead). Phase-contrast photomicrograph showing whole-cell recording electrode on a **(B)** unattached HUCB-NSC and **(C)** attached HUCB-NSCD cultured with 2% FBS and dBcAMP/CPT for 12 days. Note processes (arrows) extending from soma of HUCB-NSCD. HUCB-NSCDs immunopositive for **(D)** β-tubulin III mouse primary Ab, anti-mouse FITC-conjugated secondary Ab (differentiated 4 days in the presence of dBcAMP/CPT; arrows show neurites extending from soma of some cells), **(E)** neurofilament 200-kD mouse primary Ab, goat anti-mouse Alexa-555–conjugated secondary antibody (differentiated 12 days in the presence of dBcAMP/CPT, arrows show neurites extending from soma), and **(F)** neurofilament 70-kD mouse primary Ab, goat anti-mouse FITC-conjugated secondary antibody (differentiated 12 days, arrows show neurites extending from soma). Scale bar for A, D, E, F: 50 μm; for B and C: 20 μm. Abbreviations: Ab, antibody; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; HUCB, human umbilical cord blood; NSC, neural stem cell; NSCD, differentiated neural stem cell.

MCs, whereas two of three GABAergic receptor subtypes were expressed in HUCB-NSCs. Only one of two glutamate receptor subtypes, metabotropic 6, was expressed in HUCB-MCs; however, both the metabotropic and kainate 4 subtypes were expressed in HUCB-NSCs and HUCB-NSCDs. Glycine receptor beta was not expressed in HUCB-MCs but was detected in HUCB-NSCs and HUCB-NSCDs. Thus, the transition from HUCB-MC to HUCB-NSC was associated with increased expression of GABA, glycine, and glutamate receptor subtypes. No change in expression of cholinergic, dopaminergic, and serotonergic receptor subtypes was seen from MC to HUCB-NSC or from HUCB-NSC to HUCB-NSCD.

Of the seven ion channel receptor subtypes seen in HUCB-NSCDs, three were members of the potassium family, three were members of the sodium family, and one was a member of the transient receptor potential cation channel. Inward-rectifying potassium channels (Kir) and calcium-activated potassium ($I_{K[Ca]}$) were consistently expressed in HUCB-MC, HUCB-NSC, and HUCB-NSCD, but the KQT potassium channel subtype was not expressed in MCs. Voltage-gated sodium channels type X alpha and type XII alpha and non–voltage-gated sodium channel type 1 beta subtypes were present in HUCB-NSCs and HUCB-NSCDs; however, the voltage-gated type XII and non–voltage-gated 1 beta subtypes were not expressed in HUCB-MCs. The transient receptor potential cation channel was not expressed in HUCB-MCs but was present in HUCB-NSCs and HUCB-NSCDs.

Immunolabeling of Neurotransmitter Receptors

HUCB-NSCDs were maintained under differentiating conditions for 14 days and then immunolabeled for neurotransmitter receptors commonly expressed in the CNS, namely glutamate, GABA, glycine, serotonin, DA, and acetylcholine receptors. Many (88% ± 6.5%) HUCB-NSCD cells showed strong immunolabeling for kainate GluR2 receptor subunit; small puncta were observed over the soma and over the long, thin processes extending from the cell body (Figs. 2A, 2C). HUCB-NSCD immunopositive for GluR2 also coexpressed β-tubulin III (Figs. 2B, 2C), an early neuronal maker [22]. β-Tubulin III immunolabeling was strongly expressed in the processes extending from the soma, whereas minimal labeling was seen in the nuclear region. Almost all (93% ± 7%) HUCB-NSCDs were immunopositive for GABA-AR (Figs. 2D, 2F). These cells were also immunopositive for β-tubulin III, seen as thin strands in the soma and in the processes extending from the cell body (Figs. 2E, 2F). Because immature neurons in the CNS often express GABA, we examined whether HUCB-NSCDs express this neurotransmitter and thus could be regulated by GABA in an autocrine or paracrine fashion. Strong GABA immunolabeling was observed in 90% ± 2.5% of NSCD, as shown in Figure 2G. The high percentage of HUCB-NSCDs immunopositive for GABA and GABA-AR is consistent with an autocrine/paracrine mechanism.

Table 1. Expression of neuronal voltage-gated or ligand-gated receptor genes in dBcAMP/CPT differentiated with HUCB-MC, HUCB-NSC, and HUCB-NSCD

Neurotransmitter receptor genes	HUCB-MC	HUCB-NSC	HUCB-NSCD
Cholinergic receptor, nicotinic, alpha polypeptide 5	P; $p = .008$	P; $p = .0004$	P; $p = .0008$
Cholinergic receptor, nicotinic, beta polypeptide 3	M; $p = .059$	P; $p = .005$	P; $p = .024$
Cholinergic receptor, nicotinic, epsilon polypeptide	P; $p = .0002$	P; $p = .0002$	P; $p = .0002$
Dopamine receptor D2	P; $p = .0069$	P; $p = .0043$	P; $p = .0049$
Dopamine receptor D5	P; $p = .0009$	P; $p = .0049$	P; $p = .0049$
GABA-A receptor, alpha 2	A; $p = .063$	A; $p = .135$	P; $p = .01$
GABA-A receptor, 1	A; $p = .365$	P; $p = .0013$	P; $p = .0054$
GABA-A receptor, gamma 3	A; $p = .1$	P; $p = .033$	P; $p = .017$
Glutamate receptor, ionotropic, kainite 4	A; $p = .69$	M; $p = .059$	P; $p = .05$
Glutamate receptor, metabotropic 6	P; $p = .008$	P; $p = .008$	P; $p = .007$
Glycine receptor, beta	A; $p = .735$	P; $p = .0026$	P; $p = .016$
5-Hydroxytryptamine (serotonin) receptor 1C	M; $p = 0.056$	P; $p = .008$	P; $p = .009$
5-Hydroxytryptamine (serotonin) receptor 2A	P; $p = .0075$	P; $p = .03$	P; $p = .013$
5-Hydroxytryptamine (serotonin) receptor 3A	P; $p = .046$	M; $p = .056$	M; $p = .056$
Ion channel genes	MC	NSC-ND	NSC-D
Potassium inwardly rectifying channel, subfamily J, member 2	P; $p = .0065$	P; $p = .0013$	P; $p = .0038$
Potassium voltage-gated channel, KQT-like subfamily, member 2	A; $p = .41$	P; $p = .017$	M; $p = .059$
Potassium large conductance calcium-activated channel, subfamily M, beta member 4	P; $p = .0047$	P; $p = .002$	P; $p = .007$
Sodium channel, voltage-gated, type X, alpha	P; $p = .014$	P; $p = .035$	P; $p = .045$
Sodium channel, voltage-gated, type XII, alpha	A; $p = .061$	P; $p = .044$	P; $p = .009$
Sodium channel, non voltage-gated 1, beta	A; $p = .14$	P; $p = .05$	P; $p = .0011$

Genes with detectable expression (P, present; $p < .056$; M, marginal; $.056 < p < .06$) and no detectable expression receiving an absent call (A, absent; $p \geq .06$).

Abbreviations: GABA, γ-aminobutyric acid; HUCB, human umbilical cord blood; MC, mononuclear cell; NSC, neural stem cell; NSCD, differentiated neural stem cell.

Figure 3 shows HUCB-NSCD cells double-labeled with To-Pro 1, which stains the nuclei, and antibodies against GlyR (A), nicotinic AChR (B), 5-HT1CR (C), and DA (D) receptors. Immunolabeling for all neurotransmitter receptors examined was absent from negative controls in which the primary Ab was omitted (data not shown). GlyR immunolabeling was present in approximately 20% ± 2.5% of HUCB-NSCD. Labeling appeared as fine or granular puncta on the soma as well as on the processes extending from the cell body (Fig. 3A). Nicotinic AChR immunolabeling were observed on the soma, often as large puncta, and on neurites (Fig. 3B). Nicotinic AChR immunolabeling was seen on approximately 39.3% ± 2.5% of HUCB-NSCDs. 5HT1CR immunolabeling was present as granular puncta on the soma and fine labeling on neurites; immunolabeling was observed on 90.3% ± 3.5% of HUCB-NSCD. DA receptor D2 immunolabeling was present on 85% ± 2.5% of HUCB-NSCD cells; patches of granular labeling were often observed on the soma and neurites (Fig. 3D). None of the above immunolabeling was observed when the specific primary antibodies were omitted (not shown).

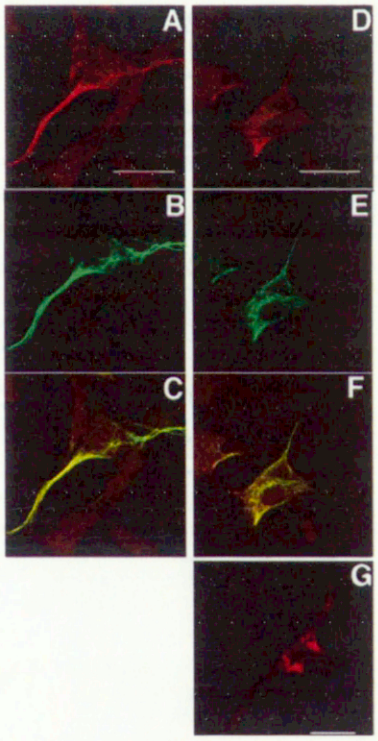

Figure 2. (A): Photomicrograph showing immunolabeling of glutamate receptor using polyclonal antiglutamate receptor 2 antibody, goat anti-rabbit Cy3 secondary Ab. **(B):** Same cell as in **(A)** immunolabeled for β-tubulin III using monoclonal Ab and goat anti-mouse FITC secondary Ab. **(C):** Merger of **(A)** and **(B)**. **(D):** Immunolabeling of GABA-A receptor using polyclonal antibody and goat anti-rabbit Cy3 secondary Ab. **(E):** Same cell as in **(D)** immunolabeled for β-tubulin III using monoclonal Ab and goat anti-mouse FITC secondary Ab. **(F):** Merger of **(D)** and **(E)**. **(G):** Immunolabeling of GABA using a monoclonal anti-GABA and goat anti-mouse Alexa-555 secondary Ab. Scale bar: 50 uM. Abbreviations: Ab, antibody, FITC, fluorescein isothiocyanate; GABA, γ-aminobutyric acid.

Electrophysiology of Nondifferentiated and Differentiated HUCB-NSCs

Whole-cell patch-clamp recordings were made from floating HUCB-NSCs (Fig. 1B) and from HUCB-NSCDs attached to poly-lysine/laminin–coated plates and treated with dBcAMP/CPT for 1–28 days (Fig. 1C). The mean membrane potential of HUCB-NSCs was −48 ± 23 mV (n = 14), whereas the mean membrane potential of HUCB-NSCDs treated with dBcAMP/CPT (Sigma, 300 μM) acid for 5–28 days was −51 ± 20 mV (n = 57). Although the membrane potential of HUCB-NSCD was slightly greater than HUCB-NSC, this difference was not statistically significant (t-test).

An inward rectifier potassium current, Kir [32], was present in HUCB-NSCs and HUCB-NSCDs (Fig. 4A). Consistent with the expression of the Kir gene (Table 1), we recorded the Kir current in almost all HUCB-NSCs and HUC-NSCDs (n = 116). Under whole-cell recording conditions, Kir was activated during hyperpolarizing voltage steps (−50 mV holding potential, −140 to +30 mV, 10-mV step). Kir increased in amplitude with voltage steps more negative than −70 mV (Fig. 4A); no current was observed at voltages more positive than −70 mV when the external K^+ concentration was 7 mM (Fig. 4B, open circle). To confirm that Kir

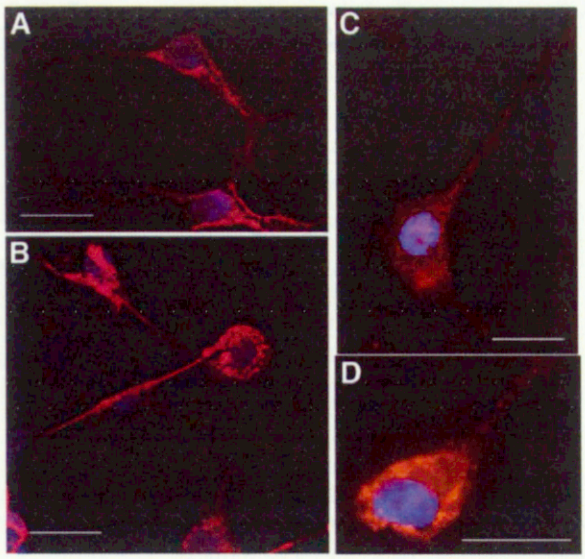

Figure 3. Immunolabeling of **(A)** glycine, **(B)** acetylcholine-nicotinic, **(C)** 5-HT, and **(D)** D2 dopamine receptors in human umbilical cord blood differentiated neural stem cells. Goat anti-mouse Alexa 555 secondary antibody used to detect acetylcholine and 5-HT receptors; goat anti-rabbit Cy3 used to detect glycine and D2 receptors. Nuclei stained with ToPro-1. Scale bar: 50 μM. Abbreviation: 5-HT, 5-hydroxytryptamine.

was selectively permeable to K^+, we varied the external K^+ and measured the reversal potential. When the external K^+ concentration was changed from 7 to 30 mM, the reversal potential of Kir shifted from −70 to −40 mV (Fig. 4B, $n = 4$). These data are consistent with the predicted reversal potential for potassium calculated from the Nernst equation. Kir was completely eliminated by external Cs^+ (5 mM, Fig. 4C, $n = 59$), Ba^{2+} (5 mM, Fig. 4D, $n = 5$), or Cd^{2+} (0.1 mM. Fig. 4E, $n = 10$), known antagonists of Kir [33–35]; Kir quickly recovered after Cs^+, Ba^{2+}, or Cd^{2+} (0.1 mM, Fig. 4E) was washed out ($n = 46$). The amplitude of Kir was largely unaffected by the I_K antagonists 4-AP (Fig. 4F) or TEA (15 mM) (data not shown) [36]. The average amplitude of Kir induced by stepping the voltage from −50 to −140 mV was −0.33 ± 0.18 nA ($n = 14$) in floating HUCB-NSCs and −0.7 ± 0.5 nA ($n = 80$) in attached HUCB-NSCDs that had been differentiated for 5 days or more (Fig. 4B). This difference was statistically significant (*t*-test, $p < .01$).

Outward Rectifying I_{K+} Increases with Differentiation

I_{K+} was not detected in floating HUCB-NSCs. However, when HUCB-NSCDs were differentiated for 5–28 days, I_{K+} was observed along with Kir. I_{K+} was present in approximately 40% (40 of 105) of

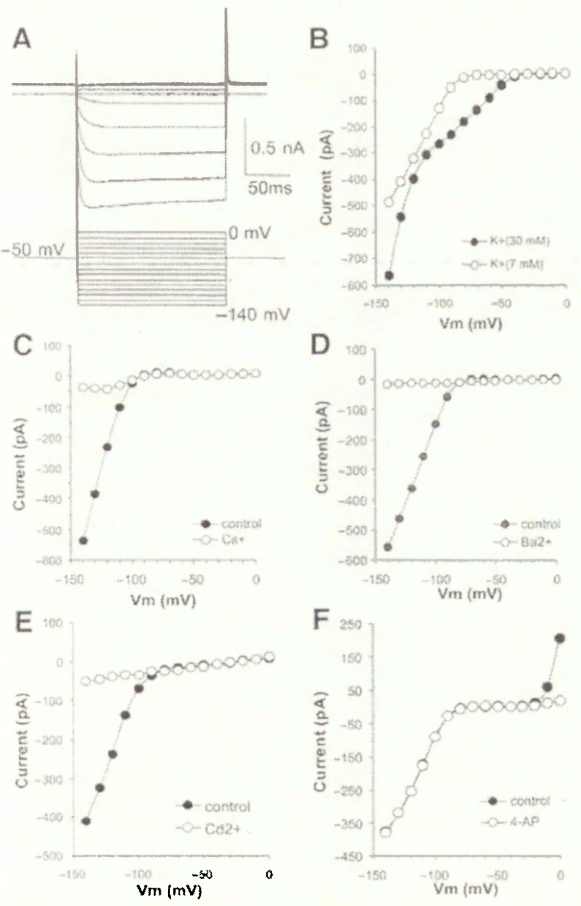

Figure 4. Inward rectifier potassium current (Kir) induced from human umbilical cord blood differentiated neural stem cells. **(A):** Typical Kir current traces induced by negative voltage steps (−50 to −140 mV, 10-mV step). Command voltage shown below. **(B):** *I*/V curve of Kir recorded from cell with 7 mM or 30 mM K^+ in the bath solution. Note reversal potential of Kir switches from approximately −70 mV with 7 mM K^+ to −30 mV with 30 mM K^+. **(C):** Kir was reversibly blocked by external Cs^+ (5 mM). **(D):** Kir was reversibly blocked by external Ba^{2+} (5 mM). **(E):** Kir was reversibly blocked by external Cd^{2+} (0.1 mM). **(F):** Addition of 4-AP did not change the *I*/V curve for Kir.

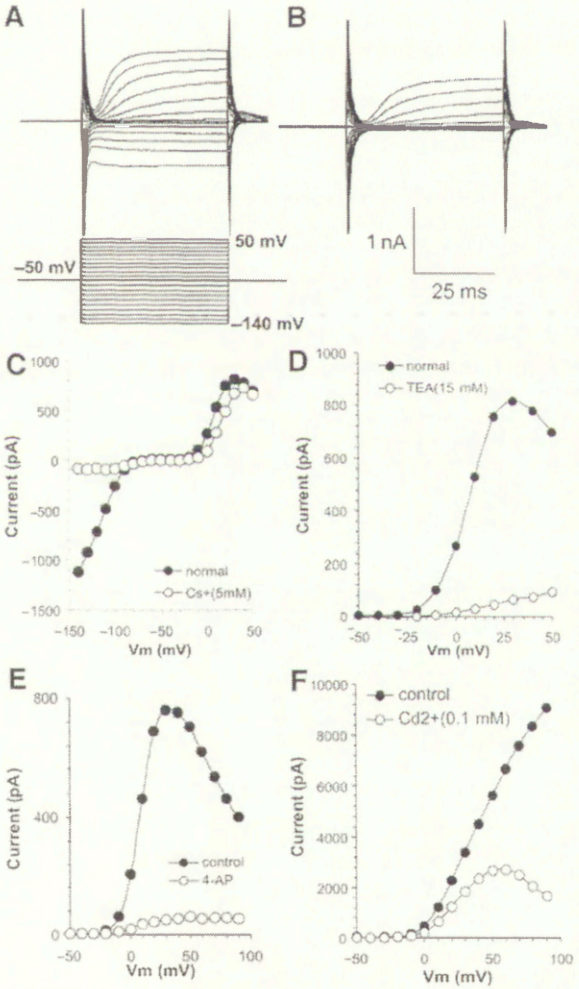

Figure 5. Outward potassium current (I_{K+}) and Kir current present in human umbilical cord blood differentiated neural stem cells. **(A):** Kir and I_{K+} were induced in voltage clamp (holding potential −50 mV, voltage step from −140 to +50 mV, 10-mV step). **(B):** Cs^+ (5 mM) can block Kir but not I_{K+}. **(C):** *I*/V curve of I_{K+} and Kir currents (●): Kir was blocked by Cs^+ but not I_{K+} (○). **(D):** I_{K+} was activated around −30 mV (●) and was reversibly blocked by tetraethylammonium (TEA) (15 mM, ○). **(E):** I_{K+} was reversibly blocked by external 4-AP (1 mM). **(F):** I_{K+} was partly blocked by external Cd^{2+} (0.1 mM).

HUCB-NSCD when the voltage was stepped from −50 to +80 mV (−50-mV holding potential, 10-mV step, 50 ms) (Fig. 5A). To isolate I_{K+} from Kir, Cs^+ was added to the bath solution (Fig. 5B), revealing a slowly activating I_{K+} at voltages more positive than −10 mV (Figs. 5B, 5C; $n = 24$). I_{K+} could be blocked by external TEA (15 mM) (Fig. 5D, $n = 4$) or 4-AP (1 mM) (Fig. 5E, $n = 4$), consistent with the criteria of a slowly activating, outward-rectifying potassium current [33]. I_{K+} was partially blocked by Cd^{2+} (100 μM, Fig. 5F, $n = 10$), suggesting that a calcium-activated potassium current makes a small contribution to the current measured at voltages more positive than +30 mV (Figs. 5D, 5F). This interpretation is consistent with expression of a gene for a calcium-activated potassium channel (Table 1).

The percentages of HUCB-NSCs and HUCB-NSCDs expressing Kir and I_{K+} under undifferentiated conditions and differentiated conditions (attached and treated with dBcAMP/CPT) for 2–5 days or more are shown in Figure 6A. The percentage of cells expressing Kir showed little change over time; however, the percentage of cells expressing I_{K+} increased from 0% (0 of 14) in the undifferentiated condition to approximately 19% (7 of 36) in differentiation conditions.

The amplitude of Kir showed only a slight increase from undifferentiated to differentiated conditions; Kir amplitude was 0.33 ± 0.18 nA ($n = 14$) in undifferentiated cells and increased to 0.61 ± 0.5 nA ($n = 28$) in cells differentiated 2–5 days and to 0.69 ± 0.5 nA ($n = 71$) in cells differentiated >5 days (Fig. 6B). Cells expressing I_{K+} were first observed after being maintained under differentiating conditions for 2–5 days. I_{K+} amplitude was 0.4 ± 0.6 nA ($n = 7$) after being differentiated for 2–5 days and 1.5 ± 1.9 nA ($n = 35$) when differentiated for more than 5 days. These results indicate that differentiating conditions enhanced the expression of I_{K+} but have little effect on Kir expression or amplitude.

Excitability of NSCs

In some HUCB-NSCDs ($n = 8$), current pulses (0 to +200 pA, 140 ms; Fig. 7A) induced an action potential–like response [37] consisting of a rapid depolarization followed by a partial repolarization. To identify the ionic conductance mediating this action potential–like response, TEA was added to the bath solution to block I_{K+}; this eliminated the repolarizing phase, leaving behind a steady depolarization (Fig. 7B). The depolarization could not be blocked by 300 nM tetrodotoxin (TTX) ($n = 8$), a sodium channel blocker (data not shown) that blocks TTX-sensitive I_{Na+} but not TTX-resistant I_{Na+} at the concentration used here [38]. Although NSCDs were differentiated for up to 4 weeks, we failed to detect I_{Na+}, a hallmark of mature neurons that generate action potentials. These results suggest that the action potential–like response seen in HUCB-NSCDs (Fig. 6A) arises from the interaction of Kir and a slowly activating I_{K+}. We cannot completely exclude the possibility that a TTX-resistant sodium current or voltage-gated calcium current is involved in this action potential–like response; however, this seems unlikely given that the response activates slowly (~7 ms).

Neurotransmitter Receptors

To determine if the neurotransmitter receptors identified by gene arrays and immunocytochemistry were functional, we performed whole-cell patch-clamp recordings while applying receptor agonists and antagonists. To test for N-methyl-D-asparate(NMDA) receptors, we applied NMDA (1 mM) to HUCB-NSCDs at a holding potential of −50 mV. Application of NMDA failed to induce a current in the four cells tested (data not shown). Application of NMDA also failed to alter the amplitude of Kir (data not shown). To test for non-NMDA glutamate receptors, we applied KA to

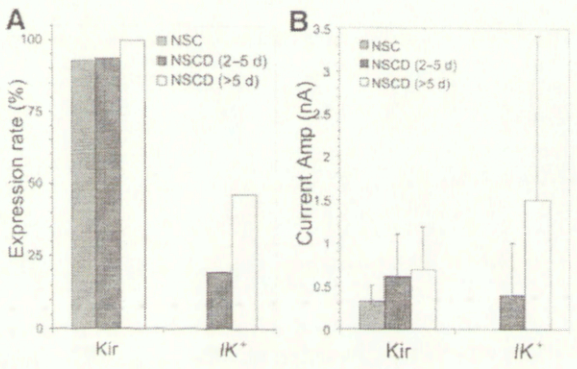

Figure 6. Comparison of Kir and I_{K+} in HUCB-NSC versus HUCB-NSCD. **(A):** Kir was recorded from nearly all (~95%) HUCB-NSCs and HUCB-NSCDs. I_{K+} only expressed in HUCB-NSCD. I_{K+} was absent from HUCB-NSC; only a few HUCB-NSCDs (7 of 36) expressed I_{K+} after 2–5 days of attachment, whereas approximately 46% (35 of 73) of HUCB-NSCD expressed I_{K+} after more then 5 days of attachment. **(B):** The average amplitude of Kir from HUCB-NSC was approximately 0.3 nA versus 0.7 nA for HUCB-NSCD (t-test, $p < .01$). I_{K+} amplitude increased from 0.4 ± 0.6 nA ($n = 7$) to 1.5 ± 1.9 nA ($n = 35$) in cells differentiated for 2–5 days versus >5 days. Abbreviations: HUCB, human umbilical cord blood; NSC, neural stem cell; NSCD, differentiated neural stem cell.

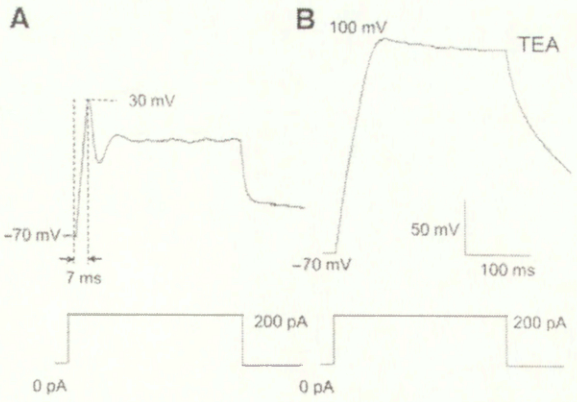

Figure 7. (A): Depolarizing response of human umbilical cord blood differentiated neural stem cells in response to a current pulse (0 to +200 pA, 140 ms). **(B):** Tetraethylammonium (TEA), which blocks I_{K+}, eliminates the onset spike and increases the width of the depolarizing response.

HUCB-NSCDs. At a holding potential of –70 mV, KA induced a nondesensitizing, inward current in some HUCB-NSCDs (4 of 45; Fig. 8A). The KA-induced current was totally blocked by CNQX, a non-NMDA receptor antagonist (Fig. 8B). The KA-induced current recovered when CNQX was washed out (Fig. 8C).

Application of KA (0.5 mM) onto HUCB-NSCD suppressed the amplitude of Kir by 22% ± 7% (n = 8, 8 of 10) induced by a voltage step (–50 to –140 mV) (Fig. 8D). To confirm that this effect was mediated through non-NMDA receptors, we applied KA in the presence of CNQX (0.1 mM). CNQX completely blocked the suppressive effect of KA on Kir amplitude (Fig. 8E); Kir quickly recovered when CNQX was washed out (data not shown).

To test for the presence of functional glycine receptors, HUCB-NSCDs were maintained at a holding potential of –50 mV to avoid activating Kir. Under these conditions, glycine (1 mM) failed to induce a current (data not shown). To determine if glycine had an effect on the amplitude of Kir, glycine was perfused onto cells while stepping the voltage from a holding potential of –50 to –140 mV. Glycine suppressed the amplitude of Kir by up to 80% (Fig. 9A) in most cells (five of six). To confirm that this effect was mediated by glycine receptors, we perfused glycine in the presence of strychnine (0.1 mM), a glycine receptor antagonist. Strychnine completely blocked the effect of glycine on Kir amplitude (data not shown); Kir quickly recovered when strychnine was washed out.

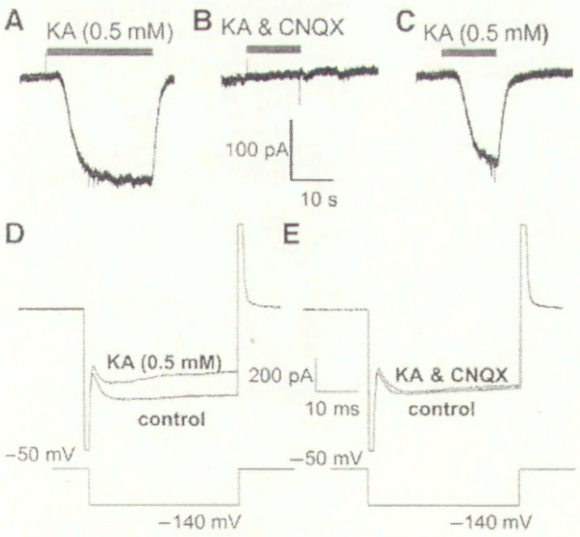

Figure 8. **(A):** KA (0.5 mM) induced an inward current from human umbilical cord blood differentiated neural stem cells. **(B):** The inward current was totally blocked by CNQX, a non-NMDA receptor antagonist. **(C):** CNQX-induced suppression was eliminated when KA was applied alone. **(D):** KA (0.5 mM) partly blocked the Kir induced by voltage step (below). **(E):** CNQX (0.1 mM) blocked the KA (0.5 mM)-induced suppression of Kir induced by voltage step (below). Abbreviations: CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; KA, kainic acid; NMDA, N-methyl-D-asparate.

To test for the presence of GABA receptors, HUCB-NSCDs were maintained at –50 to avoid activating Kir. Under these conditions, GABA (1 mM) failed to induce a current in any cells (n = 8, data not shown). To determine if GABA had an effect on the amplitude of Kir, GABA was perfused onto cells while stepping the voltage from a holding potential of –50 to –140 mV (Fig. 9B). Application of GABA (1 mM) reduced the amplitude of Kir by 58% ± 30% in most cells (five of eight).

To test for the presence of functional ACh receptors, HUCB-NSCDs were maintained at a holding potential of –50 mV to avoid activating Kir. Under these conditions, ACh (1 mM) failed to induce a current (data not shown). To determine if ACh had an effect on the amplitude of Kir, ACh was perfused onto cells while stepping the voltage from a holding potential of –50 to –140 mV (Fig. 10A). ACh suppressed the amplitude of Kir (Fig. 10A) in 5 of 13 cells; the average reduction was 33% ± 22%. To assess the effect of ACh in more detail, the holding potential was set to –100 mV to produce a sustained activation of Kir, and then ACh or nicotine was perfused onto cells. ACh (Fig. 10B) and nicotine (Fig. 10C) suppressed Kir; Kir recovered after washout of these agonists.

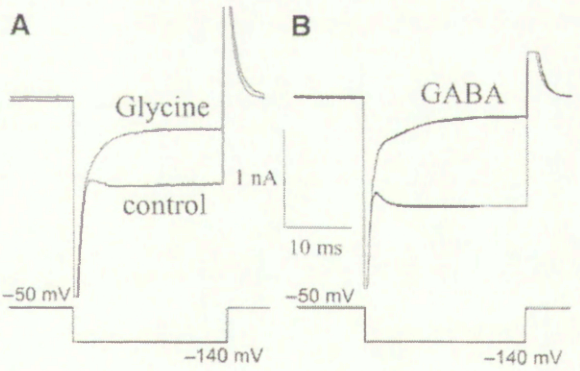

Figure 9. Both **(A)** glycine (1 mM) and **(B)** GABA (1 mM) suppressed the Kir induced by a negative voltage step (–50 to –140 mV). Abbreviation: GABA, γ-aminobutyric acid.

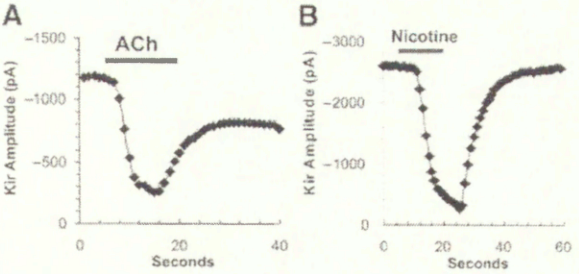

Figure 10. Human umbilical cord blood differentiated neural stem cells clamped to –140 mV to induce Kir. **(A):** ACh (1 mM) partially blocks Kir; Kir partly recovers after ACh was washed out. **(B):** Nicotine (1 mM) greatly reduces Kir; Kir recovers after nicotine washed out. Abbreviation: ACh, acetylcholine.

To test for the presence of serotonin receptors, HUCB-NSCD was maintained at a holding potential of −50 mV to avoid activating Kir. Under these conditions, 5-HT failed to induce a current (data not shown). To determine if 5-HT had an effect on the amplitude of Kir, 5-HT was perfused onto cells while repeatedly stepping the voltage from a holding potential of −50 to −140 mV (Fig. 11A). 5-HT greatly reduced the amplitude of Kir (Fig. 11A) in 5 of 13 cells; the average reduction was 65% ± 22%. To confirm that this effect was mediated through 5-HT receptors, 5-HT was applied in the presence of L-278,276, a 5-HT receptor antagonist (0.5 mM). L-278,276 greatly reduced the suppressive effect of 5-HT on Kir amplitude (Fig. 11B).

To test for the presence of DA receptors, HUCB-NSCDs were maintained at a holding potential of −50 mV to avoid activating Kir. Under these conditions, DA failed to induce a current (data not shown). To determine if DA had an effect on Kir amplitude, cells were maintained at a holding potential of −100 mV to activate Kir, and DA (1 mM) was perfused onto cells (Fig. 11C); DA gradually reduced the amplitude by 59% ± 16% ($n = 5$) of Kir in 5 of 13 cells.

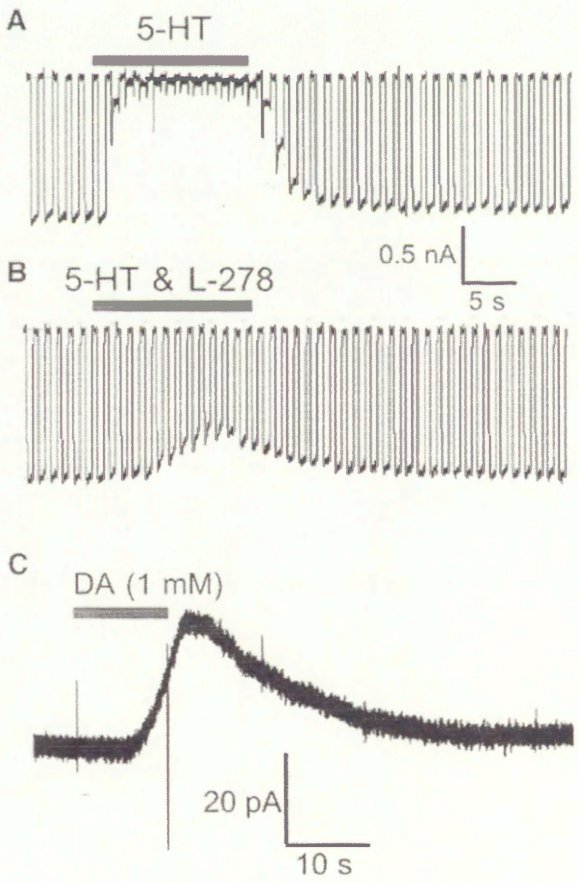

Figure 11. Human umbilical cord blood differentiated neural stem cells. **(A):** 5-HT suppresses Kir amplitude induced by negative voltage step (−50 to −140 mV); Kir amplitude recovers after 5-HT washed out. **(B):** 5-HT–induced suppression of Kir largely blocked by L-278,276, a 5-HT receptor antagonist. **(C):** Kir activated at holding potential of −100 mV. DA (1 mM) suppresses Kir amplitude; amplitude recovers after DA washed out. Abbreviations: 5-HT, 5-hydroxytryptamine; DA, dopamine.

Discussion

The main finding of this study is that when HUCB-NSCs are maintained under differentiating conditions, they express neurotransmitter receptors, ion channels, and electrophysiological properties similar to those seen in immature neurons or glia. The results provide additional evidence on the neural-like features of HUCB-NSCs and HUCB-NSCDs. Our immunocytochemical results show that polylysine/laminin-attached HUCB-NSCDs grown in 2% FBS and treated with dBcAMP/CPT differentiate almost exclusively (>80%) into neuron-shaped cells expressing β-tubulin III, a neuron-specific filament protein characteristic of developing neurons (unpublished data). Only a few cells expressed astrocytic (GFAP) or oligodendrocytic (GalC) markers, indicating that under these conditions, HUCB-NSCDs preferentially develop toward a neuronal phenotype. The preferential differentiation of HUCB-NSCs to neuronal-like cells could be related to their fetal origin, because ontogenetically younger stem cells give rise more frequently to neurons than to astrocytes. Microarray analysis (Table 1) identified other neuronal markers in proliferating, nonattached HUCB-NSCs and polylysine/laminin-attached HUCB-NSCDs; these included genes encoding a diverse set of voltage-dependent potassium and sodium channels and neurotransmitter receptors (ACh, GABA, glutamate, glycine, 5-HT, and DA). Many of these genes (GABA-A and glycine A receptors, glutamate kainite 4 receptor, potassium voltage-gated KQT-like channel, sodium voltage-gated type XII alpha channel) were not expressed in HUCB-MCs, which served as a starting cell population for HUCB-NSCs. In addition, expression of some neuronal genes (GABA-A alpha 2 and glutamatergic kainate receptor 4) increased in HUCB-NSCDs, indicating further differentiation toward a neuronal phenotype under differentiation conditions.

Voltage-Gated Ion Channels

There is growing evidence that potassium channels play an important role in early embryonic development [39] and later stages of differentiation [40, 41]. We observed two types of potassium channels. Kir and I_{K+} in HUCB-NSCs. Kir channels were constitutively expressed in both proliferating HUCB-NSCs (93%) and attached (94%–99%) HUCB-NSCDs (Fig. 4), results consistent with the gene expression data in Table 1. Kir channels play an important role in cell proliferation during early kidney development [42]. Treatment of primitive human hematopoietic progenitor cells ($CD34^+$, $CD38^-$) with stem cell factor (SCF) and interleukin-3 (IL-3) enhances the expression of Kir channels and promotes the expansion of these cells into lineage-restricted precursors [32, 34].

Antisense oligonucleotides against Kir blocked the expression of Kir channels in primitive hematopoietic progenitors stimulated with SCF and IL-3 and prevented the expansion and differentiation of these cells. Similarly, growth-stimulated proliferation of microglia was inhibited by the Kir blocker Ba^{2+} [43]. These results suggest that Kir channels play an important role in proliferation and differentiation of stem cells. Future studies directed at blocking Kir channels would help to elucidate the role of these potassium channels in the differentiation of HUCB-NSCs.

Outward potassium currents are present in many cell types [44]; however, we did not observe outward-rectifying I_{K+} in undifferentiated HUCB-NSCs. Interestingly, treatment of attached cells with dBcAMP/CPT resulted in the expression of outward I_{K+} in approximately half of the cells cultured for >5 days (Fig. 4). The outward-rectifying I_{K+} was partially blocked by 4-AP and TEA, criteria consistent with a delayed, outward-rectifying potassium current [33]. In our dBcAMP/CPT-differentiated HUCB-NSCDs, more than 80% expressed β-tubulin III; these results suggest that many of the HUCB-NSCDs expressing the delayed, outward I_{K+} had differentiated toward neural progenitors. Our results are similar to those seen in stem cells derived from the subventricular zone of postnatal rat; potassium currents were observed only in attached, EGF-differentiated cells with neuronal morphology and not in dividing, undifferentiated cells [45].

The I_{K+} seen in differentiated HUCB-NSCs was partially blocked by Cd^{2+}, suggesting that calcium-activated potassium current may make a small contribution to the outward-rectifying potassium current. The gene expression data (Table 1), however, showed evidence for a large-conductance, calcium-activated potassium channel in both undifferentiated and differentiated HUCB-NSCs. It is unclear why calcium-activated potassium currents were not seen in undifferentiated HUCB-NSCs, but their absence suggests that other factors are required for these channels to be functionally expressed in HUCB-NSCs.

Although our gene microarray data provide evidence for voltage-gated sodium channels (types X and XII) in HUCB-NSCDs, we did not find any evidence of voltage-gated sodium currents or action potentials, a hallmark of mature neurons, in HUCB-NSCs or HUCB-NSCDs. Although HUCB-NSCD expressed many neuronal markers, the absence of rapidly activating inward sodium currents after differentiation with dBcAMP/CPT suggests that other factors or conditions are required before these cells can generate action potentials. Human NSCs also lack voltage-gated sodium current; however, sodium currents could be induced in these cells after transfection with NeuroD, a neurogenic transcription factor [46], or after priming for 2 weeks with the cocktail of bFGF, heparin, and laminin [47]. Rat embryonic stem cells fired action potential exclusively after transfection with Nurr1, and when transplanted into the rat striatum, they differentiate toward functional dopaminergic neurons [4]. Adult NSCs isolated from rat hippocampus also developed action potentials when cocultured with primary neurons and astrocytes [48]. In bone marrow–derived, multipotent adult progenitor cells, spiking behavior and voltage-gated currents were observed after coculture of neural-committed progenitors with fetal mouse brain astrocytes [49]. On the other hand, human bone marrow–derived NSCs induced to differentiate using neuromorphogens developed voltage-dependent potassium channels but failed to develop voltage-dependent sodium current required for action potentials [50]. Further studies are needed to determine if other factors or conditions can lead to the further differentiation and generation of sodium currents and action potentials in HUCB-NSCs.

In preliminary studies, we looked for evidence of voltage-gated calcium current (data not shown) but failed to detect any. This result is consistent with the absence of voltage-gated calcium channels in our gene microarray data (Table 1). Our inability to detect high voltage–activated calcium currents could be due to the recording conditions and, specifically, the presence of fluoride in the recording pipette, which blocks high voltage–activated calcium channels in some cells [51]. However, in other cells, fluoride has been reported to block the rundown of high voltage–activated calcium currents [52].

Ligand-Gated Ion Channels and Neurotransmitters

Differentiated HUCB-NSCs could conceivably be used to repair damaged neural circuits in the brain and spinal cord; however, the integration of HUCB-NSCs into a neural network requires that cells express ligand-gated receptors that can respond to neurotransmitters released from presynaptic inputs. Neurotransmitters acting though their cognate receptors can also regulate cell proliferation, direct neuronal migration, and promote differentiation in the developing nervous system [53].

Many neurotransmitter receptors were identified on HUCB-NSCs. Microarray analysis indicated that kainate receptors (Table 1) but not NMDA receptors were expressed on differentiated and nondifferentiated HUCB-NSCs but not on HUCB-MCs. Whole-cell recordings revealed functional, kainate-sensitive, non-NMDA glutamate receptors on differentiated HUCB-NSCs, consistent with immunolabeling results (Fig. 2). NMDA-induced currents were not detected in whole-cell recordings, consistent with our microarray data. KA induced inward currents in 10% of differentiated HUCB-NSCs and suppressed Kir in most HUCB-NSCs. The latter effect has been attributed to a receptor-mediated influx of sodium, which blocks the intracellular face of the channel [54]. Functional ionotropic glutamate receptors emerge during terminal cell division and early neuronal differentiation of rat neuroepithelial cells [55] and NSCs from hippocampus [48]. The influx of calcium through kainate receptors promotes dendrite outgrowth [56]. In addition to their obvious role in rapid neurotransmission, kainate receptors inhibit the proliferation and lineage progression in oligodendrocyte progenitors by increasing intracellular sodium and inhibiting outward potassium currents [57].

Surprisingly, our microarray analysis revealed metabotropic glutamate receptor 6 (mGluR6) in both HUCB-NSCs and HUCB-MCs (Table 1). mGluR6 is thought to be exclusively expressed in the retina, where it induces a hyperpolarization in bipolar cells in response to glutamate [58]. In addition, mGluR6 expression is upregulated in the retina under conditions that induce apoptosis [59]. Activation of mGluR6 inhibits adenylate cyclase, leading to a decrease in cAMP, one of the factors added to the cultures to enhance differentiation [60].

Glycine receptor beta mRNA was expressed in undifferentiated and differentiated HUCB-NSCs. In addition, differentiated HUCB-NSCs coexpressed GlyR and β-tubulin III, an early neural marker, and responded to exogenous glycine by suppressing Kir amplitude. Glycine receptors and their mRNAs have been identified on stem cells derived from rodent striatum [61], neonatal oligodendrocyte progenitors [62], and human corneal limbal stem cells [29]. The functional role of glycine receptors in HUCB-NSCs is unclear; however, in the retina, taurine or the combination of glycine and GABA, acting through glycine receptors expressed on neonatal retinal progenitor cells, promotes exit from the cell cycle and significantly increases the number of rod photoreceptors [63, 64]. These results suggest that glycine receptors could regulate cell proliferation and influence the fate of HUCB-NSCs.

GABA-A receptor mRNAs (α2, 1, and γ3) were identified in differentiated HUCB-NSCs. Moreover, nearly all HUCB-NSCDs immunopositive for β-tubulin III, an early neural marker, were also immunopositive for GABA-AR and GABA, suggesting an autocrine/paracrine role for GABA. Exogenous GABA also suppressed the voltage-gated Kir in HUCB-NSCDs. The function of GABA in the developing nervous system is poorly understood; however, there is growing evidence that GABA, acting through GABA-A receptors, serves as a trophic factor and influences cell proliferation, differentiation, migration, and synapse development [65]. Activation of GABA-A receptors depolarizes cells in proliferative zones of the neocortex and suppresses cell division [66], and GABA promotes chemotactic and chemokinetic responses in embryonic cortical cells [67]. Moreover, postnatal cells in the striatum synthesize and release GABA, creating an autocrine/paracrine mechanism that controls their proliferation [68].

All studied cell populations (HUCB-MC, HUCB-NSC, and HUCB-NSCD) expressed mRNAs for three types of serotonin receptors (1C, 2A, and 3A). In addition, nearly all differentiated HUCB-NSCs were immunopositive for the 5-HT1C receptor, and application of serotonin suppressed Kir through its receptor. The function role of serotonin receptors on HUCB cells is not well understood; however, serotonin has been shown to promote the survival of cortical progenitor cells with glutamatergic characteristics [69]. In the adult hippocampus, serotonin acting through 5HT2A receptors regulates cell proliferation [70, 71]. 5-HT1C receptors are expressed on human fetal glioma cell lines, and application of serotonin was shown to modulate proliferation, migration, and tumor invasion [72].

Nicotinic AChR (alpha 5, beta 3, and epsilon) were expressed on HUCB-MC, HUCB-NSC, and HUCB-NSCD (Table 1). HUCB-NSCDs were immunopositive for nicotinic AChR beta, and perfusion of ACh and nicotine suppressed Kir. The functional roles of these AChRs are not well understood; however, ACh has been implicated in the proliferation and differentiation of neural progenitor cells. ACh and functional nAChR are present on neural progenitors in embryonic mouse cerebral cortex, suggesting a role in development [73]. Oligodendrocyte progenitor cells also express nAChR, but expression disappears after cells differentiate into oligodendrocytes or astrocytes [74].

Because of the therapeutic potential in Parkinson's disease, there is considerable interest in stem or progenitor cells that express DA and its receptors. DA D2 and D5 receptor mRNAs were expressed on HUCB-MCs as well as HUCB-NSCs and HUCB-NSCDs. DA receptor D2 immunolabeling was also seen on HUCB-NSCDs, and DA application suppressed Kir amplitude in these cells. In the neostriatum, DA D1 and D2 receptors repress and enhance neurogenesis, respectively [75]. Moreover, DA acting through D1 receptors promotes neural differentiation by stimulating neurite outgrowth and growth cone formation [76, 77]. All together, these results suggest that the DA receptors on HUCB-NSCs may be involved in cell proliferation and differentiation.

Summary

Our results show that neural stem–like cells derived from the nonhematopoietic fraction of HUCB and expand as a stable, clonogenic line over a long time, retaining their capacity to differentiate into neuron-like cells that express neuron-specific cytoskeletal markers, numerous neurotransmitter receptors, and several functional voltage-gated channels. Kir channels are expressed in most HUCB-NSCs and HUCB-NSCDs; however, I_{K+} was observed only in some HUCB-NSCDs. Although the bulk of the evidence suggests that HUCB-NSCDs are progressing along a neuronal lineage, the absence of voltage-gated sodium channels clearly indicates that this process is incomplete and that other factors or conditions are required for these cells to fully differentiate into neurons. One approach that might further advance differentiation of HUCB-NSCDs toward more mature neurons would be to coculture these cells with astrocytes [48] or organotypic brain slices [78]. During early development, neurotransmitters as a class of secreted molecules could also influence proliferation, migration, and differentiation [63, 64, 67, 70–72]. An alternative method that could provide important clues regarding the specific roles neurotransmitters play in proliferation and development would be to culture HUCB-NSCs or HUCB-NSCDs with neurotransmitter agonists or antagonists for ACh, GABA, glutamate, glycine, serotonin, and DA. Finally, a third approach would be to transfect HUCB-NSCs with neurogenic transcription factors or treat them with other morphogens that promote neuronal phenotypes [4, 46].

Acknowledgments

W.S. and L.B. contributed equally to this study. This research was supported by grants from the NIH (P01 DC03600 to R.J.S., R01 DC06630 to R.J.S., NS46321-01 to M.K.S.), Oishei Foundation (to M.K.S.), NSF (IBN-9728923 to M.K.S.), American Parkinson Disease Association (to M.K.S.), and Polish State Committee for Scientific Research (K053/P05/2003 to K.D.-J.).

References

1 Eriksson PS, Perfilieva E, Bjork-Eriksson T et al. Neurogenesis in the adult human hippocampus. Nat Med 1998;4:1313–1317.

2 Magavi SS, Leavitt BR, Macklis JD. Induction of neurogenesis in the neocortex of adult mice. Nature 2000;405:951–955.

3 Hallbergson AF, Gnatenco C, Peterson DA. Neurogenesis and brain injury: managing a renewable resource for repair. J Clin Invest 2003;112:1128–1133.

4 Kim JH, Auerbach JM, Rodriguez-Gomez JA et al. Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease. Nature 2002;418:50–56.

5 Pluchino S, Furlan R, Martino G. Cell-based remyelinating therapies in multiple sclerosis: evidence from experimental studies. Curr Opin Neurol 2004;17:247–255.

6 Richardson RM, Fillmore HL, Holloway KL et al. Progress in cerebral transplantation of expanded neuronal stem cells. J Neurosurg 2004;100:659–671.

7 Toda H, Takahashi J, Mizoguchi A et al. Neurons generated from adult rat hippocampal stem cells form functional glutamatergic and GABAergic synapses in vitro. Exp Neurol 2000;165:66–76.

8 Englund U, Bjorklund A, Wictorin K et al. Grafted neural stem cells develop into functional pyramidal neurons and integrate into host cortical circuitry. Proc Natl Acad Sci U S A 2002;99:17089–17094.

9 Muraro PA, Cassiani Ingoni R, Martin R. Hematopoietic stem cell transplantation for multiple sclerosis: current status and future challenges. Curr Opin Neurol 2003;16:299–305.

10 Windrem MS, Nunes MC, Rashbaum WK et al. Fetal and adult human oligodendrocyte progenitor cell isolates myelinate the congenitally dysmyelinated brain. Nat Med 2004;10:93–97.

11 Kopen GC, Prockop DJ, Phinney DG. Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. Proc Natl Acad Sci U S A 1999;96:10711–10716.

12 Sanchez-Ramos J, Song S, Cardozo-Pelaez F et al. Adult bone marrow stromal cells differentiate into neural cells in vitro. Exp Neurol 2000;164:247–256.

13 Li Y, Chen J, Wang L et al. Intracerebral transplantation of bone marrow stromal cells in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's disease. Neurosci Lett 2001;316:67–70.

14 Mezey E, Chandross KJ, Harta G et al. Turning blood into brain: cells bearing neuronal antigens generated in vivo from bone marrow. Science 2000;290:1779–1782.

15 Weimann JM, Charlton CA, Brazelton TR et al. Contribution of transplanted bone marrow cells to Purkinje neurons in human adult brains. Proc Natl Acad Sci U S A 2003;100:2088–2093.

16 Cogle CR, Yachnis AT, Laywell ED et al. Bone marrow transdifferentiation in brain after transplantation: a retrospective study. Lancet 2004;363:1432–1437.

17 Corti S, Locatelli F, Strazzer S et al. Neuronal generation from somatic stem cells: current knowledge and perspectives on the treatment of acquired and degenerative central nervous system disorders. Curr Gene Ther 2003;3:247–272.

18 Kim DK, Fujiki Y, Fukushima T et al. Comparison of hematopoietic activities of human bone marrow and umbilical cord blood CD34 positive and negative cells. STEM CELLS 1999;17:286–294.

19 Sanchez-Ramos JR, Song S, Kamath SG et al. Expression of neural markers in human umbilical cord blood. Exp Neurol 2001;171:109–115.

20 Storms RW, Goodell MA, Fisher A et al. Hoechst dye efflux reveals a novel CD7(+)CD34(-) lymphoid progenitor in human umbilical cord blood. Blood 2000;96:2125–2133.

21 Zigova T, Song S, Willing AE et al. Human umbilical cord blood cells express neural antigens after transplantation into the developing rat brain. Cell Transplant 2002;11:265–274.

22 Buzanska L, Machaj EK, Zablocka B et al. Human cord blood-derived cells attain neuronal and glial features in vitro. J Cell Sci 2002;115:2131–2138.

23 Sanchez-Ramos JR. Neural cells derived from adult bone marrow and umbilical cord blood. J Neurosci Res 2002;69:880–893.

24 Hung SC, Cheng H, Pan CY et al. In vitro differentiation of size-sieved stem cells into electrically active neural cells. STEM CELLS 2002;20:522–529.

25 Lu D, Sanberg PR, Mahmood A et al. Intravenous administration of human umbilical cord blood reduces neurological deficit in the rat after traumatic brain injury. Cell Transplant 2002;11:275–281.

26 Willing AE, Vendrame M, Mallery J et al. Mobilized peripheral blood cells administered intravenously produce functional recovery in stroke. Cell Transplant 2003;12:449–454.

27 Yoo ES, Lee KE, Seo JW et al. Adherent cells generated during long-term culture of human umbilical cord blood CD34$^+$ cells have characteristics of endothelial cells and beneficial effect on cord blood ex vivo expansion. STEM CELLS 2003;21:228–235.

28 Buzanska L, Habich A, Jurga M et al. Human cord blood-derived neural stem cell line - possible implementation in studying neurotoxicity. Toxicology In Vitro 2005 (in press).

29 Seigel GM, Sun W, Salvi R et al. Human corneal stem cells display functional neuronal properties. Mol Vis 2003;9:159–163.

30 Sun W, Ding D, Wang P et al. Substance P inhibits potassium and calcium currents in inner ear spiral ganglion neurons. Brain Res 2004;1012:82–92.

31 Sun W, Salvi RJ. Dopamine modulates soduim currents in cochlear spiral ganglion neurons. Neuroreport 2001;12:803–807.

32 Shirihai O, Attali B, Dagan D et al. Expression of two inward rectifier potassium channels is essential for differentiation of primitive human hematopoietic progenitor cells. J Cell Physiol 1998;177:197–205.

33 Hille B. Ionic Channels of Excitable Membrane. Sunderlang, MA: Sinauer Associates Inc., 1992.

34 Shirihai O, Merchav S, Attali B et al. K$^+$ channel antisense oligodeoxynucleotides inhibit cytokine-induced expansion of human hemopoietic progenitors. Pflugers Arch 1996;431:632–638.

35 Hu Q, Shi YL. Characterization of an inward-rectifying potassium current

in NG108-15 neuroblastoma x glioma cells. Pflugers Arch 1997;433:617–625.

36 Kuryshev YA, Haak L, Childs GV et al. Corticotropin releasing hormone inhibits an inwardly rectifying potassium current in rat corticotropes. J Physiol 1997;502:265–279.

37 Tourneur Y. Action potential-like responses due to the inward rectifying potassium channel. J Membr Biol 1986;90:115–122.

38 Elliott AA, Elliott JR. Characterization of TTX-sensitive and TTX-resistant sodium currents in small cells from adult rat dorsal root ganglia. J Physiol 1993;463:39–56.

39 Day ML, Pickering SJ, Johnson MH et al. Cell-cycle control of a large-conductance K^+ channel in mouse early embryos. Nature 1993;365:560–562.

40 Patil N, Cox DR, Bhat D et al. A potassium channel mutation in weaver mice implicates membrane excitability in granule cell differentiation. Nat Genet 1995;11:126–129.

41 Neusch C, Weishaupt JH, Bahr M. Kir channels in the CNS: emerging new roles and implications for neurological diseases. Cell Tissue Res 2003;311:131–138.

42 Braun GS, Horster MF, Huber SM. Kirl.1 expression in embryonic kidney epithelia. Biochem Biophys Res Commun 2003;312:1191–1195.

43 Schlichter LC, Sakellaropoulos G, Ballyk B et al. Properties of K^+ and Cl^- channels and their involvement in proliferation of rat microglial cells. Glia 1996;17:225–236.

44 Rudy B. Diversity and ubiquity of K channels. Neuroscience 1988;25:729–749.

45 Feldman DH, Thinschmidt JS, Peel AL et al. Differentiation of ionic currents in CNS progenitor cells: dependence upon substrate attachment and epidermal growth factor. Exp Neurol 1996;140:206–217.

46 Cho T, Bae JH, Choi HB et al. Human neural stem cells: electrophysiological properties of voltage-gated ion channels. Neuroreport 2002;13:1447–1452.

47 Wu P, Tarasenko YI, Gu Y et al. Region-specific generation of cholinergic neurons from fetal human neural stem cells grafted in adult rat. Nat Neurosci 2002;5:1271–1278.

48 Song H, Stevens CF, Gage FH. Astroglia induce neurogenesis from adult neural stem cells. Nature 2002;417:39–44.

49 Jiang Y, Henderson D, Blackstad M et al. Neuroectodermal differentiation from mouse multipotent adult progenitor cells. Proc Natl Acad Sci U S A 2003;100(suppl 1):11854–11860.

50 Hermann A, Gastl R, Liebau S et al. Efficient generation of neural stem cell-like cells from adult human bone marrow stromal cells. J Cell Sci 2004;117:4411–4422.

51 Bertollini L, Biella G, Wanke E et al. Fluoride reversibly blocks IIVA calcium current in mammalian thalamic neurones. Neuroreport 1994;5:553–556.

52 Breakwell NA, Behnisch T, Publicover SJ et al. Attenuation of high-voltage-activated $Ca2^+$ current run-down in rat hippocampal CA1 pyramidal cells by NaF. Exp Brain Res 1995;106:505–508.

53 Nguyen L, Rigo JM, Rocher V et al. Neurotransmitters as early signals for central nervous system development. Cell Tissue Res 2001;305:187–202.

54 Schroder W, Seifert G, Huttmann K et al. AMPA receptor-mediated modulation of inward rectifier K^+ channels in astrocytes of mouse hippocampus. Mol Cell Neurosci 2002;19:447–458.

55 Maric D, Liu QY, Grant GM et al. Functional ionotropic glutamate receptors emerge during terminal cell division and early neuronal differentiation of rat neuroepithelial cells. J Neurosci Res 2000;61:652–662.

56 Metzger F, Wiese S, Sendtner M. Effect of glutamate on dendritic growth in embryonic rat motorneurons. J Neurosci 1998;18:1735–1742.

57 Gallo V, Zhou JM, McBain CJ et al. Oligodendrocyte progenitor cell proliferation and lineage progression are regulated by glutamate receptor-mediated K^+ channel block. J Neurosci 1996;16:2659–2670.

58 Gerber U. Metabotropic glutamate receptors in vertebrate retina. Doc Ophthalmol 2003;106:83–87.

59 Dyka FM, May CA, Enz R. Metabotropic glutamate receptors are differentially regulated under elevated intraocular pressure. J Neurochem 2004;90:190–202.

60 Knopfel T, Grandes P. Metabotropic glutamate receptors in the cerebellum with a focus on their function in Purkinje cells. Cerebellum 2002;1:19–26.

61 Nguyen L, Malgrange B, Belachew S et al. Functional glycine receptors are expressed by postnatal nestin-positive neural stem/progenitor cells. Eur J Neurosci 2002;15:1299–1305.

62 Belachew S, Rogister B, Rigo JM et al. Cultured oligodendrocyte progenitors derived from cerebral cortex express a glycine receptor which is pharmacologically distinct from the neuronal isoform. Eur J Neurosci 1998;10:3556–3564.

63 Young TL, Cepko CL. A role for ligand-gated ion channels in rod photoreceptor development. Neuron 2004;41:867–879.

64 Renteria RC, Johnson J, Copenhagen DR. Need rods? Get glycine receptors and taurine. Neuron 2004;41:839–841.

65 Owens DF, Kriegstein AR. Is there more to GABA than synaptic inhibition? Nat Rev Neurosci 2002;3:715–727.

66 LoTurco JJ, Owens DF, Heath MJ et al. GABA and glutamate depolarize cortical progenitor cells and inhibit DNA synthesis. Neuron 1995;15:1287–1298.

67 Behar TN, Li YX, Tran HT et al. GABA stimulates chemotaxis and chemokinesis of embryonic cortical neurons via calcium-dependent mechanisms. J Neurosci 1996;16:1808–1818.

68 Nguyen L, Malgrange B, Breuskin I et al. Autocrine/paracrine activation of the GABA(A) receptor inhibits the proliferation of neurogenic polysialylated neural cell adhesion molecule-positive (PSA-NCAM+) precursor cells from postnatal striatum. J Neurosci 2003;23:3278–3294.

69 Dooley AE, Pappas IS, Parnavelas JG. Serotonin promotes the survival of cortical glutamatergic neurons in vitro. Exp Neurol 1997;148:205–214.

70 Malberg JE, Duman RS. Cell proliferation in adult hippocampus is decreased by inescapable stress: reversal by fluoxetine treatment. Neuropsychopharmacology 2003;28:1562–1571.

71 Banasr M, Hery M, Printemps R et al. Serotonin-induced increases in adult cell proliferation and neurogenesis are mediated through different and common 5-HT receptor subtypes in the dentate gyrus and the subventricular zone. Neuropsychopharmacology 2004;29:450–460.

72 Merzak A, Koochekpour S, Fillion MP et al. Expression of serotonin receptors in human fetal astrocytes and glioma cell lines: a possible role in glioma cell proliferation and migration. Brain Res Mol Brain Res 1996;41:1–7.

73 Schneider AS, Atluri P, Shen Q et al. Functional nicotinic acetylcholine receptor expression on stem and progenitor cells of the early embryonic nervous system. Ann N Y Acad Sci 2002;971:135–138.

74 Rogers SW, Gregori NZ, Carlson N et al. Neuronal nicotinic acetylcholine receptor expression by O2A/oligodendrocyte progenitor cells. Glia 2001;33:306–313.

75 Ohtani N, Goto T, Waeber C et al. Dopamine modulates cell cycle in the lateral ganglionic eminence. J Neurosci 2003;23:2840–2850.

76 Schmidt U, Pilgrim C, Beyer C. Differentiative effects of dopamine on striatal neurons involve stimulation of the cAMP/PKA pathway. Mol Cell Neurosci 1998;11:9–18.

77 Schmidt U, Beyer C, Oestreicher AB et al. Activation of dopaminergic D1 receptors promotes morphogenesis of developing striatal neurons. Neuroscience 1996;74:453–460.

78 Benninger F, Beck H, Wernig M et al. Functional integration of embryonic stem cell-derived neurons in hippocampal slice cultures. J Neurosci 2003;23:7075–7083.

PUBLIKACJA 3

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Toxicology in Vitro 19 (2005) 991–999
Toxicology in Vitro
www.elsevier.com/locate/toxinvit

Human cord blood-derived neural stem cell line—Possible implementation in studying neurotoxicity

L. Bużańska, A. Habich, M. Jurga, J. Sypecka, K. Domańska-Janik *

NeuroRepair Department, Medical Research Center, Polish Academy of Sciences, Pawińskiego 5 Str, 02-106 Warsaw, Poland

Received 9 May 2005; accepted 17 June 2005
Available online 9 August 2005

Abstract

Neural stem cell line developed from human umbilical cord blood (HUCB-NSC) [Bużańska et al., 2003. Journal of Neurochemistry 85, 33] is an ethically uncontroversial source of stem cells, able to differentiate into neuronal, astrocytic and oligodendroglial lineages. Developmental fate decisions of HUCB-NSC can be experimentally manipulated in vitro by the presence of trophic factors, mitogenes and neuromorphogenes, but can also be influenced by neurotoxins. In this report two-dimensional (2-D) and three-dimensional (3-D) HUCB-NSC cultures are introduced as useful models for testing developmental neurotoxicity. For 2-D culture models we established a standardized method for the assessment of the growth rate and cell differentiation in 96-well plates. The proliferative capacity of the HUCB-NSC was monitored by the MTT test while their ability to differentiate into neural-like cells by immunocytochemistry of β-tubulin III and MAP-2 for neurons, GFAP and S-100β for astrocytes and GalC for oligodendrocytes. The 3-D culture of HUCB-NSC is represented by neurospheres. Proliferation and migration of the intermediate precursors from attached neurospheres are shown to be controlled and altered by various growth factors and further modulated by the extracellular matrix component—fibronectin. Thus, neurospheres derived from the HUCB-NSC line can represent a suitable model of the activation of dormant stem cells residing in their niche, and can be used for neurotoxic studies.

Keywords: Human neural stem cell line; Cord blood-derived; Neural differentiation; Neurotoxicity

1. Introduction

Somatic stem cells (sSC) are defined as cells that have the ability to perpetuate themselves through self-renewal and to generate mature cells of a particular tissue through differentiation (Palm et al., 2000; Poulsom et al., 2002; Corti et al., 2003). Studies carried out in the last decade have revealed unexpected potential of brain neurogenesis and the existence of neural (N)SC even in adulthood. Moreover, the pool of early progenitors is likely to decline in time (Kempermann et al., 2000; Schaffer and Gage, 2004). The prospect neural stem cells have to offer (NSC) for the cell therapy of the neurodegenerative disorders is now widely accepted and stem cell research is mainly focused on this aspect of NSC application (Bjorklund and Lindval, 2000; Kim et al., 2002; Pluchino et al., 2003). However, the NSC culture can be considered as model system for the screening in vitro the putative toxic factors and their influence for the viability and differentiation in neuronal cells (Storch and Schwarz, 2002). Thus, stem and precursor cells can be a potential target either for various neurotoxic compounds or for neuroprotective drug treatments. However, one of the most serious limitations for the use of human NSC to in vitro study is their limited availability because of complex tissue localization and/or irreversible growth inhibition typical for any type of sSCs in long-term culture (Svendsen et al., 1998; Vescovi et al., 1999; Caldwell et al., 2001). In our study we overpass

* Corresponding author. Tel./fax: +48 22 6086510.
E-mail address: kd-j@cmdik.pan.pl (K. Domańska-Janik).

0887-2333/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tiv.2005.06.036

both of these limits by developing the first human cord blood-derived cell line (HUCB-NSC) which exhibits classical neural stem cell properties and grows in culture for more than 45 passages over 3 years (Bużańska et al., 2003). As reported recently, stem/progenitor cells derived from human umbilical cord (HUCB) or human bone marrow (HBM), although hematopoietic in nature, under certain conditions can change their natural fate and display neural features (Woodbury et al., 2000; Sanchez-Ramos et al., 2001; Bużańska et al., 2002; Jiang et al., 2002; Zigova et al., 2002; Mezey et al., 2003). Identification of the molecular mechanism underlying phenotypic conversion of the cord blood mononuclears into neuronal, astrocytic and oligodendrocytic lineage is not possible at present and remains to be established. The most probable scenarios of this phenomenon are currently discussed but also questioned as a possibly unstable event, resulting from cell fusion or merely leading to functional insufficiency of the newly appearing cells (Terada et al., 2002; Ying et al., 2002). Thus, we do gather compelling evidence that this doesn't occur in our case. Besides expression of the typical markers commonly used to identify NSC and to characterize differentiated brain-building cells we have shown the expression of stem and neuron-specific genes, functional proteins, neuron-like electrophysiological properties and stable, diploid karyotype during long-term expansion of the cells in vitro (Bużańska et al., 2003; Sun et al., 2005). Moreover, their ability to form the neurospheres proposed as a hallmark of natural NSC niche, provides additional argument for genuine neural character of HUCB-NSC line.

In this report we present 2-D and 3-D culture models of HUCB-NSC for testing developmental neurotoxicity. Standardization of this uncontroversial neural stem cells developmental model system for studying the growth rate, apoptosis and differentiation is proposed. Then, a broad spectrum of putative toxic factors and relevant neuroprotective compounds can be screened in vitro according to the cell type and developmental stage of culture.

2. Materials and methods

2.1. 2-D HUCB-NSC culture in 96-well plates: a standardization of the method for the assessment of the growth rate

Adherent cultures were trypsinized and pooled with the floating cells, counted and seeded on 96-well plates (NUNC) at density $1–5 \times 10^5$ cells/cm^2 with the aim of determining an optimal growth conditions. Cultures were grown in F12/DMEM + 2%FBS + ITS medium up to 14 days. On the 1st, 5th, 10th and 14th day the MTT colorimetric assay (0.5 mg/ml final concentration) assay was performed to measure growth rate. The method is based on the reduction of the tetrazolium salt MTT (-3-(4,5-dimethythiazol-2-yl)-2,5-diphenol-tetrazoliumbromide) (Sigma). Resulting blue formazan salts were dissolved by replacing the culture medium with 100 μl of DMSO per well. Optical density (OD) was quantified by ELISA plate-reader (measurement filter 570 nm versus reference filter 630 nm). Monitoring of culture development by MTT method allowed to evaluate the growth rate (amount of formazan produced is proportional to the number of living cells) and to choose an optimal culture density for possible neurotoxicity tests. Data were gathered from four independent experiments and standard deviation has been calculated.

2.2. Attachment induced differentiation and immunostaining of 2-D HUCB-NSC culture

HUCB-NSC cells (both floating and adherent) were seeded on 96-well plates at 2×10^4 cells/cm^2 density and cultured in F12/DMEM + 2%FBS + ITS medium for 14 days. Then cultures were briefly washed twice with PBS and fixed for 20 min in 4% paraformaldehyde/PBS. Blocking solution, containing 10% normal goat serum in PBS, was applied for 1 h, 25 °C. For the GalC immunostaining, cells were incubated overnight with mouse monoclonal primary antibody (culture supernatant obtained from R-mAb hybridoma cells IgG3; gift of B.Zalc, INSERM U-495, Paris, 1:30) and for 1 h with goat anti-mouse IgG FITC (Sigma) secondary antibody (1:500). For cytoskeletal markers, cells were permeabilized for 0.5 h with solution containing 0.01% Triton X-100 and 5% normal goat serum in PBS, then incubated for 1 h, 25 °C with either monoclonal goat anti-mouse primary antibody rinsed against: β-tubulin III (Sigma, 1:1000), Microtubule associated Protein 2 (anti-MAP-2, mouse monoclonal 1:1000, Sigma), Neurofilament Heavy 200 kD (anti NF-200, mouse monoclonal, 1:400, Chemicon), S100β (rabbit polyclonal, 1:100, Swan) or glial fibrillary acidic protein (anti GFAP, rabbit polyclonal 1:400, Dakopatts, Glostrup, Denmark). Anti β-tubulin III was detected with goat anti-mouse monoclonal IgG_{2b} + FITC (Pharmingen 1:400), MAP-2 and NF-200 with anti-mouse monoclonal anti-IgG1 + TxR (Pharmingen, 1:300), anti-GFAP and S100β detected with goat anti-rabbit IgG + Cy3 (Pharmingen 1:500) or IgG FITC (Pharmingen 1:100). Cell nuclei were visualized by incubation with Hoechst 33258. The labeled cultures were examined under fluorescence microscope Axiovert 25. Images were captured by the Videotronic

CCD-4230 camera and processed by image analysis system KS300.

2.3. Culture of HUCB-NSC derived neurospheres (3-D cultures)

Neurospheres derived from HUCB-NSC line have been propagated over one year in serum-free medium: DMEM/F12 supplemented with B27 (1:50), EGF 10 ng/ml, AAS (1:100). In these conditions neurospheres grow as free floating spheres (Fig. 4A) in average diameter of 150 μm.

2.4. Transition of neurospheres to dividing progenitors

Floating neurospheres were shifted to 24-well plates coated with poly-L-lysine in low serum medium: DMEM/F12 supplemented with B27 (1:50), ITS (1:100) and 2% FBS and incubated overnight. After 16 h floating (non-attached) neurospheres were removed and media were changed to low serum medium supplemented with mitogenes: LIF/CNTF (both 10 ng/ml), lithium (1 mM), BDNF (10 ng/ml). Only neurospheres over 100 μm diameters were taken into consideration and further cultured for 7 days. After 3 days half of the medium was changed. At day 7 of culture the area of spreading cells from individual neurosphere was measured using Zeiss LSM 510 microscope and software. The area of the attached neurosphere core was then subtracted from the total area occupied by the progeny. After fixation immunocytochemistry for Ki67 protein was performed. Number of Ki67+ cells was evaluated by Zeiss LSM 510 confocal microscope. For the estimation of proliferation rate only Ki67+ cells outside the attached core were counted. This number was divided by neurosphere core area (mm^2) to exclude neurosphere size-dependent differences. All nuclei were stained with Hoechst. Measurements were made on at least 10 neurospheres.

2.5. Immunostaining of 3-D cultures

Neurospheres and their spread progeny were fixed in 4% PFA and permeabilized with 1% Triton X-100 for 20 min, and blocked in 5% normal goat serum solution. Then monoclonal IgG1 anti-Ki67 antibody was applied (1:100, Novocastra Labs overnight) and anti-IgG1 secondary antibody conjugated with TXRD fluorochrome (1:300; Chemicon).

3. Results

3.1. 2-D HUCB-NSC culture in 96-well plates: a standardization of the method for the assessment of the growth rate

The method based on the reduction of the tetrazolium salt MTT into the blue formazan allowed to evaluate the growth rate (amount of formazan produced is proportional to the number of living cells) of HUCB-NSC line in the paradigm suitable for monitoring possible neurotoxicant—induced changes. For 14 days of the experiment the number of living cells when plated in 96-well plates at densities: 10,000, 20,000, 30,000 and 40,000 (cells/cm^2) was monitored and OD was evaluated (Fig. 1A). OD value was calculated into the cell number and plotted as a function of time in culture (Fig. 1B). Cells plated at densities 10,000 and 20,000 were growing exponentially and did not reach confluency through 14 days of experiment, while plated at 30,000 and 40,000 were confluent at day 7 (reaching approx. 7–8 × 10^6 cells/cm^2) and further growth was inhibited (Fig. 1A and B). Then an optimal plating density for the 14 day long experiments was estimated at 0.8–2 × 10^5 cells/cm^2 (2600–6600 cells per well). Phase contrast images of these cells are shown in Fig. 2A–C. On the first day cells are mostly rounded and proliferating (Fig. 2A, arrow), on the fifth day approximately half of the cell population spreads and starts to differentiate (Fig. 2B, arrow), while the rest stay round and proliferating. In the end of experiment (day 14th), the culture forms confluent monolayer (Fig. 2C).

3.2. Attachment induced differentiation and immunostaining of 2-D culture

After 14 days of experiment in the cell population plated at 2 × 10^5 cells/cm^2 neuronal markers β-tubulin (Fig. 3A) and NF-200 (Fig. 3B) are expressed at 28% and

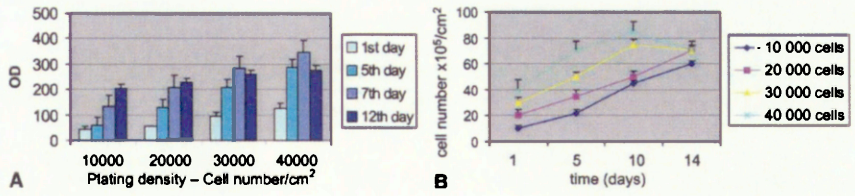

Fig. 1. Correlation of the growth rate with plating density of HUCB-NSC 2-D culture when grown in 96-well plates, $n = 4$. (A) Bar chart of Optical Density (OD) estimated in MTT test as a function of plating density for different time points of experiment. (B) Linear chart of estimated cell number as a function of time for different plating densities.

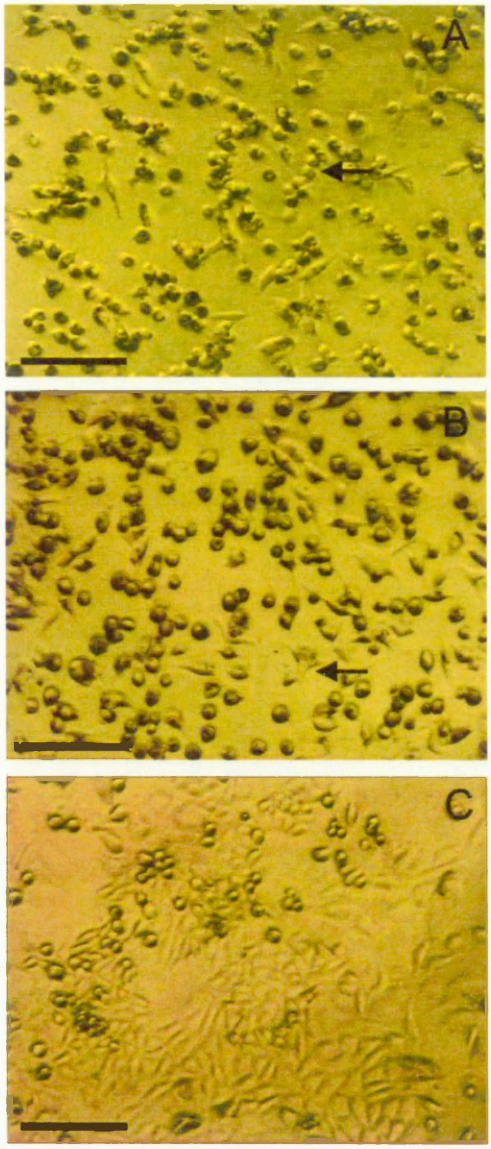

Fig. 2. Phase contrast images of HUCB-NSC plated at 20,000 cells/cm^2 and growing in 2-D culture for 14 days. Time after plating: (A) 1 day (arrow-rounded, dividing cells). (B) 5 days (arrow-attached differentiated cell). (C) 14 days. Scale bar 100 μm.

7.5% respectively, astrocytic markers GFAP (Fig. 3C) and S-100b (Fig. 3D) at 8.8% and 11.4%, while oligodendrocytes are expressed at 1.1% (Fig. 3E). More advanced neuronal marker MAP-2 is not expressed at this stage of differentiation. However, spontaneous, the attachment induced differentiation of HUCB-NSC in standard conditions seems to promote the neurogenic lineage.

3.3. Mitogenes stimulate proliferation and migration of cells within the neurospheres

HUCB-NSC neurospheres adhere to poly-L-lysine or fibronectin exclusively in the presence of serum (Fig. 4B and C respectively). Moreover, they proliferate, migrate and differentiate spontaneously in a few hours after adhesion. When neurospheres are cultured 7 days in the presence of lithium and LIF/CNTF they reveal significantly higher rate of proliferation (Ki67+) than those cultured in BDNF or kept in low serum medium without any additions (Fig. 5B). Interestingly, BDNF itself even lower the rate of proliferation from neurospheres (Fig. 5B). Cells from the attached neurosphere spread into larger area in the presence of lithium or LIF/CNTF as compared to cultures in standard media or supplemented with BDNF (Fig. 5A).

3.4. Fibronectin inhibits proliferation and migration of cells from attached neurosphere

Only 20% of the whole amount of neurospheres adhered firmly when plated on fibronectin-coated surface whether about 50% did so on poly-L-lysinated slides. Also the rate of proliferation and migration of the cells from the attached neurospheres was significantly lower on the fibronectin-coated surface (compare Fig. 4D and E with Fig. 5C and D). Moreover, there was not a significant influence of mitogenes when neurospheres were grown on fibronectin (Fig. 5C and D).

4. Discussion

In our recent study we have shown, that neural stem cells isolated from human umbilical cord blood (Bużańska et al., 2002), though not genetically manipulated, can give rise to clonal human umbilical cord blood-neural stem cell (HUCB-NSC) line, which has been grown in culture for more than 3 years (in continuous culture up to 55 passages and in additional subcloned lines) (Bużańska et al., 2003). These cells can be maintained in serum-free condition as a proliferating population of floating, non-differentiated cells forming aggregates (Fig. 6B) or neurospheres (Figs. 4 and 6A) as well as a mixed, floating and adherent cell culture in low (2%) serum condition (Fig. 6C).

The availability of the human NSC line in culture allows a detailed examination of the compounds that influence the dynamics of SC expansion. In general it would be determined by the balance between SC self-renewal, the fate choice decision, rate of committed precursors proliferation, their terminal differentiation and apoptotic elimination of the cells. The variety of extrinsic and intrinsic regulatory signals that govern these processes have been identified and characterized so far (Moon et al., 2002; Willert et al., 2003; Sato et al., 2004; Sherley, 2002). However our understanding of these processes is still not complete, especially in the context of SCs developmental regulation. Nevertheless, as the total number of NSC in their specific niches has been reported

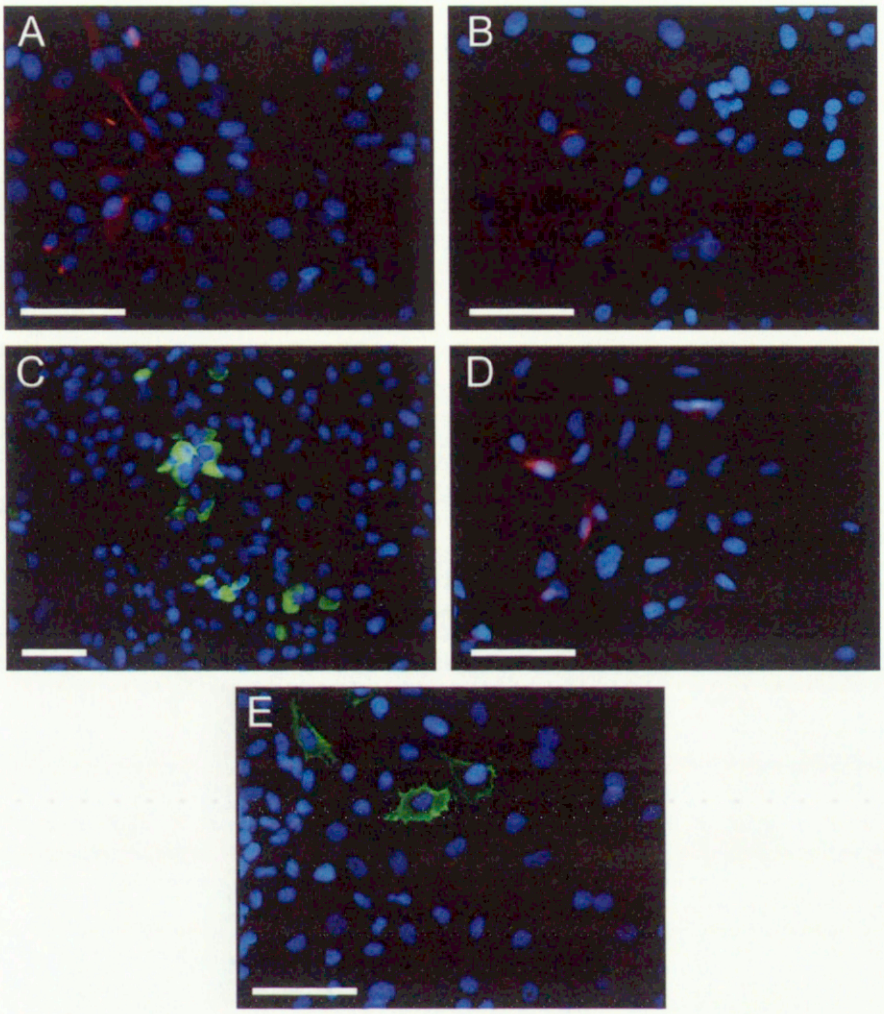

Fig. 3. Immunodetection of neural-specific markers in HUCB-NSC plated at 20,000 cell/cm^2 in 96-well plates and cultured for 14 days: (A) β-Tubulin III and (B) NF-200 for neuronal cells; (C) S100β and (D) GFAP for astrocytic cells; (E) GalC for oligodendroglial cells.

to decline in time (due to aging, stresses, diseases, toxicity etc.) it would suggest physiopathological importance of such regulation (Sommer and Rao, 2002).

The possible fate of somatic SC on the way from undifferentiated, quiescent progenitors to mature progeny can be summarized as follows (Fig. 6). Undifferentiated sSCs may remain "dormant" in their niche for years (Fig. 6A), then after receiving adequate stimulation enter the cell cycle and self-renew by symmetric mode of SC division increasing the undifferentiated SC pool (Fig. 6A). They can also divide asymmetrically and differentiate into specific lineage, then mature into the adult phenotypes according to physiological needs.

We can use our HUCB line for modeling NSC and early progenitor populations halted on different steps of their developmental continuum (Fig. 6A–D, left row inserts).

At first we can monitor quiescent NSC behavior taking advantage of their ability to form the neurospheres in vitro (Fig. 6A), which can be taken as an approximation of NSCs residing in the tissue-specific niche (Jurga and Domanska-Janik, 2004; Doetsch, 2003) and after stimulation giving rise to the cohorts of proliferating intermediate precursors (Fig. 6B and C). Contemporary, mechanistic knowledge about a transition from the quiescent to dividing states of neural SC is still fragmentary and incomplete. In our experiments we have estimated the influence of neural-specific mitogenes and growth factors as well as ECM component-fibronectin on proliferating capacity of neurospheres after their activation by adhesion to substratum in vitro.

The further development of NSC, involves undifferentiated, self-renewing progenitors, proliferating precursors on different levels of their commitment leading finally to matured cells which can be monitored separately by manipulation with cell culturing medium. In that respect HUCB-NSC line can be cultured as a population of non-differentiated, floating and proliferating cells, when

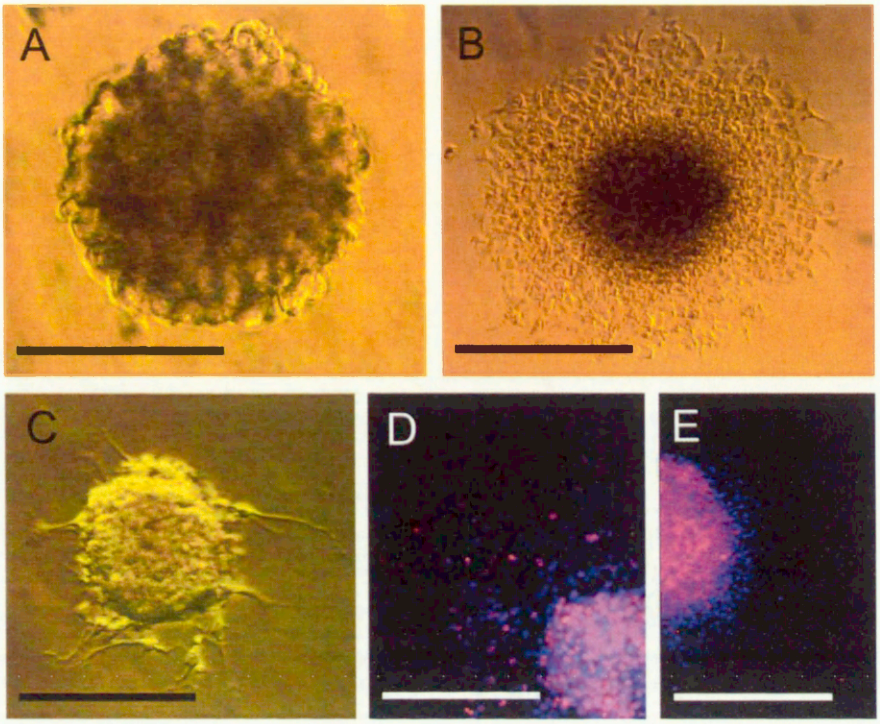

Fig. 4. Free floating single neurosphere derived from HUSB-NSC line (A; phase contrast). Neurosphere attached to poly-L-lysine coated wells in low serum medium (B; phase contrast), fibronectin-coated wells (C; phase contrast). Immunostaining for Ki67 (red)—marker of dividing cells plated on poly-L-lysine and fibronectin-coated wells (D and E respectively). Blue—cell nuclei stained with Hoechst. Scale bar 100 μm. (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article).

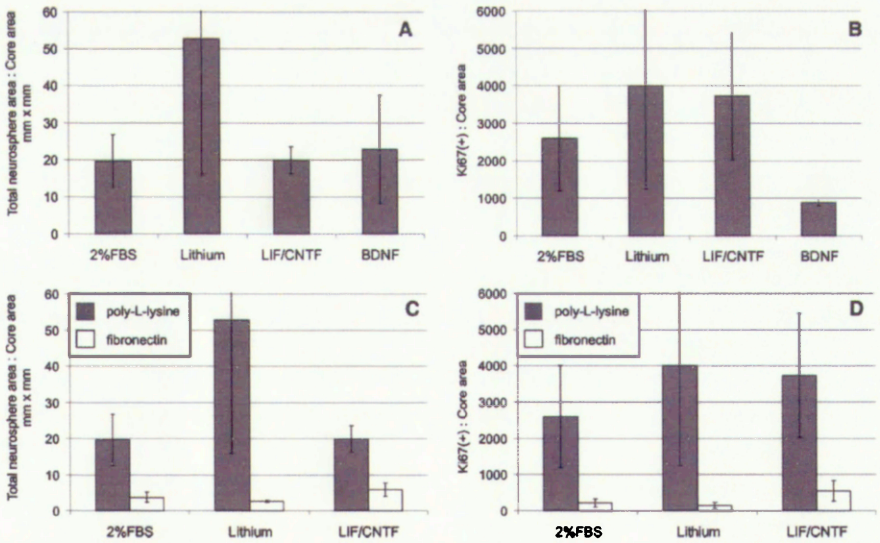

Fig. 5. Migration (A) and proliferation (B) of neural progenitors from neurospheres attached to poly-L-lysine in 2% FBS medium and cultured in the presence of mitogenes: lithium, LIF/CNTF, BDNF, $n = 10$. Proliferation (C) and migration (D) of neurospheres progenitors attached to poly-L-lysine vs fibronectin.

maintained in serum-free conditions in the presence of LIF and EGF (Fig. 6B). Low-serum, mitogene-free medium promotes heterogeneous population of proliferating, floating and adherent cells already committed to neural phenotypes (Fig. 6C). The low serum medium supplemented with neuromorphogenes like RA, BDNF or dBcAMP (Bużańska et al., 2003 or 2002) can give rise to differentiated neurons, oligodendrocytes and astrocytes (Fig. 6D).

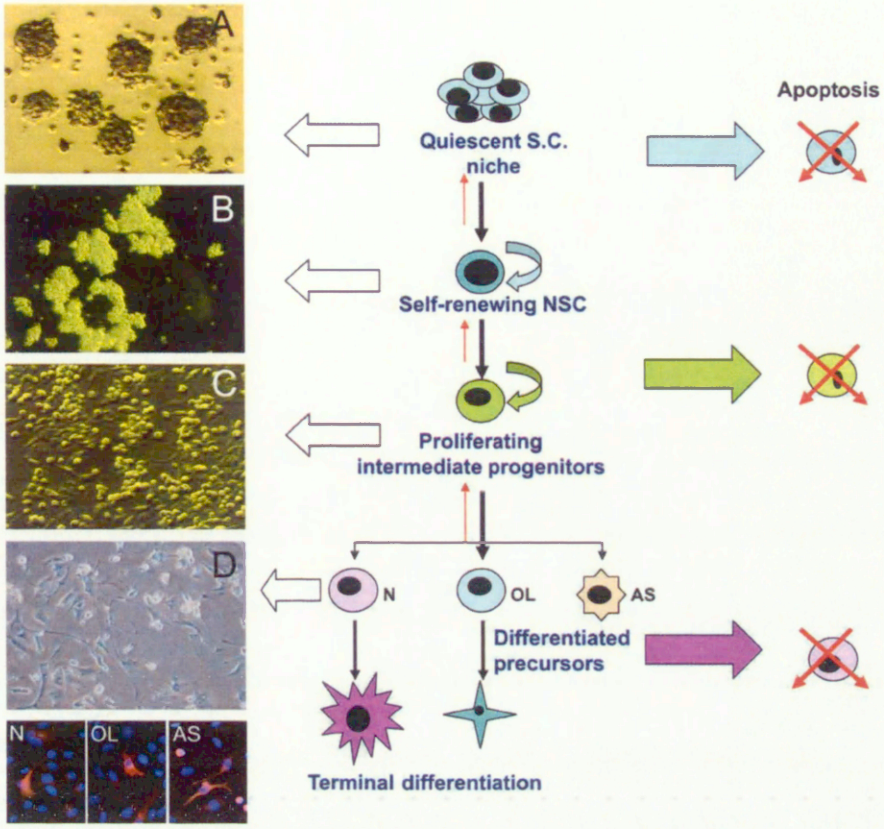

Fig. 6. Developmental steps of HUCB-NSC toward neural lineages. Phase contrast (A–D). (A) HUCB-NSC derived neurospheres—quiescent stem cell niche; (B) Self-renewing, floating HUCB-NSC in defined serum-free medium; (C) Intermediate, neural committed HUCB-NSC grown in low serum medium at floating and adherent fraction. (D) HUCB-NSC derived neuronal, astrocytic and oligodendroglial precursors differentiated in media supplemented with neuromorphogens. Immunocytochemistry: (N) β-tubulin III, (OL) GalC, (AS) GFAP for neuronal, oligodendrocytic and astrocytic cells respectively.

This experimental paradigm would allow dissecting and defining the cells of the highest susceptibility (i.e. neurons, astrocytes, oligodendrocytes as well as their precursors) to various signals from the changing environment.

Thus we can ask questions about factors and toxins which could influence the size of stem and intermediate progenitor pools along their developmental continuum. In general it would rely on the dynamics of exit from cell cycle (apoptosis, terminal differentiation) and rate of expansion (proliferation) on any developmental stage. However, due to exponential expansion of the individual SC, elimination of a single stem or early dividing progenitor will have a distinctive effect on the final number of differentiated progeny.

Proliferating capacity of neural cells in brain depends on interplay between various positive and negative signals and their transduction pathways, some of them listed on the diagram (Fig. 7) have been explored in our study. At the *extracellular level* they contain a plethora of incoming signals from growth factors (GF), neutrophins, extracellular matrix (ECM), hormones and neurotransmitters. We have shown for example, that in attached neurospheres BDNF inhibits proliferation of neural progenitors, while LIF/CNTF and lithium support cell divisions and migration (Figs. 4 and 5). *Apart from* mitogens, different kinds of extracellular matrix components like fibronectin or poly-L-lysine can influence cell proliferation and migration in our 3-D neurosphere model. Again, fibronectin as compared to poly-L-lysine seems to inhibit these processes (Fig. 4C).

The incoming signals induce specific responses from *cell interior*, activating specific pathways (like shown here the mitogene-activated kinases pathway—MAPK) and many other signaling cascades induced by key kinases and molecules with common *convergence* on the level of the cell cycle regulators: β-catenin, cyclines and their specific kinases, cycle targeting proteins p53 or Rb, etc. Accordingly, in our experiments inhibition by lithium of GSK-3β kinase, which is a negative regulator of β-catenin (key Wnt signalling pathway protein, Sato et al., 2004) was shown to promote proliferation of early progenitor cells derived from stimulated neurospheres (Fig. 5).

Another protein that attracted our attention on recently is an Id1—the proliferation promoting inhibitor

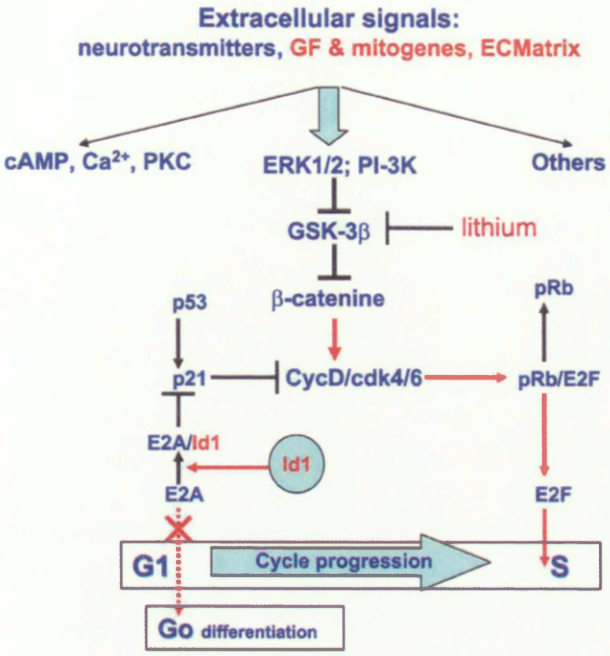

Fig. 7. Extracellular and intracellular proliferation signals.

of cell differentiation. This protein, being a truncated form of bHLH transcriptional factor, can negatively interact with other proteins of bHLH structure (among them the neurogenins and E2A—the cell cycle regulating protein family, Benezra et al., 1990; Zebedee and Hara, 2001). Recently we have confirmed the ability of Id1 to inhibit progenitor cells differentiation in neurospheres as well as neuronal differentiation at an early proliferating progenitor level (Jurga and Buzanska, 2003).

Previous analyses of mRNA and protein expression (DNA microarray and immunocytochemistry respectively) as well as electrophysiological studies confirmed stem-like and/or neural-like characteristics of HUCB-NSC (Bużańska et al., 2003; Sun et al., 2005). The proliferation signaling in these cells were indicated by activation of genes implicated in Wnt, Notch and Lif/Jak/Stat signaling pathways (Bużańska et al., 2003) important for the maintenance of self-renewal capacity of stem cells (Shimazaki et al., 2001; Sato et al., 2004; Espinosa et al., 2003).

Contrary to proliferation, apoptosis will efficiently reduce cell pool size at every level of development. But again, the molecular mechanism for cell death execution will be stage and cell-type specific. Currently, the most information about developmental apoptosis in CNS comes from the analysis of mutant mice deficient of particular death signaling compounds (Kuida et al., 1998; Gilmore et al., 2000). From these data it has been defined that apoptotic complex of caspases 9, 3 and Apaf1 together with the mitochondria-released Cytochrome C, are central play markers in developmental apoptosis of neuroepithelium. Inhibition of them leads to huge enlargement of neurogenic ventricular zone in brain. In contrast, Bcl2 family proteins seem not be involved in regulation of apoptosis in neurogenic VZ, opposite to their profound effect upon death/survival of differentiated neurons. It is also not known at present if quiescent SC or exclusively their proliferating progeny can be a target for apoptotic signals. In the floating, non-differentiated population of HUCB-NSC pro-apoptotic (i.e. casp3, casp9, apaf1, p53, atm, fadd) and anti-apoptotic (i.e. bcl2l1, bclx, bag3, act1) genes are expressed at the house-keeping, low level, as shown by DNA microarray (Bużańska et al., 2003). Whether they can be induced upon cell differentiation or during exposition to an unfriendly environment (toxins) remains to be determined.

Acknowledgements

Sponsored by Polish State Committee for Scientific Research (K 045/P05/2002 and K/P05/2003).

References

Benezra, R., Davis, R.L., Lockshon, D., Turner, D.L., Weintraub, H., 1990. The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. Cell 6, 49–59.

Bjorklund, A., Lindval, O., 2000. Cell replacement therapies for central nervous system disorders. Nature Neuroscience 3, 537–544.

Bużańska, L., Machaj, E.K., Zablocka, B., Pojda, Z., Domanska-Janik, K., 2002. Human cord blood-derived cells attain neuronal and glial features in vitro. Journal of Cell Science 15, 2131–2138.

Bużańska, L., Stachowiak, E., Stachowiak, M.K., Domańska-Janik, K., 2003. Neural stem cell line derived from human umbilical cord blood—morphological and functional properties. Journal of Neurochemistry 85 (Suppl. 2), 33.

Caldwell, M.A., He, X., Wilkie, N., 2001. Growth factors regulate the survival and fate of cells derived from human neurospheres. Nature Biotechnology 19, 475–479.

Corti, S., Locatelli, F., Strazzer, S., Guglieri, M., Comi, G.P., 2003. Neuronal generation from somatic stem cells: current knowledge and perspectives on the treatment of acquired and degenerative central nervous system disorders. Current Gene Therapy 3, 247–272.

Doetsch, F., 2003. A niche for adult neural stem cells. Current Opinion in Genetics and Development 13, 543–550.

Espinosa, L., Ingles-Esteve, J., Aguilera, C., Bigas, A., 2003. Phosphorylation by glycogen synthase kinase-3 beta down-regulates Notch activity, a link for Notch and Wnt pathways. Journal of Biological Chemistry 278, 32227–32235.

Gilmore, E.C., Nowakowski, R.S., Caviness Jr., V.S., Herrup, K., 2000. Cell birth, cell death, cell diversity and DNA breaks: how do they all fit together? Trends in Neurosciences 23, 100–105.

Jiang, Y., Jahagirdar, B.N., Reinhardt, R.L., Schwartz, R.E., Keene, C.D., Ortiz-Gonzalez, X.R., Reyes, M., Lenvik, T., Lund, T., Blackstad, M., Du, J., Aldrich, S., Lisberg, A., Low, W.C., Largaespada, D.A., Verfaillie, C.M., 2002. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 418, 41–49.

Jurga, M., Buzanska, L., 2003. The influence of Id1 protein on human stem cells differentiation into neuronal pathway. Journal of Neurochemistry 85 (Suppl. 2), 33.

Jurga, M., Domanska-Janik, K., 2004. Neurospheres of human umbilical cord blood derived neural stem cells—in vitro model of stem cell niche. FENS forum Abstracts 2. A040.16.

Kempermann, G., van Praag, H., Gage, F.H., 2000. Activity-dependent regulation of neuronal plasticity and self repair. Progress Brain Research 127, 35–48.

Kim, J.H., Auerbach, J.M., Rodriguez-Gomez, J.A., Velasco, I., Gavin, D., Lumelsky, N., Lee, S.H., Nguyen, J., Sanchez-Pernaute, R., Bankiewicz, K., McKay, R., 2002. Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease. Nature 418, 50–56.

Kuida, K., Haydar, T.F., Kuan, C.Y., Gu, Y., Taya, C., Karasuyama, H., Su, M.S., Rakic, P., Flavell, R.A., 1998. Reduced apoptosis and cytochrome *c*-mediated caspase activation in mice lacking caspase 9. Cell 94, 325–337.

Mezey, E., Key, S., Vogelsang, G., Szalayova, I., Lange, G.D., Crain, B., 2003. Transplanted bone marrow generates new neurons in human brains. Proceedings of the National Academy of Sciences of the USA 4, 1364–1369.

Moon, R.T., Bowerman, B., Boutros, M., Perrimon, N., 2002. The promise and perils of Wnt signaling through beta-catenin. Science 296, 1644–16466.

Palm, K., Salin-Nordstrom, T., Levesque, M.F., Neuman, T., 2000. Fetal and adult human CNS stem cells have similar molecular characteristics and developmental potential. Molecular Brain Research 31, 192–195.

Pluchino, S., Quattrini, A., Brambilla, E., Gritti, A., Salani, G., Dina, G., Galli, R., Del Carro, U., Amadio, S., Bergami, A., Furlan, R., Comi, G., Vescovi, A.L., Martino, G., 2003. Injection of adult neurospheres induces recovery in a chronic model of multiple sclerosis. Nature 422, 688–694.

Poulsom, R., Alison, M.R., Forbes, S.J., Wright, N.A., 2002. Adult stem cell plasticity. Journal of Pathology 197, 441–456.

Sanchez-Ramos, J.R., Song, S., Kamath, S.G., Zigova, T., Willing, A., Cardozo-Pelaez, F., Stedeford, T., Chopp, M., Sanberg, P.R., 2001. Expression of neural markers in human umbilical cord blood. Experimental Neurology 171, 109–115.

Sato, N., Meijer, L., Skaltsounis, L., Greengard, P., Brivanlou, A.H., 2004. Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. Nature Medicine 10, 55–63.

Schaffer, D.V., Gage, F.H., 2004. Neurogenesis and neuroadaptation. Neuromolecular Medicine 5, 1–9.

Sherley, J.L., 2002. Asymmetric cell kinetics genes: the key to expansion of adult stem cells in culture. Stem Cells 20, 561–572.

Shimazaki, T., Shingo, T., Weiss, S., 2001. The ciliary neurotrophic factor/leukemia inhibitory factor/gp130 receptor complex operates in the maintenance of mammalian forebrain neural stem cells. Journal of Neuroscience 21, 7642–7653.

Sommer, L., Rao, M., 2002. Neural stem cells and regulation of cell number. Progress in Neurobiology 66, 1–18.

Storch, A., Schwarz, J., 2002. Neural stem cells and neurodegeneration. Current Opinion in Investigational Drugs 3, 774–781.

Sun, W., Buzanska, L., Salvi, R., Domanska-Janik, K., Stachowiak, M.K., 2005. Voltage-sensitive and ligand-gated channels in differentiating fetal neural stem cells isolated from the non-hematopoietic fraction of human umbilical cord blood. Stem Cells 23, 93–102.

Svendsen, C.N., ter Borg, M.G., Armstrong, R.J., Rosser, A.E., Chandran, S., Ostenfeld, T., Caldwell, M.A., 1998. A new method for the rapid and long term growth of human neural precursor cells. Journal of Neuroscience Methods 85, 41–153.

Terada, N., Hamazaki, T., Oka, M., Hoki, M., Mastalerz, D.M., Nakano, Y., Meyer, E.M., Morel, L., Petersen, B.E., Scott, E.W., 2002. Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. Nature 416, 542–545.

Vescovi, A.L., Parati, E.A., Gritti, A., Poulin, P., Ferrario, M., Wanke, E., Frolichsthal-Schoeller, P., Cova, L., Arcellana-Panlilio, M., Colombo, A., Galli, R., 1999. Isolation and cloning of multipotential stem cells from the embryonic human CNS and establishment of transplantable human neural stem cell lines by epigenetic stimulation. Experimental Neurology 156, 71–83.

Willert, K., Brown, J.D., Danenberg, E., Duncan, A.W., Weissman, I.L., Reya, T., Yates III, J.R., Nusse, R., 2003. Wnt proteins are lipid-modified and can act as stem cell growth factors. Nature 423, 448–452.

Woodbury, D., Schwarz, E.J., Prockop, D.J., Black, I.B., 2000. Adult rat and human bone marrow stromal cells differentiate into neurons. Journal of Neuroscience Research 61, 364–370.

Ying, Q.L., Nichols, J., Evans, E.P., Smith, A.G., 2002. Changing potency by spontaneous fusion. Nature 416, 545–548.

Zebedee, Z., Hara, E., 2001. Id proteins in cell cycle control and cellular senescence. Oncogene 20, 8317–8325.

Zigova, T., Song, S., Willing, A.E., Hudson, J.E., Newman, M.B., Saporta, S., Sanchez-Ramos, J., Sanberg, P.R., 2002. Human umbilical cord blood cells express neural antigens after transplantation into the developing rat brain. Cell Transplantation 11, 265–274.

PUBLIKACJA 4

STEM CELLS AND DEVELOPMENT 15:391–406 (2006)

In Focus: Neural Stem Cells

Neural Stem-like Cell Line Derived from a Nonhematopoietic Population of Human Umbilical Cord Blood

LEONORA BUŻAŃSKA,[1,2] MARCIN JURGA,[1] EWA K. STACHOWIAK,[2] MICHAL K. STACHOWIAK,[2] and KRYSTYNA DOMAŃSKA-JANIK[1]

ABSTRACT

The ability of stem and progenitor cells to proliferate and differentiate into other lineages is widely viewed as a characteristic of stem cells. Previously, we have reported that cells from a CD34$^-$ (nonhematopoietic) adherent subpopulation of human cord blood can acquire a feature of multipotential neural progenitors in vitro. In the present study, using these cord blood-derived stem cells, we have established a clonal celi line termed HUCB-NSCs (human umbilical cord blood-neural stem cells) that expresses several neural antigens and has been grown in culture for more than 60 passages. During this time, HUCB-NSCs retained their growth rate, the ability to differentiate into neuronal-, astrocyte-, and oligodendrocyte-like cells and displayed a stable karyotype. DNA microarray analysis of HUCB-NSCs revealed enhanced expression of selected genes encoding putative stem and progenitor celi markers when compared to other mononuclear cells. dBcAMP-induced HUCB-NSCs were further differentiated into more advanced neuronal cells. This is the first report of the establishment and characterization of a nontransformed HUCB-NSC line that can be grown continuously in a monolayer culture and induced to terminal differentiation. These cells should further our understanding of the regulatory mechanisms involved in NSC self-renewal and differentiation.

INTRODUCTION

GROWTH AND DIFFERENTIATION of somatic stem cells are more restricted than that of the embryonic stem (ES) cells. Somatic stem cells are generally thought to only give rise to cell types characteristic of their origin tissue. However, recent studies have suggested that stem and progenitor cells isolated from specific tissue may form specialized cell types normally present in other tissue types when cultured under certain conditions or transplanted into those heterologous tissues (1–8). Typically, somatic stem cells are present in limited amounts and, thus, are difficult to isolate and expand in vitro. Their widespread use has been largely prevented by our inability to grow these cells in vitro in sufficient quantities. Neural stem cells (NSCs) isolated from the central nervous system (CNS) and expanded in vitro with mitogenic growth factors have only shown a limited lifespan. After a finite number of cell divisions, these cells spontaneously differentiate and/or enter an irreversible growth arrest (9). This is true for both neuraly differentiated ES cells and fetal or adult brain-derived progenitor strains. These cells can be propagated as replicating cell aggregates called neurospheres (10,11), but they undergo pro-

[1]NeuroRepair Department, Medical Research Center, 02-106 Warsaw, Poland.
[2]Department of Pathology and Anatomical Sciences, SUNY at Buffalo, Buffalo, NY 14214.
Dr. Bużańska was a visiting assistant professor at SUNY when this work was performed.

gressive functional inactivation with time (12,13). To overcome this problem, one approach has been to use immortalized NSCs transformed with proto-oncogenes and expand them in long-term monolayer cultures (14). Recently, Conti et al. (15) reported a successful long-term expansion of the NSCs in monolayer cultures without prior transformation and immortalization.

In our previous studies (1), and in a more recent report by McGuckin et al. (16), we showed that expanding populations of neural stem/progenitor-like cells had been selected from the human cord blood nonhematopoietic ($CD34^-$ and $CD45^-$) mononuclear fraction. Those nestin- and glial fibrillary acidic protein (GFAP)-expressing progenitors were differentiated predominantly (<80%) into neuron-, astrocyte-, or oligodendrocyte-like cells identified by immunocytochemical staining (1). Additionally, pluripotent ES-like or mesenchymal-type cells with multilineage differentiation potential have been identified in human cord blood (17,18). These human umbilical cord blood (HUCB)-derived cells could be further expanded in the presence of mitogens and induced to differentiate into diverse germ lines, which includes multipotent neural progenitors.

In the present study, we characterize the HUCB-NSC cell line and provide evidence regarding its clonal and nonimmortalized properties. By establishing optimal culturing conditions, we successfully expanded the HUCB-HSCs for more than 60 passages, while maintaining their exponential growth, differentiation potential and stable karyotype. We show that the HUCB-NSC line in its nondifferentiated stage express stem- and neural-specific genes and proteins, whereas dBc-AMP stimulated differentiation-induced expression of specific mature neuron-like markers. In a parallel electrophysiological study, the differentiated HUCB-NSC line was also shown to acquire neuron-like features with functional voltage and ligand gated channels, similar to those identified in other immature neuronal systems (19).

MATERIALS AND METHODS

Expansion of HUCB-NSCs in different culture conditions

The mononuclear $CD34^-$ fraction of HUCB was isolated as previously described (1). Briefly, the mononuclear fraction of cord blood was obtained on a Ficoll/Hypaque gradient and antigen depleted for $CD34^+$ cells using immunomagnetic beads (MilteneyiBiotek anti-CD34 Isolation Kit). Cells were further cultured as an adhesive monolayer in Iscoves' modified Dulbecco medium (IMDM; Gibco, Paisley, UK) supplemented with 10% fetal calf serum (FBS; Gibco) (1). After growth in such conditions for 6 weeks, cells were stimulated with epidermal growth factor (EGF, 10 ng/ml; Sigma, Manchester, UK) in Dulbecco's modified Eagle medium (DMEM; Gibco). The NSC line was established by sequential passaging only floating cells from EGF-stimulated culture. Such cultures were further expanded in: (1) serum-free DMEM/F12 (Gibco), supplemented with B27 (1:50; Gibco) EGF(10 ng/ml; Sigma), basic fibroblast growth factor (bFGF; 10 ng/ml; Sigma), leukemia inhibitory factor (LIF; 10 ng/ml; Sigma), and antibiotic antimycotic solution (AAS; 1:100, Gibco); (2) low serum DMEM/F12 containing, 2% FBS, insulin-transferrin-selenium (ITS, 1:100, Gibco), AAS (1:100); and (3) high serum/mitogen DMEM/F12 containing 10% FBS, EGF, bFGF (10 ng/ml), and AAS (1:100). The nonadhesive, floating culture in the serum-free medium was propagated by splitting in half and refilling the medium every 7 days. The mixed cultures (with adherent and floating cells) in the low-serum or high-serum/mitogen media were propagated by collecting only floating cells and transferring them into separate flasks or by trypsynization of the whole fraction (1:4 ratio). Attached cells were trypsinized and transferred in a 1:4 ratio every 30 days. All cultures were maintained at 37°C, 5% CO_2, and 95% humidity.

The ability of HUCB-NSCs to form single-cell derived clones was estimated by plating the cells at a clonal density of 10 cells/cm^2 into 25-cm^2 culture flasks and observing selected areas under an inverted microscope (Zeiss Axiovert 25; Carl Zeiss, Oberkochen, Germany) starting with one cell in the field. Alternatively, single cells were plated in individual wells of a 96-well plate in DMEM/F12, 2% FBS, ITS (1:100), EGF, and bFGF (10 ng/ml). Cell division was followed in selected wells. Phase-contrast images of the cells in growing cultures or developing clones were taken and saved with the Videotronic Infosystems CCD-4230 camera coupled to an Axiovert 25 and operated by a computer-assisted image analysis system (KS300, Carl Zeiss).

Transient transfection with EGFP

Transient transfection was performed using a Gene-Pulser II (Bio-Rad; Hercules, CA) electroporating system. Serum-free grown HUCB-NSCs were collected by centrifugation. Approximately 10^6 cells were suspended in 400 μl of DMEM/F12 serum-free medium and placed in an electroporation cuvette (BioRad). DNA (15 μg) (pEGFP-N1, Clontech; Mountain View, CA) was added and the cells were electroporated with pulses of 250 V at 950 μF. After electroporation, the cells were chilled on ice for 15 min before transferring into serum-free medium.

Karyotypic analysis of HUCB-NSCs

Karyotypic analysis of early (9th) and late passages (42nd) of HUCB-NSCs was performed at the Cytogenetics Laboratory, Institute of Oncology in Warsaw, Poland, as described by Pienkowska-Grela et al. (20). Briefly, cells were incubated overnight with colcemid (0.5 μg/ml). Metaphase spreads were prepared following standard procedures for fixation with methanol-acetic acid. Chromosomes were G-banded by Wright or Giemsa staining. Karyotypes were analyzed and classified according to the International System for Human Cytogenetic Nomenclature. At least 20 metaphase spreads for the early (>10) and late (<25) passages were analyzed.

Culture growth

To estimate the growth of cultures, we plated 5×10^5 cells in 25-cm^2 flasks at conditions described above and followed them for an additional 22 days. Every 4 days, the flask contents were collected by centrifugation (floating culture) or by trypsinization and centrifugation (attached and floating cultures). The cells were incubated with Trypan Blue (1:1; Sigma) and counted using a Bürker chamber (Blau Brand). Trypan Blue-stained cells were excluded from the counting. Mean values with standard deviations were calculated from four independent experiments each using early (>10) and late (<25) passages and different culture conditions (serum-free, low serum, or high serum/mitogen). Statistical evaluation of data was performed with the ANOVA test (graph Pad Prism 4.1, GraphPad Software, San Diego, CA).

Expression of neural markers in nondifferentiated cells

Expression of neural markers was analyzed in cells growing in serum-free, low-serum, and high-serum conditions. Cells grown in serum-free medium and floating cells from low-serum medium were either cytospun at 400 rpm for 3 min on glass slides as a dispersed cell population, or were plated on poly-L-lysine-coated coverslips as whole spheres/aggregates. The cytospun cells were dried for 30 min at room temperature and fixed with 4% paraformaldehyde (PFA) (Sigma) for 20 min and then processed for immunocytochemistry. Whole spheres/aggregates were allowed to attach for 16 h or 4 days, then fixed with 4% PFA, and immunostained. The whole population of low-serum growing cultures was examined for expression of neural markers upon attachment (5×10^4 cells/cm^2) to poly-L-lysine-coated coverslips for 24 h in their growing medium.

Experimental design for differentiation of HUCB-NSCs

For retinoic acid (RA)-induced differentiation, HUCB-NSCs were plated for 4 days at 5×10^4 cells/cm^2 in a 24-well tissue culture plate on poly-L-lysine (Sigma)-coated glass coverslips in Neurobasal Medium (Gibco) supplemented with 10% FBS, AAS (1:100, Sigma), and 0.5 μM RA (Sigma). HUCB-NSC differentiation in co-cultures with rat brain cells was performed as previously described (1). Briefly, HUCB-NSCs were prelabeled with 25 μM tracker (5 chloromethyl-fluorescein-diacetate; Molecular Probes; Paisley, UK) and plated for 4 days on rat cortical primary cultures (5×10^4 cells/cm^2). For dBCAMP-induced differentiation, cells were plated for 7 or 14 days on poly-L-lysine-coated glass coverslips or 25-cm^2 culture bottles at 5×10^4 cells/cm^2 in low-serum medium supplemented with dBcAMP/CPT 300 μM; Sigma) (Table 1).

Immunocytochemistry

Cells were fixed for 30 min in 4% PFA and immunostained for neural markers. Nonspecific binding of

TABLE 1. DIFFERENTIATION OF HUCB-NSCs

Cells	*Culture medium*	*Conditions of differentiation*	*Purpose*
HUCB-NSCs, passages 9 and 25	High serum/ mitogens	Neurobasal medium 10% FBS + 0.5 μM RA	To compare differentiation potential of early and late HUCB-NSC passages
HUCB-NSCs, prelabeled with cell tracker, passages 9 and 25	High serum/ mitogens	Co-cultures with rat brain cortical cells in DMEM, 10% FBS	To compare differentiation potential of early and late HUCB-NSC passages
HUCB-NSCs, passage 25, floating cells	Low serum	DMEM/F12, 2% FBS and ITS (1:100) + 300 μM dBcAMP/CPT	To show neuronal maturation of HUCB-NSCs at the mRNA and protein level

antibodies was blocked for 1 h with 5% normal goat serum (NGS; Gibco). The following primary antibodies were used: anti-human nestin (1:1,000, gift of Dr. U. Lendahl, Karolinska Institute, Stockholm), anti β-tubulin III (1:300; Sigma), anti-MAP2 (1:1,000; Sigma), anti-Tau protein (MAPT, microtubule-associated protein TAU, 1:100; Chemicon, Temecula, CA), anti-neurofilament heavy 200 kD (NF-200, 1:100; Chemicon), anti-α-internexin (1:100; Chemicon), anti-galactosylceramide (GalC, 1:50), culture supernatant obtained from R-mAb hybridoma cells (IgG_3; gift of B. Zalc, INSERM U-495, Paris), anti-cow glial fibrillary acidic protein (GFAP, 1:100; Dakopatts, Glostrup, Denmark), anti-synaptic vesicle 2 (SV2, 1:10; Hybridoma; Iova), anti-γ-aminobutyric acid (GABA) receptor-associated protein 1 (1:100; Chemicon), anti-glutamate decarboxylase 67 (GAD67, 1:100; Chemicon), anti-tyrosine hydroxylase (1:5,000; Chemicon), anti-dopamine D2A receptor (1:100; Chemicon), anti-calretinin (1:2,500, gift of Dr. J. Kuznicki, International Institute of Molecular Biology, Warsaw), anti-latexin IgY (1:200; GenWsay Biotech, San Diego, CA), and anti-FGFR1 (1:100, affinity purified) (21). Primary antibodies were applied overnight at 4°C. The secondary antibodies conjugated to Texas Red (TxR) or fluorescein is othicocyanate (FITC), anti-mouse IgG FITC (Sigma), anti-mouse IgG_{2a}-TxR, anti-mouse IgG_3-TxR, or anti-rabbit IgG-TxR (Pharmingen; San Diego, CA), and rabbit anti-chicken IgY Alexa Fluo 555 (GenWsay Biotech; San Diego, CA), were diluted 1:100 in the same solution as the primary antibody and applied for 1 h at room temperature (RT). To detect the nuclei, cultures were incubated with 5 μM Hoechst 33258 (Sigma) for 20 min at RT. Slides were mounted in Fluoromount-G (SouthernBiotech; Galveston, TX) and analyzed with fluorescent microscopes (Nikon Diaphot, Axiovert 25, or Axioskop 2, Zeiss) or confocal microscopes (BioRad MRC 1024, Biorad Laboratories; Hercules, CA or Carl Zeiss. The percentage of cord blood cells positive for neuron-, astrocyte, or oligodendrocyte-specific antigens was estimated relative to the total number of HUCB-NSCs in the field examined. Data represent the means of at least three independent cord blood cell samples, each counted in triplicate (at least 600 cells per count). The ANOVA test (Graph Pad Prism 4.1, GraphPad Software, San Diego, CA) was used to evaluate the significance of differences between controls and experimental samples.

DNA microarray analysis

DNA microarray analysis was performed on three samples: (1) HUCB-MC, control mononuclear cell fraction of freshly isolated, CD34 immuno-depleted HUCB cells (Stemgenix, Buffalo, NY) and grown for 2 days in IMDM, 10% FBS (Gibco); (2) HUCB-NSC, nondifferentiated, floating cells harvested from the culture growing in low-serum medium; (3) HUCB-NSCD, cells differentiated for 4 weeks on poly-L-lysine-coated coverslips in the presence of low-serum medium supplemented with 300 μM CPT-dBcAMP analog (Sigma). Two independent experimental runs were performed from HUCB-NSC passages 24 and 25. Total RNA was isolated using Trizol Reagent (Invitrogen) from approximately 4 million cells in each experimental group. Synthesized cDNA samples were hybridized to Affymetrix "HG-U133" Set A and B (www.affymetrix.com) Gene Chips with 45,000 probe sets representing more than 39,000 transcripts derived from approximately 33,000 human genes (Gene Core Laboratory, Roswell Park Cancer Institute, Buffalo, NY). Streptavidin-phycoerytrin stained gene chips were scanned with a Genechip System confocal scanner (Agilent, Affymetrix Inc.) at 3 μm resolution. Quality of the original data was checked by the dChip program (Harvard University). Each individual probe set had to match the theoretical hybridization pattern for the gene and the number of outliers (genes that do not match the pattern) was established. Most of the DNA chips examined had approximately 0.1% outliers, and only one (HUBC-MC) had approximately 3% but still less than the commonly accepted border value of 5%, and thus qualified them for further analysis.

The raw data were transferred to Microsoft Excel files for later analysis. The relative abundance of each gene transcript in the absolute analysis was assessed according to its p value calculated with the statistical algorithms in the Affymetrix Microarray Suite. Present (P), marginal (M), and absent (A) calls for gene expression were defined as $p \leq 0.05$, $0.05 < p < 0.06$, and $p \geq 0.06$, respectively. In differentiation experiments, genes assigned as "induced" were scored as "expressed" in differentiated HUCB-NSCs while being "nonexpressed" by undifferentiated HUCB-NSCs. In the comparative analyses, the average chip intensity was measured (dChip program), and the results were normalized to the chip with the median set as the overall brightness (median cell intensity). The Data Mining Tool Program (Affymetrix) was used to statistically evaluate the differences in gene expression between samples. The genes being compared from different samples were assigned to the "Fold Change Ratio" with the definition of fold change: FC = 2^ (Signal Log Ratio), where Signal Log Ratio is the $\log_2$ ratio of difference in expression level between the two arrays compared. Four cross-comparisons between the chip data from two independent experimental runs for each sample were performed with the Data Mining Tool. Two-fold or greater differences in gene expression with $p \leq 0.05$ were considered "significant." Raw gene chip data of the comparative analysis between samples are available at http://www.cmdik.pan.pl/zespoly/pnm/index.html.

RESULTS

Stable karyotopye of long-term cultured HUCB-NSCs

To determine whether long-term-cultured HUCB-NSCs undergo chromosomal changes, we analyzed the karyotypes of cells that were continuously cultured for over 3 years. After 40–42 passages, the HUCB-NSCs maintained a normal chromosomal pattern of 46, XY (Fig. 1). The presence of chromosome Y demonstrated that the isolated HUCB-NSC line was derived from the foetal cells (male) present in the cord blood.

Neural commitment of HUCB-NSCs revealed by phenotype, growth rate, and molecular properties in different culture conditions

The neural progenitor fraction was obtained from human cord blood (1). In the present study, the antigen-purified ($CD34^-$, $CD45^-$) cell population obtained from the human cord blood (1) was expanded in continuous cultures for more than 60 passages. Our first goal had been to establish long-term culturing conditions under which the abilities of undifferentiated cells to proliferate, self-renew, and differentiate into all neural subtypes could be preserved. Toward this aim, we compared several characteristics of the HUCB-NSCs maintained in three types of culture conditions.

In the first condition (serum-free medium supplemented with LIF, EGF and bFGF; Table 1), the growing HUCB-NSCs did not attach to the dish. These floating cells remained nondifferentiated and formed spheres/aggregates that were round and regular in shape (Fig. 2A, arrow) or were irregular and loosely packed (Fig. 2A, arrowhead, Fig. 2B). Some cells on the surface of the loose aggregates began to differentiate morphologically by forming short processes. Those changes were readily observed in pEGFP-N1-transfected cultures in which the differentiating surface cells as well as the round nondifferentiated cells inside the spheres expressed enhanced green fluorescent protein (EGFP) (Fig. 2B, arrow).

In the second culture condition (low-serum cultures; Table 1), cells that attached to the dish became flattened and grew short processes. The single floating cells (Fig. 2D, arrow), or floating spheres/aggregates (Fig. 2D, ar-

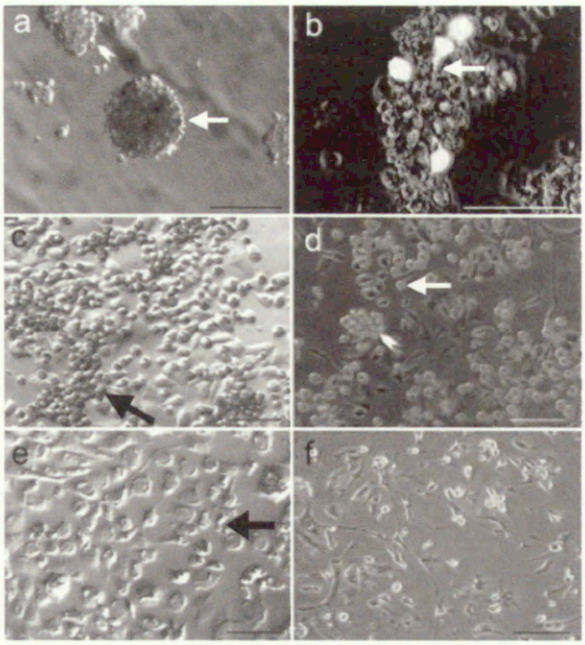

FIG. 2. Phase-contrast images of HUCB-NSCs in different culture conditions. (**a** and **b**) Serum-free medium. Free-floating cells clustered spontaneously into loosely packed small aggregates (**a**, arrowhead) or more regular entities resembling typical neurospheres (**a**, arrow). Floating cell aggregates containing a few cells transiently transfected with EGFP (**b**, arrow) are shown by phase-contrast and fluorescence microscopy (**b**). Note, that some EGFP-labeled cells at the surface of the aggregate started to extend protrusions (**b**, arrow). (**c** and **d**) Low-serum medium-grown cells containing a mixture of floating and adherent cell populations. Proliferating cells were either loosely attached to the monolayer (**c**, arrow) or were free floating in the medium as single cells (**d**, arrow) or aggregates (**d**, arrowhead). (**e**) The cells in high-serum medium expanded mainly as an adherent layer with heterogeneous morphology. (**f**) Adherent cells from (**d**) dBcAMP differentiated (7 days) acquired more defined neuronal morphology with long membrane projections and neurite-like extension. Scale bar, 100 μm.

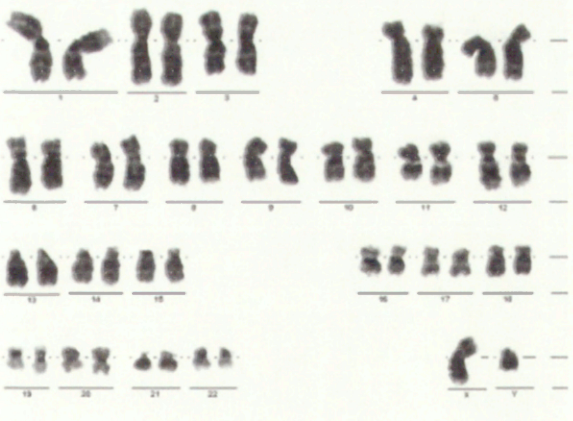

FIG. 1. Karyotype of HUCB-NSC at 42nd passage. Long-term culture and expansion of HUCB-NSCs did not change their chromosomal make-up. Cells from early (>10) and late (<25) passages show a typical chromosomal pattern (46, XY) without apparent abnormalities.

rowhead) showed no morphological differentiation. Also, some nondifferentiated cells were found attached to the adherent cell layer (Fig. 2C, arrow). Free floating cells harvested from either serum-free or low-serum conditions are morphologicaly identical and reveal similar properties regarding protein expression and electrophysiological responses (19,22).

Comparative DNA analysis of the free-floating, nondifferentiated cells grown in low-serum conditions (referred to as HUCB-NSC in Table 2) and of the reference population of freshly isolated mononuclear fraction (HUCB-MC) revealed enhanced expression of genes related to self-renewal, including *Fzd8*, *Fzd7*, *Wnt5a*, *Ctnmb1*, *Lif*, *LifR*, *Erbb2*, *Jak3*, *Stat6*, *Bmp4*, and other genes involved in Wnt, Lif, and transforming growth factor-β (TGF-β) signaling pathways (Table 2). Stem-like genes that distinguish NSCs from ES cells are those involved in Notch and FGF signaling pathways (23). In these two categories, genes that were significantly upregulated in HUCB-NSCs included *Notch2*, *Notch3*, *Notch4*, *Jag2*, *Dll3*, *Hey1*, *Pnn1* and 2, *Adam15*, *17*, and *23*, *Fgf2*, *Pdgf*, *Nrg1*, and their receptors *FgfR1* (the most abundant, up to 600-fold increase), *FgfR3*, *PdgfR*, and *Erbb2*. *Sox2* mRNA, which marks neural commitment (23), was also highly expressed in HUCB-NSCs, but was absent in HUCB-MC.

In our third culture condition (high-serum cultures; Table 1), the majority of cells were adherent (Fig. 2E). In addition, rare small aggregates or single floating cells were observed (Fig. 2E, arrow). In this condition, the attached cells were more spread and had longer processes than in the low serum (compare Fig. 2B,C and Fig. 2E).

The growth rate of cultures in high serum differed from those in serum-free and low-serum conditions (Fig. 3). Initially, the high-serum cultures expanded more rapidly than the low- or no-serum cultures. At day 4, the difference between low- and high-serum cultures was statistically significant ($p \leq 0.05$). From day 12, the growth of the high-serum cultures became arrested whereas low- and serum-free cultures continued to expand. Consequently, on day 20, the number of cells in high-serum cultures became significantly smaller than in serum-free or low-serum cultures (Fig. 3; $p \leq 0.05$). In effect, during the subsequent 20 days, the number of cells maintained in serum-free and low-serum conditions increased over 10-fold. Their estimated cell doubling time was approximately 4 days and the rates at which the number of cells increased in serum-free and low-serum conditions were not significantly different (Fig. 3; $p \leq 0.05$). Additionally, we compared the growth of cultures from the 9–10th and 25–26th HUCB-NSC passages in the three tested conditions and found no statistically significant

Table 2. Examples of NSC-Related Genes Expressed at Higher Levels in HUCB-NSCs Relative to HUCB-MC (Fold Change >2)

Gene	*Description*	*Fold change*
FGFR1	Fibroblast growth factor receptor 1	600
PDGFRA	Platelet-derived growth factor alpha polypeptide	382
FGFR3	Fibroblast growth factor receptor 3	96
BMP4	Bone morphogenetic protein 4	67
NRG1	Neuregulin 1	67
HEY1	Hairy/enhancer-of-split 1	48
KIAA1735	Frizzled receptor signaling pathway	37
BMPR1	Bone morphogenetic protein receptor, type 1	23
FGF1	Fibroblast growth factor 1	23
NOTCH3	Notch homolog 3	19
PDGFA	Platelet-derived growth factor alpha polypeptide	11
FZD8	Frizzled homolog 8	10
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2	10
LIF	Leukemia inhibitory factor	8
ADAM15	A disintegrin and metalloproteinase domain 15	7
JAG2	Jagged 2	4
FZD7	Frizzled homolog 7	3
PEN-2	Presenilin enhancer 2	2
WNT5A	Wingless 5A	2
LIFR	Leukemia inhibitory factor receptor	2
SOX2	SRY (sex determining region Y)-box 2	2
NF 200	Neurofilament heavy	2

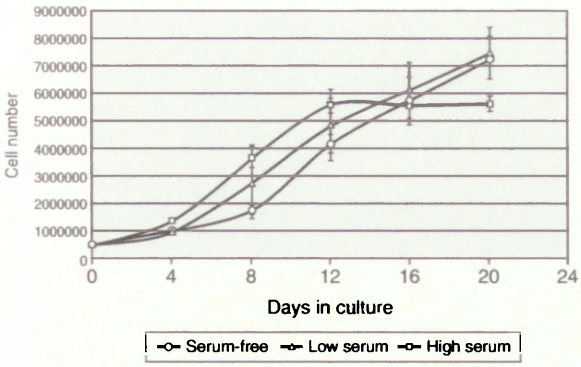

FIG. 3. Comparison of the growth of HUCB-NSC cultures in high-serum, low-serum, and serum-free conditions.

differences between the early and late passages ($p < 0.05$; not shown). Thus, HUCB-NSCs showed similar growth rates in all tested conditions in compared passages. Both early and late passaged cells responded to cell contact inhibition when grown in monolayers in the presence of 10% serum, which further indicated their nontransformed phenotype.

The HUCB-NSC line is clonogenic

Single cells isolated from the floating population of the long-term expanded (32–42 passages) HUCB-NSC line grown in low-serum conditions, produced clones of multipotent neural progenitors. The cloning efficiency for these cells was estimated at approximately 10% of the total number of cells (8 clones for 80 plated single cells) (Fig. 4. A–F). As shown in Fig. 4F, a clone grown for 30 days contained approximately 1,000 cells, implying one cell division per 3 days.

Clones subcultured from low-serum medium on poly-L-lysine-coated coverslips, and grown in this medium for an additional 14 days, multiplied forming a rounded group of nondifferentiated cells immunopositive for the stem/progenitor marker GFAP (Fig. 4G, red) in the center of the clone. Cells at the edges of the clone spread out, extended processes (Fig. 4G–I), and differentiated upon attachment to the surface into neuronal-like cells that expressed β-tubulin III (Fig. 4G,H,I, green), astrocyte-like cells immunolabeled for S100β (Fig. 4I, red), and oligodendrocyte-like cells immunolabeled for GalC (Fig. 4H, red). Some of the morphologically nondifferentiated cells co-expressed β-tubulin III and GFAP (Fig. 4G, arrow).

HUCB-NSCs grown in low-serum conditions express neural markers

High expression of pro-neural genes including nestin (Nes), GFAP, and neurofilament heavy (*Nf-200*) was shown by RT-PCR in nondifferentiated HUCB-NSCs in our earlier study (22). The expression of Nestin and NF-200 mRNA was also detected in our microarray assay. Consistent with the microarray results, the floating HUBC-NSCs (attached to glass slides by cytospinning) immunoreacted with antibodies against nestin (94 ± 2% of cells), GFAP (38 ± 8%), and NF-200 (neurofilament heavy 200 kD) (18 ± 2%) as shown in Fig. 5A,B,D,E, and Fig. 5G,H, respectively. Similar results were obtained with the floating cells grown in serum-free cultures (not shown). Although the filamentous appearance of NF-200 (Fig. 5H) was clear in these nondifferentiated cells, the nestin immunostaining lacked the filamentous structure (Fig. 5B). Similarly, in the cytospun floating HUCB-NSCs, the GFAP lacked the filamentous appearance characteristic of GFAP polymers (Fig. 5E). These nondifferentiated cells were negative for lineage-specific markers such as β-tubulin III and MAP2 for neurons, S100β for astrocytes, and Gal-C for oligodendrocytes. In contrast, cells plated on poly-L-lysine-coated cover-slips for 24 h showed distinct filamentous immunostainings. The filamentous nestin was found in 15 ± 2% (Fig. 5C) and filamentous GFAP in 23 ± 6% of plated cells (Fig. 5F), while S100B was detected in 8 ± 1%. Immunostaining against GalC revealed only 2 ± 1% positive cells. The expression of β-tubulin III and NF-200 (marker proteins characteristic of immature and mature neurons) was observed in 30 ± 5% and 29 ± 4% of cells, respectively. Microtubule-associated protein 2 (MAP2), expressed during advanced neuronal differentiation, was absent at this stage of HUCB-NSC differentiation, regardless of whether only the cytospun floating fraction or the whole population of poly-L-lysine-attached cells were examined. Significant expression of the mature neuron-specific marker, MAP2, upon attachment to poly-L-lysine as well as induction of the early neuronal specific β-tubulin III, astrocyte-specific S100B, and oligodendrocyte-specific GalC immunoreactivity were consistent with the results of mRNA microarray analysis in HUCB-NSCs. Although Map2 was not expressed in nondifferentiated HUCB-NSCs, β-tubulin III, S100β, and GalC all received "present" albeit "low" expression calls.

HUCB-NSCs maintain unchanged differentiating potential in long-term cultures

Cord blood-selected HUCB progenitors had been previously shown to differentiate into cells expressing neural-specific structural proteins upon treatment with 0.5 μM (RA) or co-cultured with rat brain cortical cells (1). Here, we estimated the differentiation of the early and late passages after the line expansion. Expression of neuronal β-tubulin III, astrocytic/neural stem cells GFAP, and oligodendrocytic GalC was evaluated immunocytochemically (Table 3). The relative number of

TABLE 3. COMPARISON OF THE HUCB-NSC NEURAL DIFFERENTIATING POTENTIAL FROM EARLY (<10) AND LATE (>25) PASSAGES

	Passage <10	*Passage >25*
Control medium (FBS 10% + EGF)		
β-Tubulin III-positive cells	6% ± 1.3%	4.7% ± 1.8%
GFAP-positive cells	1.8% ± 0.3%	2.3% ± 0.6%
GALC-positive cells	2.2% ± 0.4%	2% ± 0.6%
Differentiating medium (RA)		
β-Tubulin III-positive cells	27.6% ± 5.4%	28.3% ± 0.5%
GFAP-positive cells	14% ± 3.2%	15.3% ± 2.1%
GALC-positive cells	7% ± 0.5%	7.4% ± 0.7%
Co-culture with brain cortical cells		
β-Tubulin III-positive cells	38% ± 4.2%	35.5% ± 5.1%
GFAP-positive cells	30% ± 3.4%	28.3% ± 4.4%
GALC-positive cells	11% ± 0.6%	10.7% ± 0.5

the cells expressing β-tubulin III, GFAP, or GalC was not significantly different in early (<10) and late (>25) HUCB-NSC passages (Table 3) and was similar to the newly isolated HUCB-NPs fraction (1). Thus, during the prolonged continuous culture, the differentiation potential of HUCB-NSCs remained stable.

dBcAMP differentiated HUBC-NSCD express genes and proteins typical of neuronal lineage

dBcAMP was reported as a potent stimulator of the neural progenitor developmental pathway (24). Hence, to induce more advanced neuronal differentiation of HUCB-NSCs, low-serum-grown cells were attached to a poly-L-lysine/laminin-coated surface and maintained in the presence of 300 μM dBcAMP/CPT for 1–4 weeks. The differentiated cells were referred to as HUCB-NSCD. Cells differentiating for 7 days in dBcAMP showed predominantly neuron-like morphologies with long processes, as illustrated by the phase-contrast image (Fig. 1F). A high number of these cells expressed neuronal cytoskeleton markers β-tubulin III (80 ± 7% of cells; Fig. 5J, Fig. 6A,C,D,I,J,K, green) and NF-200 (73 ± 9% of cells; Fig. 6A, red), whereas only 23 ± 6% and 5 ± 1% were S100β (Fig. 5K) and Gal-C (Fig. 5L) positive, respectively. Nestin-positive cells appeared in a marginal amount (0.5%). Other cytoskeletal proteins, including NF-200 and α-internexin, were also immunodetected, consistent with their mRNA presence shown by DNA array analysis (Table 2). NF-200, an early neural marker, was found in nondifferentiated cells (Fig. 5G,H) and was also co-expressed in dBcAMP differentiated cells along with β-tubulin III (Fig. 6A). α-Internexin had a filamentous appearance and was detected in the cytoplasm of 30 ± 2% of the differentiated cells (Fig. 6B). MAP2, specific for mature neurons, was expressed by 30% of the

FIG. 4. Phase-contrast images of a sequence of divisions of HUCB-NSCs forming a single-cell-derived clone (**a–f**). Cells were plated as a single cell per well in a 96-well plate. The clone was grown for: 3 (**b**), 9 (**c**), 12 (**d**), 21 (**e**), and 30 (**f**) days. Note that some of the clone cells were migrating from the place of origin. Immunocytochemical detection of neural markers in a low-serum-grown clone expanded for 14 days (**g–i**), (**g**). Expression of β-tubulin III (green) and GFAP (red). Note that most of the nondifferentiated, rounded cells located in the center of the clone express GFAP. Some of the more differentiated cells co-express GFAP and β-tubulin III (orange, arrow). (**h**) Expression of β-tubulin III (green) and S100β (red) late astrocytic marker. Some neuron-like cells migrated from the clone. (**i**) Expression of β-tubulin III (green) and GalC (red). Differentiated cells are located at the edge of the clone. Cell nuclei are stained blue with Hoechst 33258. Scale bar, 50 μm.

FIG. 5. Expression of early neural markers of low-serum-grown HUCB-NSC: nondifferentiated, unattached, cytospun cells (**a,b,d,e,g,h**) and whole-cell population plated for 24 h on poly-L-lysine-coated cover slips (**c,f,i**). (**a,b,** and **c,** red) Antinestin immunostaining, in nonattached HUCB-NSCs (**a,b**), filamentous organization of the nestin protein was clearly seen in attached cells (**c**). (**d,e,** and **f,** red) Anti-GFAP immunostaining, filamentous organization of GFAP is not visible in cytospun cells unattached (**e**) and visible in attached for 24 h (**f**). (**g,h,** and **i,** red) Anti-neurofilament heavy (NF-200) immunostaining with defined filamentous organization of NF-200 both in floating (**h**) and attached (**i**) cells. Scale bar for a, d, g, 50 μm: b, c, e, f, h, i, 25 μm. Expression of neural structural markers in HUCB-HSC low-serum-grown cells differentiated for 7 days in the presence of dBcAMP (**j,k,l**). Immunostaining with antibodies against β-tubulin III (**j,** green), S100β (**k,** red), and GalC (**l,** red). Cell nuclei were stained blue with Hoechst 33258. Scale bar, **j,k,l,** 50 μm.

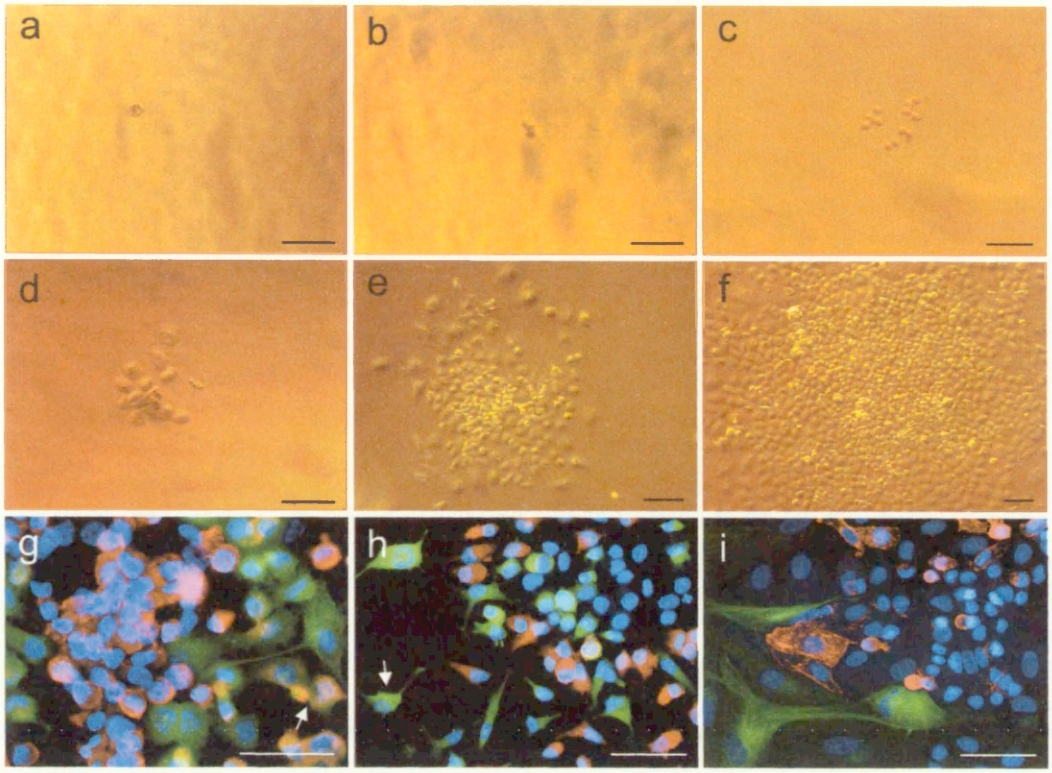

FIG. 4.

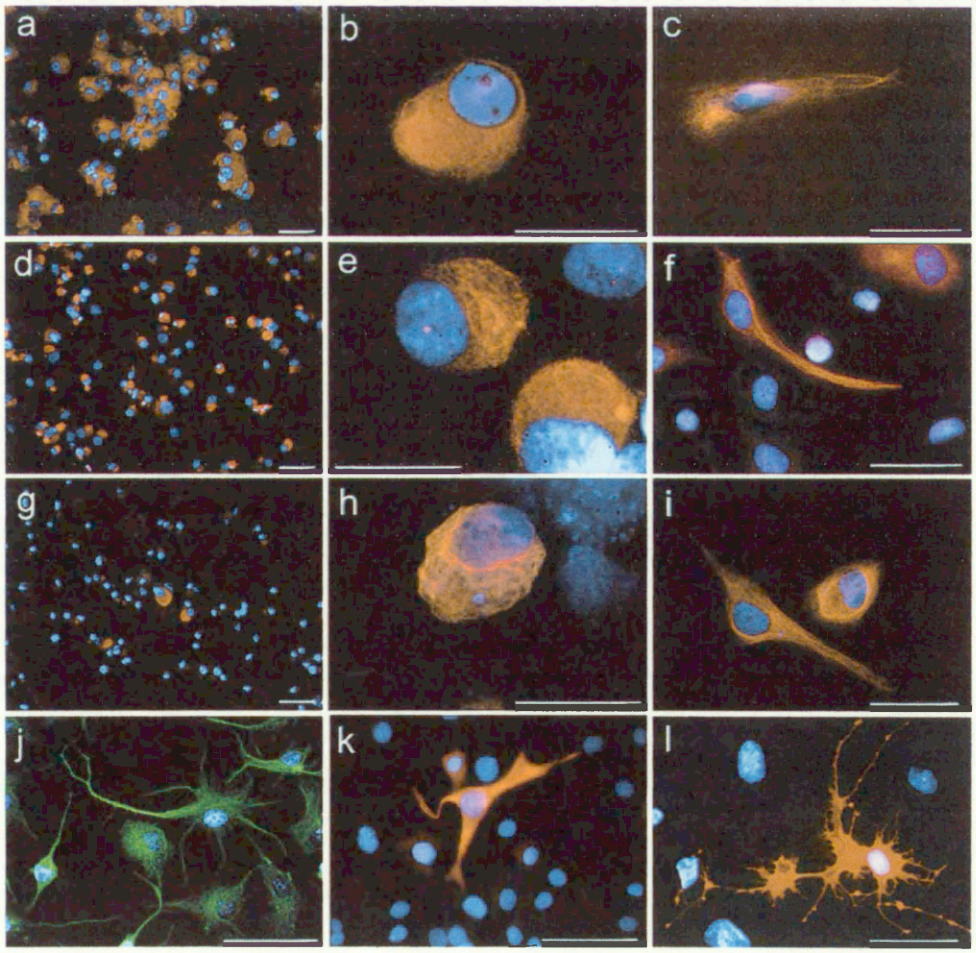

FIG. 5.

dBcAMP differentiated HUCB-NSCD, but was not expressed in nondifferentiated HUCB-NSCs. This is consistent with the exclusive detection of MAP2 RNA by DNA microarray analysis in HUCB-NSCD as compared to HUCB-NSCs and HUCB-MCs. The MAP2 protein was co-expressed with β-tubulin III and localized to neuronal processes (Fig. 6C).

To verify the neuronal lineage of the differentiating cells, we co-immunostained cells with a β-tubulin III antibody and with antibodies against proteins involved in specific neuronal functions. One such protein was the neuron-specific Ca^{2+} binding protein calretinin. Expression of the calretinin gene was significantly up-regulated in differentiated HUCB-NSCD (Table 4). The calretinin protein was co-expressed with β-tubulin III in 38 ± 2% of HUCB-NSCD (Fig. 6D). Synaptic vesicle 2A (SV2A) is known to be an integral membrane glycoprotein of synaptic vesicles in growing and mature neurons. We detected SV2 immunoreactivity co-expressed with the dopamine receptor protein Dr2a in 65 ± 4% of HUCB-NSC. Patch-like expression of SV2A in the cytoplasm of HUCB-NSCD is illustrated in the confocal image of the differentiated cells (Fig. 6E). The *Sv2a* gene was also overexpressed by HUCB-NSCD (Table 4). One of the most up-regulated genes in HUCB-NSC was the *Latex* gene (36-fold change) (Table 4). Latexin, a subtype-specific molecular marker of glutamatergic neurons (25), was also detected by immunostaining (Fig. 6F) in 70 ± 2% of the differentiated cells. The GABA(A) receptor associated protein (GABA-RAP) and glutamate decarboxylase 1, 67 kD (GAD67), both involved in synaptic transmission, were also detected by immunostaining (Fig. 6, G and H, respectively).

These findings were consistent with the significant increase in the expression of genes encoding GABA(A) receptor associated proteins *GabarapL3*, *-L1*, *and -L2* and with the detection of *Gad67* RNAs by DNA microarray analysis (Table 4). The microtubule-associated protein, Tau, characteristic of mature neurons, was expressed by 50 ± 4% of HUCB-NSCD. Differentiated HUCB-NSCD also expressed the *Mapt* gene, which encodes Tau. Confocal immunoimages of co-stained β-tubulin III and Tau (Fig. 6I) revealed co-expression of these two neuronal proteins in HUCB-NSCD. Immunostaining for tyrosine hydroxylase was detected in 80 ± 5% of the differentiated cells (Fig. 6J, red) and was co-localized with β-tubulin III (Fig. 6J, green). Fibroblast growth factor receptor 1 (FGFR1) was expressed in the whole population of tested cells; however, its intracellular localization changed depending upon the stage of differentiation. In control HUCB-NSC, FGFR1 immunoreactivity was evenly distributed thought the cells (Fig. 6K). In contrast, in dB-cAMP differentiated HUCB-NSCD, the greatest FGFR1 accumulation was observed in the nucleus (Fig. 6L). The FGFR1 gene was highly expressed in both HUCB-NSC and HUCB-NSCD (Tables 2 and 4).

DISCUSSION

In this report, we have presented evidence that genetically nontransformed, HUCB-derived neural clonogenic progenitors (1) can give rise to a stable, neural-like stem line that can be maintained in continuous culture for over 60 passages. More or less defined subpopulations of umbilical cord blood (1,26–28), as well as other cells isolated from bone marrow (3,29,30) and epithelial (31) or adipose (32) tissues, have been reported to undergo phenotypic metamorphosis and, upon specific in vitro or in vivo stimulation, to display neural-like properties. However, to best of our knowledge, long-term maintenance and propagation of these cells as stable lines has not been reported. Of note, the recently described fetal brain or ES derived NSC lines capable of long-term growth in monolayer cultures (15) had been obtained from adherent primary cultures with subsequent EGF treatment and further passaging of only floating, nondifferentiated cells in EGF- and bFGF-containing media. A similar protocol had been used for the expansion of HUCB-derived neural progenitors (1) toward a stable line (HUCB-NSCs) described here.

As already mentioned in the Results section, the HUCB-derived line has several characteristics of NSC/NP cells, i.e., high clonogenicity (up to 10% of isolated single cells gave rise to secondary multipotent clones), ability to form neurosphere-like aggregates (33), and ability to differentiate into neuron-, astrocyte-, and oligodendrocyte-like cells. The HUCB-derived line contains a normal human karyotype without identifiable chromosomal changes often observed during oncogenic transformation. Moreover, after transplantation of the HUCB-NSCs into immunodeficient nonobese diabetic/severe combined immunodeficient (NOD/SCID), mice we found no tumor formation (unpublished).

A number of HUCB-NSC-expressed genes encode proteins of the Wnt, Lif, Notch, and FGFR1 signaling pathways that are known to regulate stem cell self-renewal and proliferation. Those genes have been referred to as putative "stemness" genes (34–39). The neuroectodermal commitment of HUCB-NSCs was indicated by the activation of several pro-neural genes (*Sox2, NeuroD1, Otx1,* and *Msi1*) and the lack of gene expression essential for mesodermal (T gene) and endodermal differentiation (*Foxa1*), shown by microarray assays and in a parallel study by quantitative RT-PCR (23). The activation of the FGFR1 pathway was shown to be a hallmark of NSCs (22,40). *FgfR1* appeared to be one of the most up-regulated genes in telencephalon-isolated NSCs when compared to the Sox2 promoter-isolated, clonally

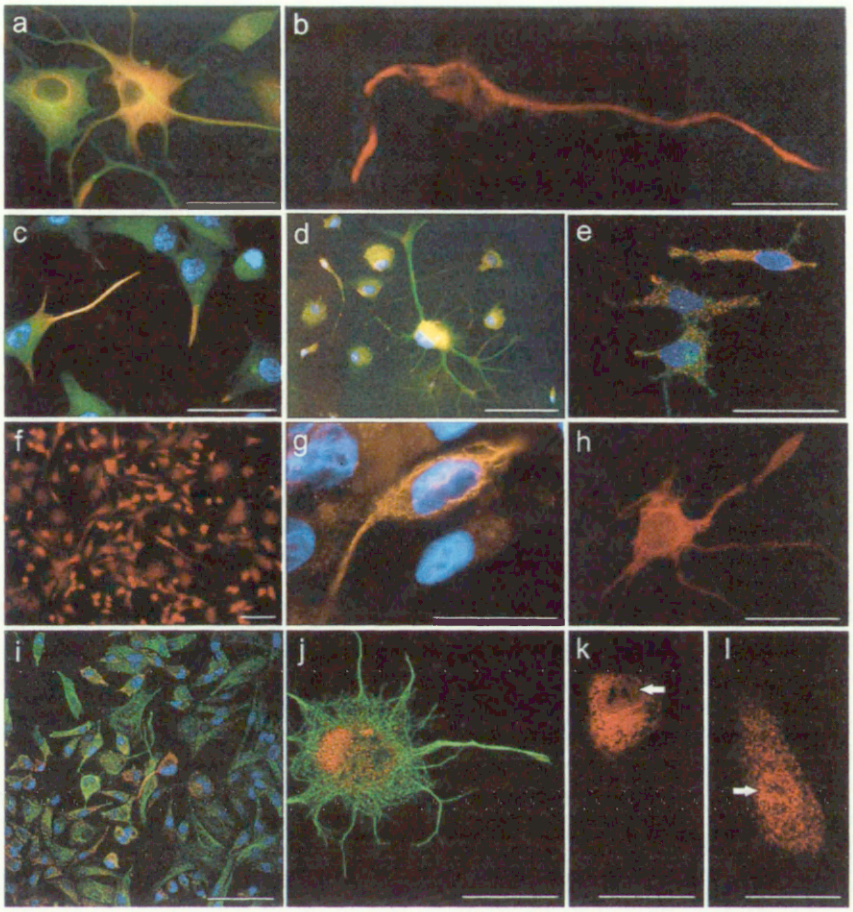

FIG. 6. Immunocytochemical detection of neuronal markers in dBcAMP-differentiated cells (**a**) β-Tubulin III (green) and NF200 (red) co-staining, (**b**) α-Internexin. (**c**) Merger of β-tubulin III (green) and MAP2 (red) immunostaining. Note that MAP2 is localized to neuronal extensions. (**d**) β-Tubulin III (green) and calretinin (red) co-staining. (**e**) Synaptic vesicle 2 (SV2, green) and dopamine receptor 2a (DR2a, red) co-staining. Note dot-like, membrane localization of SV2. (**f**) Latexin. (**g**) GABA receptor 1-associated protein (GABAR1AP) immunostaining. (**h**) Glutamate decarboxylase 67 (GAD 67) immunodetection. Note the cytoplasmic localization of GAD67. (**i**) Co-expression of β-tubulin III (green) and MAPT (microtubule associated protein TAU; red. (**j**) Co-expression fo β-tubulin III (green) and tyrosine hydroxylase (TH-red). (**k,l**) Expression of FGFR1 in control (k) and differentiated cells (l). Note the nuclear localization of FGFR1 in differentiated cells. Arrows, Cell nuclei. Scale bar, 50 μm.

related ES cells (22). As shown by our microarray analysis, *FgfR1* gene expression is increased up to 600-fold in nondifferentiated HUCB-NSCs, as compared to HUCB-MCs. A putative role of FGFR1 in self-renewal activity of stem cells can be linked with epigenetic control of cells fate by the FGF signaling pathway (41,42).

Comparison of the early and late HUCB-NSC passages showed no significant differences in their growth rates, clonogenicity, and differentiation potential. Importantly, we found no enhanced expression of the classical protooncogenes by HUCB-NSCs compared to HUCB-MCs. An additional indication that these cells remained noncancerous was the lack of overgrowth after reaching confluence. A doubling time of 4 days for HUCB-NSCs was similar to that observed in human fetal CNS-derived NSCs in both early and late passages by Vescovi et al. (11) and also in fetal and adult (hippocampus-derived) CNS stem cell cultures (43,44).

Unlike the growth of human NSCs from fetal and adult CNS tissues (11,44), the growth of the HUCB-NSC line in the absence of serum was optimal in presence LIF. In that respect, HUCB-NSCs are similar to mouse ES cells (45), which, in contrast to human cell lines (46,47), required LIF to maintain their undifferentiated state. However, according to Wright et al. (48), LIF also increases the longevity of the bFGF and EGF fetal human NSCs responsive in long-term cultures (more than 30 population doublings). Indeed, activation of the LIF/CNTFR/LIFR/gp130 signaling pathway stimulated proliferation of forebrain neural stem/progenitors by suppression of

TABLE 4. EXAMPLES OF GENES UP-REGULATED IN HUCB-NSCD RELATIVE TO HUCB-NSCS UPON DIFFERENTIATION IN THE PRESENCE OF DBCAMP (FOLD CHANGE >2)

Gene	*Description*	*Fold change*
GPR17	G protein-coupled receptor 17	41
LXN	Latexin protein	36
GABARAPL3	GABA(A) receptors associated protein like 3	18
SV2A	Synaptic vesicle glycoprotein 2A	15
PKC-alpha	Protein kinase C-alpha	12
GABARAPL1	GABA(A) receptors associated protein like 1	6
CREM	cAMP responsive element modulator	6
CALB2	Calbindin 2, 29kDa (calretinin)	4
STAT3	Signal transducer and activator of transcription 3	3
SCG2	Secretogranin II (chromograinin C)	3
SEMA3D	Semaphorin 3D	3
NPTX1	Neuronal pentraxin I, (synaptic transmission)	3
PCLO	Piccolo (presynaptic cytomatrix protein)	3
CHGB	Chromogranin B (secretogranin 1)	2
STX4A	Syntaxin 4A, (neurotransmitter transport)	2
SYNJ1	Synaptojanin 1 (synaptic vesicle endocytosis)	2
MAPKAPK2	MAPKKK cascade, protein amino acid phosphorylation	2

their restriction to a glial cell fate (49) as well as by maintenance of cell cycle progression (reviewed in ref. 35). However, with the addition of 2% serum supplemented with ITS, expansion of HUCB-NSCs was possible without supplementation of mitogens (LIF, EGF, bFGF) (Fig. 2). We speculate that in this case the endogenous growth-promoting factors can be more efficiently retained and/or released by a subset of the cells growing in a monolayer. The remaining undifferentiated cells that were transiently attached to the monolayer could be stimulated there by secreted growth factors acting in a paracrine manner. Consequently, the subpopulation of the floating cells would maintain their stem-like characteristics indicated by the expression of the early neuroectodermal/NSC stem cell markers such as nestin (50), GFAP (51), and heavy neurofilament NF200 (52), in parallel with their RNA expression. Moreover in the nondifferentiated, floating HUCB-NSCs immunostaining for nestin and GFAP proteins does not reveal the classical filamentous pattern, suggesting an immature stage of neural commitment.

Furthermore, the HUCB-NSC line maintained an unchanged ability to differentiate, even after prolonged culturing (Table 3). Treatment with dBcAMP markedly and consistently stimulated neuronal commitment with the known neuromorphogenic effects of cAMP (53–55). After long-term dBcAMP/CPT stimulation (300 μM), the cells showed advanced maturation into the neuronal lineage with high expression of neural-specific genes and proteins (Table 4) and with neural-like electrophysiological characteristic (19).

The gene expression profile of HUCB-NSCs changed substantially upon dBcAMP-induced differentiation. Genes activated in HUCB-NSCD (compared to nondifferentiated HUCB-NSCs) were mostly related to the signaling pathways, neurotransmitters, receptors, or channels (19) involved in neuronal functions. For example, GABA-ergic neuronal differentiation of HUCB-NSCD was indicated by the up-regulation of genes encoding G-coupled membrane proteins, GABA-receptors-associated proteins (GABARAPL3, GABARAPL1, GABARAPL2), and the Ca^{2+}-binding protein calretinin. In mice, calretinin expression is typical for an early postmitotic step in neuronal differentiation and defines adult hippocampal neurogenesis (56), modifies presynaptic calcium signaling (57), and is detected in striatal GABA-ergic interneurons (58). The gene that encodes the carboxypeptidase A inhibitor protein Latexin, a subtype-specific molecular marker of glutamatergic neurons (25), was markedly up-regulated in HUCB-NSCD. Latexin expression may also indicate a dopaminergic lineage differentiation because it is known to be co-expressed with Nurr1 in tyrosine hydroxylase (TH)-positive neurons (59). Our immunocytochemical data show that the TH protein is co-expressed with the neuronal marker β-tubulin III in HUCB-NSCD. The expression of both latexin and TH by approximately 80% of HUCB-NSCD indicates their co-expression in a significant number of differentiated HUCB-NSCs.

Furthermore, the increased expression of *FgfR1* in both HUCB-NSCs and HUCB-NSCD together with the exclu-

sive expression of *FgfR3*, *Fgf1*, *Fgf2*, and genes encoding CREB-binding protein (CBP), p300, and the transcriptional factors CREB and CREM are all consistent with the overexpression of typical neuronal genes known to be regulated by cAMP-responsive elements (CRE). Those genes also include *Chgb* (Chromogranin B, secretogranin1) and *Scg2* (chromogranin C, secretogranin II), known regulators of neurotransmitter and neurotrophin secretion, TH, and light neurofilament (60). This together with other results, suggests an involvement of the FGFR1 signaling pathway in the regulation of neuronal differentiation of HUCB-NSCs. The cAMP-dependent activation of FGFR1 was shown in this study by its translocation to the nucleus upon differentiation (Fig. 6K,L) and confirms observation of Stachowiak et al. (24) for human neuronal progenitor cells. The novel integrative FGFR1-related signaling pathway has been proposed to activate CBP/p300 and co-ordinate responses elicited by different cellular signals involved in neuronal differentiation (54).

In the present work, the changes in expression of certain genes encoding cytoskeletal- and synaptic neuron-specific proteins were followed by their expression at the protein level as validated by immunocytochemistry. The proteins shown to be expressed in HUCB-NSCD included β-tubulin III, neurofilament-200, α-internexin, MAPs TAU and MAP2, calretinin, synaptic vesicle 2, GABA receptor-associated protein 1, and glutamate decarboxylase 67. Transcripts for these proteins were not detected by DNA microarray assay in HUCB-MCs. This indicates the neural commitment of the HUCB-NSC line as well as its ability to differentiate into neuron-like cells equipped with neuroskeletal- as well as neurotransmitter-producing proteins and the synaptic neurotransmitter release apparatus.

The dBcAMP-treated HUCB-NSCD, similar to other differentiated NSCs derived from nervous tissue, showed activation of MAP kinase and up-regulation of several genes encoding proteins involved in synaptic transmission. This included synaptic vesicle 2, syntaxin, and synaptojanin (Table 4). Recently, we have analyzed the expression of neurotransmission-related receptors and ion channels using the DNA microarray absolute database in the context of the HUCB-NSCD electrophysiological properties. We found activation of genes encoding acetylcholine (ACh), GABA, glutamate (kainate and metabotropic), glycine, 5-hydroxytryptamine (5-HT) and dopamine (DA) receptors, and voltage-dependent potassium and sodium channels (19).

In summary, we have presented here general characteristics of the neural-like stem cell line established from the umbilical cord blood $CD34^-$ mononuclear fraction. This line contains clonogenic, multipotent, neurally committed stem cells. The phenotype of the HUCB-NSC line appears more similar to the in vitro immortalized neural cell lines (14) or NSC lines grown in monolayer (15) than to the classical "strains" grown as neurospheres from fetal or adult CNS tissues (10). Still, in defined growth conditions, HUCB-NSCs can also form neurosphere-like structures (33), a proposed hallmark of NSCs. Because similar cells have been isolated independently by other researchers (16,17), the HUCB line appears to be a reliable source for NSC-like cells with a potential therapeutic application.

Our data also contribute indirectly to the recent discussion that, at least in part, neural differentiation of cells arisen from sources other than neuronal tissue in vitro could be a matter of artifacts induced by their growth in altered culture conditions such as reduced serum level and the use of anti-oxidants or protein kinase C (PKC) activation by phorbol esters (61) (and S. Przyborski, personal communication, 2005). Moreover, these changes can be reproduced in variety of cell types, including primary fibroblasts, and by addition of chemicals eliciting cytoskeleton collapse. However, such phenotypic conversion, if it occurs, could be easily reversed by placing the cells back into their original culture conditions. Also, this artifactual "neuralization" of cell phenotype would not be followed by proneural gene expression or de novo translation of neural protein markers (62). In contrast, our cell line was grown from the beginning in culture conditions commonly used for neural and other tissues. In this putative physiological environment and without exposure to any additional treatments, our cells attained neural morphology and changed the expression of plethora of genes and proteins toward that characteristic of neural lineage. This new, neural stem/progenitor-like phenotype, once established, did not change during consecutive generations. Furthermore, the HUCB-NSC-like cells when treated with classical neuromorphogenes, such as RA, brain-derived neurotrophic factor (BDNF), and cAMP, responded typically with induction of genes and proteins characteristic of more differentiated, functional neurons. These functional properties were further confirmed by electrophysiological study showing concomitant acquisition of several functional properties characteristic of maturating neurons (19).

The identity of ancestor cells for HUCB-NSCs and whether they are a product of phenotypic conversion of MSC-like precursors (similar to BM stromal cells as reported in refs. 2, 3, and 63) or arise from immature "multidifferentiated" cells already reported in native BM (64,65) remain unknown. In addition the presence of pluripotent remnants of embryonic/primordial-germ like stem cells (18,42,66) in this immature, cord blood-derived cell population can not be excluded. The molecular mechanisms responsible for the unusual plasticity of tissue-specific stem cells of cord blood may rely on a transient state of the "multilineage open transcriptosome," as characterized by a low level of transcription, but high responsiveness to external in vitro stim-

ulation (67). The umbilical cord blood, as tissue of still primitive ontogeny, could retain a certain number of such potentially pluripotent or "flexible" progenitor cells that could be induced to neural lineage in vitro and, in addition, display ES-like, unlimited kinetics of cell proliferation. Studies addressing these questions are in progress.

ACKNOWLEDGMENTS

We thank Dr. B. Pienkowska-Grela (Institute of Oncology, Warsaw, Poland) for help in cell karyotyping and Dr. L. Stain (Roswell Park Cancer Institute, Buffalo, NY) for analysis of gene array data. This work is supported by Polish State Committee for Scientific Research (grants No.6P05A04920 and K053/P05/2003) and by the United States National Science Foundation (IBN-9728923) and National Institutes of Health (NS43621-01).

REFERENCES

1. Buzanska L, EK Machaj, B Zablocka, Z Pojda and K Domanska-Janik. (2002). Human cord blood-derived cells attain neuronal and glial features in vitro. J Cell Sci 115: 2131–2138.
2. Hess DC, T Abe, WD Hill, AM Studdard, J Carothers, M Masuya, PA Fleming, CJ Drake and M Ogawa. (2003). Hematopoietic origin of microglial and perivascular cells in brain. Exp Neurol 186:134–144.
3. Ji Jiang Y, BN Jahagirdar, RL Reinhardt, RE Schwartz, CD Keene, XR Ortiz-Gonzalez, M Reyes, T Lenvik, T Lund, M Blackstad, J Du, S Aldrich, A Lisberg, WC Low, DA Largaespada and CM Verfaillie. (2002). Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 418:41–49.
4. Korbling M, RL Katz, A Khanna, AC Ruifrok, G Rondon, M Albitar, RE Champlin and Z Estrov. (2002). Hepatocytes and epithelial cells of donor origin in recipients of peripheral blood stem cells. N Engl J Med 346: 738–746.
5. Mezey E, S Key, G Vogelsang, I Szalayova, GD Lange and B Crain. (2003). Transplanted bone marrow generates new neurons in human brains. Proc Natl Acad sci USA 4:1364–1369.
6. Okamoto R, T Yajima, M Yamazaki, T Kanai, M Mukai, S Okamoto, Y Ikeda, T Hibi, J Inazawa and M Watanabe. (2002). Damaged epithelia regenerated by bone marrow-derived cells in the human gastrointestinal tract. Nature Med 8:1011–1017.
7. Poulsom R, MR Alison, SJ Forbes and NA Wright. (2002). Adult stem cell plasticity. J Pathol 197:441–456.
8. Corti S, F Locatelli, S Strazzer, M Guglieri and GP Comi. (2003). Neuronal generation from somatic stem cells: current knowledge and perspectives on the treatment of acquired and degenerative central nervous system disorders. Curr Gene Ther 3:247–272.
9. Caldwell MA, X He and N Wilkie. (2001). Growth factors regulate the survival and fate of cells derived from human neurospheres. Nature Biotechnol 19:475–479.
10. Svendsen CN, MG ter Borg, RJ Armstrong, AE Rosser, S Chandran, T Ostenfeld and MA Caldwell. (1998). A new method for the rapid and long term growth of human neural precursor cells. J Neuroscience Meth 85:141–153.
11. Vescovi AL, EA Parati, A Gritti, P Poulin, M Ferrario, E Wanke, P Frolichsthal-Schoeller, L Cova, M Arcellana-Panlilio, A Colombo and R Galli. (1999). Isolation and cloning of multipotential stem cells from the embryonic human CNS and establishment of transplantable human neural stem cell lines by epigenetic stimulation. Exp Neurol 156:71–83.
12. Carpenter MK, C Winkler, R Fricker, DF Emerich, SC Wong, C Greco, EY Chen, Y Chu, JH Kordower, A Messing, A Bjorklund and JP Hammang. (1997). Generation and transplantation of EGF-responsive neural stem cells derived from GFAP-hNGF transgenic mice. Exp Neurol 148:187–204.
13. Jain M, RJ Armstrong, S Elneil, AE Rosser and RA Barker. (2003). Migration and differentiation of transplanted human neural precursor cells. Neuroreport 14:1257–1262.
14. Villa A, B Navarro-Galve, C Bueno, S Franco, MA Blasco and A Martinez-Serrano. (2004). Long-term molecular and cellular stability of human neural stem cell lines. Exp Cell Res 294:559–570.
15. Conti L, SM Pollard, T Gorba, E Reitano, M Toselli, G Biella, Y Sun, S Sanzone, QL Ying, E Cattaneo and A. Smith. (2005). Niche-independent symmetrical self-renewal of mammalian tissue stem cell. PLOS Biology 3:e283.
16. McGuckin CP, N Forraz, Q Allouard and R Pettengell. (2004). Umbilical cord blood stem cells can expand hematopoietic and neuroglial progenitors in vitro. Exp Cell Res 295:350–359.
17. Kogler G, S Sensken, JA Airey, T Trapp, M Muschen, N Feldhahn, S Liedtke, RV Sorg, J Fischer, C Rosenbaum, S Greschat, A Knipper, J Bender, O Degistirici, J Gao, Al Caplan, EJ Colletti, G Almeida-Porada, HW Muller, E Zanjani and P Wernet. (2004). A new human somatic stem cell from placental cord blood with intrinsic pluripotent differentiation potential. J Exp Med 200:123–135.
18. McGuckin CP, N Forraz, MO Baradez, S Navran, J Zhao, R Urban, R Tilton and L Denner. (2005). Production of stem cells with embryonic characteristics from human umbilical cord blood. Cell Prolif 38:245–255.
19. Sun W, L Buzanska, K Domanska-Janik, RJ Salvi and MK Stachowiak. (2005). Voltage-sensitive and ligand-gated channels in differentiating fetal neural stem cells isolated from the non-hematopoietic fraction of human umbilical cord blood. Stem Cells 23:931–945.
20. Pienkowska-Grela B, B Grygalewicz and U Bregula. (2002). Overrepresentation of the short arm of chromosome 12 in seminoma and nonseminoma groups of testicular germ cell tumors. Cancer Genet Cytogenet 134:102–108.
21. Hanneken A, PA Macher and A Baird. (1995). High affinity immunoreactive FGF receptors in the extracellular matrix of vascular endothelial cells. J Cell Biol 128:1221–1228.
22. Jurga M, I Markiewicz, A Sarnowska, A Habich, H Kozlowska, B Lukomska, L Buzanska and K Domanska-Janik.

(2005). Neurogenic potential of human umbilical cord blood–neural stem cells depends on previous long-term culture conditions. J Neurosci Res 83:627–637.
23. D'Amour KA and FH Gage. (2003). Genetic and functional differences between multipotent neural and pluripotent embryonic stem cells. Proc Natl Acad Sci USA 30:11866–11872.
24. Stachowiak EK, X Fang J Myers, S Dunham and MK Stachowiak. (2003). cAMP-induced differentiation of human neuronal progenitor cells is mediated by nuclear FGF receptor-1 (FGFR1). J Neurochem 84:1296–1312.
25. Takiguchi-Hayashi K. (2001). In vitro clonal analysis of rat cerebral cortical neurons expressing latexin, a subtype-specific molecular marker of glutamatergic neurons. Brain Res Dev Brain Res 14:87–90.
26. Sanchez-Ramos JR, S Song, SG Kamath, T Zigova, A Willing, F Cardozo-Pelaez, T Stedeford, M Chopp and PR Sanberg. (2001). Expression of neural markers in human umbilical cord blood. Exp Neurol 171:109–115.
27. Ha Y, JU Choi, DH Yoon, DS Yeon, JJ Lee, HL Kim and YE Cho. (2001). Neural phenotype expression of cultured human cord blood cells *in vitro*. Neuroreport 12:523–3527.
28. Zigova T, S Song, AE Willing, JE Hudson, MB Newman, S Saporta, J Sanchez-Ramos and PR Sanberg. (2002). Human umbilical cord blood cells express neural antigens after transplantation into the developing rat brain. Cell Transplant 11:265–274.
29. Kopen GC, DJ Prockop and DG Phinney. (1999). Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. Proc Natl Acad Sci USA 14:10711–10716.
30. Hermann A, R Gastl, S Liebau, MO Popa, J Fiedler, BO Boehm, M Maisel, H Lerche, J Schwarz, R Brenner and A Storch. (2004). Efficient generation of neural stem cell-like cells from adult human bone marrow stromal. J Cell Sci 117:4411–4422.
31. Toma JG, M Akhavan, KJ Fernandes, F Barnabe-Heider, A Sadikot, DR Kaplan and FD Miller. (2001). Isolation of multipotent adult stem cells from the dermis of mammalian skin. Nature Cell Biol 3(9):778–784.
32. Safford KM, KC Hicok, SD Safford, YD Halvorsen, WO Wilkison, JM Gimble and HE Rice. (2002). Neurogenic differentiation of murine and human adipose-derived stromal cells. Biochem Biophys Res Commun 294: 371–379.
33. Jurga M and M Domanska-Janik. (2004). Neurospheres of human umbilical cord blood derived neural stem cells—in vitro model of stem cell niche. FENS Lisbon, abstract book.
34. Sato N, L Meijer and L Skaltsounis. (2004). Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. Nature Med 10:55–63.
35. Metcalf D. (2005). The unsolved enigmas of leukemia inhibitory factor. Stem Cells 21:5–14.
36. Moon RT, B Bowerman, M Boutros and N Perrimon. (2002). The promise and perils of Wnt signaling through beta-catenin. Science 296:1644–1646.
37. Portin P. (2002). General outlines of the molecular genetics of the Notch signaling pathway in *Drosophila melanogaster*: a review. Hereditas 136:89–96.
38. Espinosa L, J Ingles-Esteve, C Aguilera and A Bigas. (2003). Phosphorylation by glycogen synthase kinase-3 beta down-regulates Notch activity, a link for Notch and Wnt pathways. J Biol Chem 278:32227–32235.
39. Cong F and H Varmus. (2002). Nuclear-cytoplasmic shuttling of Axin regulates subcellular localization of beta-catenin. Proc Natl Acad Sci USA 101:2882–2887.
40. Dvorak P, D Dvorakova, S Koskova, M Vodinska, M Najvirtova, D Krekac and A Hampl. (2005). Expression and potential role of fibroblast growth factor 2 and its receptors in human embryonic stem cells. Stem Cells 23: 1200–1211.
41. Hsieh J and FH Gage. (2004). Epigenetic control of neural stem cell fate. Curr Opin Genet Dev 14:461–469.
42. Rao M. (2004). Conserved and divergent paths that regulate self-renewal in mouse and human embryonic stem cells. Dev Biol 275:269–286.
43. Palm K, T Salin-Nordstrom, MF Levesque and T Neuman. (2000). Fetal and adult human CNS stem cells have similar molecular characteristics and developmental potential. Brain Res Mol Brain Res 31:192–195.
44. Svendsen CN and MA Caldwell. (2000). Neural stem cells in the developing central nervous system: implication for cell therapy through transplantation. Progr Brain Res 127:13–34.
45. Smith AG, JK Heath, DD Donaldson, GG Wong, J Moreau, M Stahl and D Rogers. (1988). Inhibition of pluripotential embryonic stem (ES) cell differentiation by purified peptides. Nature 336:688–690.
46. Reubinoff BE, MF Pera and CY Fong. (2000). Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nature Biotechnol 18:399–404.
47. Xu RH, RM Peck, DS Li, X Feng, T Ludwig and JA Thomson. (2005). Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. Nat Methods 2:185–190.
48. Wright LS, J Li, MA Caldwell, K Wallace, JA Johnson and CN Svendsen. (2003). Gene expression in human neural stem cells: effects of leukemia inhibitory factor. J Neurochem 86:179–195.
49. Shimazaki T, T Shingo and S Weiss. (2001). The ciliary neurotrophic factor/leukemia inhibitory factor/gp130 receptor complex operates in the maintenance of mammalian forebrain neural stem cells. J Neurosci 21:7642–7653.
50. Lendahl U, LB Zimmerman and RD McKay. (1990). CNS stem cells express a new class of intermediate filament protein. Cell 60:585–595.
51. Doetsch F, I Caille, DA Lim, JM Gracia-Verdugo and A Alvarez-Buylla. (1999). Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. Cell 97:703–716.
52. Goolsby J, MC Marty, D Heletz, J Chiappelli, G Tashko, D Yarnell, PS Fishman, S Dhib-Jalbut, CT Bever Jr, B Pessac and D Trisler. (2003). Hematopoietic progenitors express neural genes. Proc Natl Acad Sci USA 100:14926–14931.
53. Yan J, L Studer and RD McKay. (2001). Ascorbic acid increases the yield of dopaminergic neurons derived from basic fibroblast growth factor expanded mesencephalic precursors. J Neurochem 76:307–311.

54. Stachowiak MK, X Fang, JM Myers, SM Dunham, R Berezney, PA Maher and EK Stachowiak. (2003). Integrative nuclear FGFR1 signaling (INFS) as a part of a universal "feed-forward-and-gate" signaling module that controls cell growth and differentiation. J. Cell Biochem 90:662–691.
55. Piper DR, T Mujtaba, MS Rao and MT Lucero. (2000). Immunocytochemical and physiological characterization of a population of cultured human neural precursors. J Neurophysiol 84:534–548.
56. Brandt MD, S Jessberger, B Steiner, G Kronenberg, K Reuter, A Bick-Sander, W von der Behrens and G Kempermann. (2003). Transient calretinin expression defines early postmitotic step of neuronal differentiation in adult hippocampal neurogenesis of mice. Mol Cell Neurosci 24:603–613.
57. Edmonds B, R Reyes, B Schwaller and WM Roberts. (2000). Calretinin modifies presynaptic calcium signaling in frog saccular hair cells. Nature Neurosci 38:786–790.
58. Rymar VV, R Sasseville and KC Luk. (2004). Neurogenesis and stereological morphometry of calretinin-immunoreactive GABAergic interneurons of the neostriatum. J Comp Neurol 469:325–339.
59. Arimatsu Y, M Ishida, T Kaneko, S Ichinose and A Omori. (2003). Organization and development of corticocortical associative neurons expressing the orphan nuclear receptor. Nurr1. J Comp Neurol 466:180–196.
60. Mahata SK, NR Mahapatra, M Mahata M and DT O'Connor. (2002). Neuroendocrine cell type-specific and inducible expression of chromogranin/secretogranin genes: crucial promoter motifs. Ann NY Acad Sci 971:27–38.
61. Tint IS, EM Bonder, HH Feder, CP Reboulleau, JM Vasiliev, and IM Gelfand. (1992). Reversible structural alterations of undifferentiated and differentiated human neuroblastoma cells induced by phorbol ester. Proc Natl Acad Sci USA 89:8160–8164.
62. Bertani N, P Malatesta, G Volpi, P Sonego and R Perris. (2005). Neurogenic potential of human mesenchymal stem cells revisited: analysis by immunostaining, time-lapse video and microarray. J Cell Sci 118:3925–3936.
63. Song S and J Sanchez-Ramos. (2003). Brain as the Sea of Marrow. Exp Neurol 184:54–60.
64. Tondreau T, L Lagneaux, M Dejeneffe, M Massy, C Mortier, A Delforge and D Bron. (2004). Bone marrow-derived mesenchymal stem cells already express specific neural proteins before any differentiation. Differentiation 72:319–326.
65. Ratajczak MZ, M Kucia, R Reca, M Majka, A Janowska-Wieczorek and J Ratajczak. (2004). Stem cell plasticity revisited: CXCR4-positive cells expressing mRNA for early muscle, liver and neural cells "hide out" in the bone marrow. Leukemia 18:29–40.
66. Melton DA and C Cowan. (2004). "Stemness": definitions, Criteria, and Standards. In Lanza R., Gerhart J., Hogan B., Melton D., Pedersen R., Thomson J., West M. (eds) Handbook of Stem Cells, vol 1. Elsevier AP 25–31.
67. Liu Y and MS Rao. (2003). Transdifferentiation—fact or artifact. J Cell Biochem 88:29–40.

Address reprint requests to:
Dr. Krystyna Domańska-Janik
NeuroRepair Department
Medical Research Center
Pawińskiego 5 Str, Warsaw, Poland

E-mail: kd-j@cmdik.pan.pl

Dr. Michal K. Stachowiak
Department of Pathology and Anatomical Sciences
206 A. Farber Hall
3435 Main Street
Buffalo, NY 14214

E-mail: mks4@buffalo.edu

Received January 16, 2006; accepted February 14, 2006.

PUBLIKACJA 5

Neuro-degenerative Diseases

Neurodegenerative Dis 2006;3:19–26
DOI: 10.1159/000092088

Neuronal Differentiation of Human Umbilical Cord Blood Neural Stem-Like Celi Line

Leonora Bużańska Marcin Jurga Krystyna Domańska-Janik

NeuroRepair Department, Medical Research Institute, Polish Academy of Sciences, Warsaw, Poland

Key Words
Human umbilical cord blood · Neural stem celi line · Neuronal differentiation

Abstract
The expanding population of neural stem/progenitor cells can be selected from human cord blood nonhematopoietic (CD34-negative) mononuclear fraction. Due to repeated expansion and selection of these cells we have established the first clonogenic, nonimmortalized human umbilical cord blood neural stem-like cell (HUCB-NSC) line. This line can be maintained at different stages of neural progenitor development by the presence of trophic factors, mitogens and neuromorphogens in culture media. Neurogenic potential of HUCB-NSC was established for serum-free and low-serum cultured cells. Commitment of HUCB-NSC by serum was shown to be important for the optimal response to the signals provided by surrounding environment in vitro. Enhanced neuronal differentiation induced by dBcAMP treatment was accompanied by expression of several functional proteins including glutamatergic, GABAergic, dopamine, serotonin and acetylcholine receptors, which was shown by microarray, immunocytochemistry and electrophysiology. Electrophysiological studies, whole-cell patch-clamp recordings, revealed in differentiated HUCB-NSC two types of voltage-sensitive and several ligand-gated currents typical for neuronal cells. The above HUCB-NSC characteristic conceivably implicates that cord blood-derived progenitors could be effectively differentiated into functional neuron-like cells in vitro.

Introduction

Embryonic stem cells can proliferate exponentially in culture for long periods while maintaining their pluripotency, thus producing a sufficient amount of cells for possible therapeutic application. However, the same properties of embryonic stem cells that allow such expansion together with their well-known genomic instability may promote tumor formation in cell transplant recipients. In consequence, the alternative sources of somatic stem cells (SSC) for use in tissue engineering and cell replacement are extensively investigated. Except for the last research data [1], SSC were shown to have limited life span even in the presence of mitogenic growth factors, and are thought to be not pluripotent but multipotent. However, SSC were shown in vitro and in vivo to give rise to cells belonging to nonrelated tissues, and are thus of great therapeutic interest [2–5]. The problem, still not resolved in the case of SSC, is their limited proliferation capacity. This makes it impossible to grow SSC in large amounts in the laboratory as established and defined lines.

KARGER
Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1660–2854/06/0032–0019$23.50/0

Accessible online at:
www.karger.com/ndd

Leonora Bużańska
NeuroRepair Department, Medical Research Institute, Polish Academy of Sciences
5 Pawińskiego Street
02-106 Warsaw (Poland)
Tel. +48 22 608 6510, Fax +48 22 668 5532, E-Mail buzanska@cmdik.pan.pl

As reported recently, stem/progenitor cells derived from the human umbilical cord blood (HUCB) or human bone marrow, although hematopoietic in nature, under certain conditions can change their natural fate and display neural features [3–8]. Human cord blood, due to its primitive developmental position, was assumed to contain a subpopulation of stem cells which could retain enhanced, embryonic-like capacity to proliferate. Indeed, the expanding population of neural stem/progenitor cells was selected from human cord blood nonhematopoietic (CD34-negative) mononuclear fraction [5], and due to repeated expansion and selection of these cells the first clonogenic, nonimmortalized HUCB neural stem-like cell (HUCB-NSC) line was established [9, 10].

We have shown that HUCB-NSC could attain neuronal, astrocytic and oligodendroglial features and conceivably be differentiated into functional neuron-like cells.

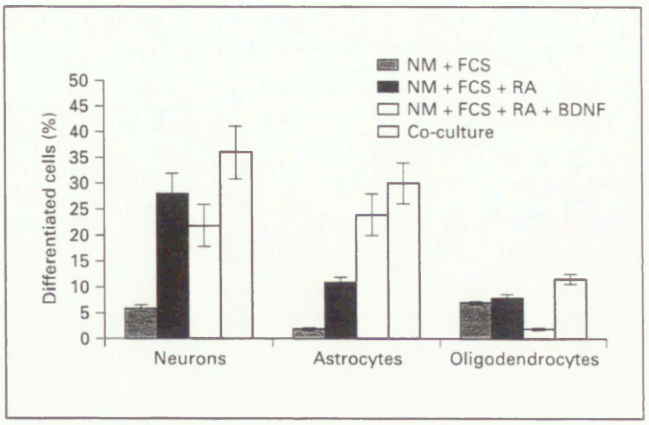

Fig. 1. Expression of neural markers: β-tubulin III (for neurons), GFAP (for astrocytes) and GalC (for oligodendrocytes) for the population of human cord blood-derived CD 34– selected fraction of mononuclears cultured and differentiated as previously described [5]. Briefly, for differentiation cells were cultured for 4 days at: neurobasal medium (NM) supplemented with 10% FCS (dark grey bars); NM with 10% FCS plus 0.5 m*M* of RA (black bars), NM with 10% FCS, 0.5 m*M* RA and 10 ng/ml BDNF (white bars); co-culture with rat primary cortical cells (light grey bars). The results are expressed as the mean ± SD of cell number from nine independent cultures and three parallel experiments [5].

HUCB-Derived SC Can Be Differentiated into Neuronal, Astrocytic and Oligodendroglia-Like Cells

In a previous study, using the method of HUCB cell subfractionation, immunomagnetic removal of CD 34+ hematopoietic SC and subsequent culturing in the presence of culture media and growth factors, a self-renewing, clonogenic cell population with neural-type precursor characteristics was generated [5]. The Nestin-expressing clones shown in this study could differentiate into neuronal, astrocytic and oligodendroglial phenotypes, thus revealing multipotential character of isolated precursors. The HUCB-derived cells showed a relatively high commitment to neuronal and astrocytic fate, at the level similar to that previously observed with fetus-derived neural stem cells [11, 12]. In the presence of 10% FBS and 0.5 μ*M* retinoic acid (RA), the cell culture differentiated at 28 ± 4, 11 ± 1 and 8 ± 0.5% into cells expressing neuronal, astrocytic and oligodendroglial markers, respectively. Addition of 10 n*M* brain-derived neurotrophic factor (BDNF) to the medium lowered the amount of neuronal and oligodendroglial cells while stimulating differentiation into astrocyte-like cells. For the co-culture experimental system, HUCB-NSC were prelabeled with fluorescent dye and traced as an inset in the culture of rat cortical cells. Such conditions appeared to be the best for promotion of neural commitment and differentiation (36 ± 5, 30 ± 4 and 11.5 ± 1% for neuronal, astrocytic and oligodendroglia-like cells, respectively) as compared to other systems tested in this study [5]. Quantification of the extent of neuronal, astrocytic and oligodendrocytic differentiation in cultures grown for 4 days in differentiation-promoting conditions is shown in figure 1. The above data suggest that the co-culture system could be an alternative to in vivo injection studies in providing optimal trophic support for neural progenitor cell differentiation.

Recently, we evaluated the morphogenic effect of different combinations of several growth factors and neuromorphogens, such as PDGF-AA, PDGF-BB, CNTF, GDNF, T3 and dBcAMP, applied to low-serum (LS; 2% serum, no mitogens) culture medium for 2 weeks. In these experiments, we have established optimal in vitro conditions to direct differentiation of HUCB-NSC into either one of three neural lineages. In appropriate media for each of these lineages we are able to direct differentiation into cells positive for neuronal markers up to 80%, for astrocytic markers up to 65% or for oligodendrocytic markers up to 12% [unpubl. data]. Examples of differentiation into neuronal, astrocytic or oligodendroglial phenotypes are presented in figure 2.

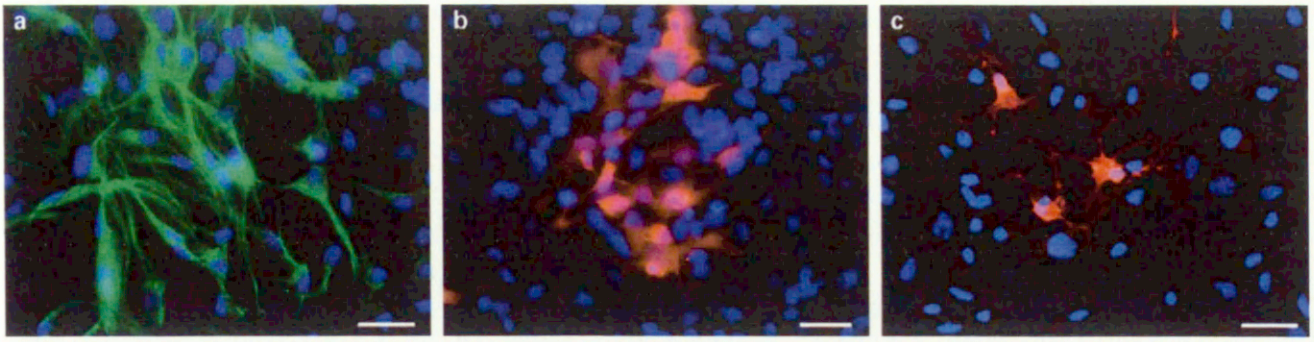

Fig. 2. Immunocytochemical images of HUCB-NSC cultured for 2 weeks in the presence of neuromorphogens containing differentiating media and expressing β-tubulin III (**a**), S-100β (**b**) and GalC (**c**). Scale bars: 50 μm.

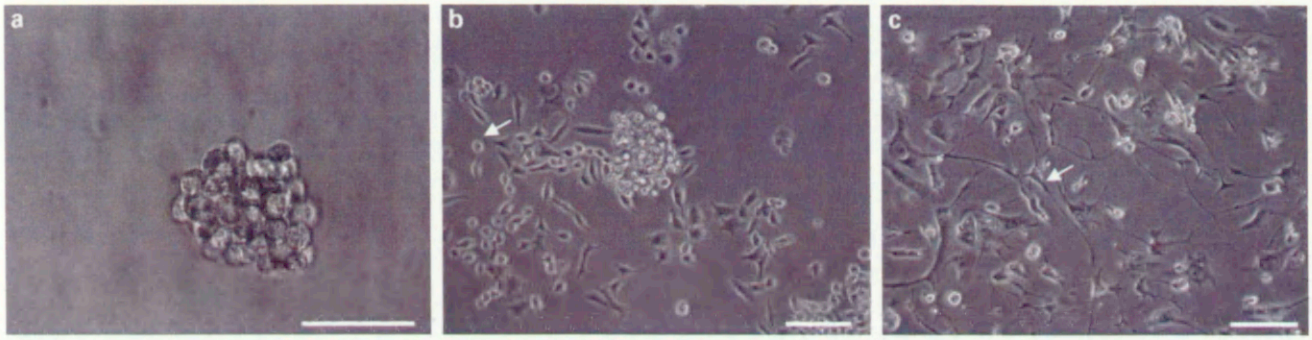

Fig. 3. Phase contrast images of HUCB-NSC cultured (12) as: nonadherent aggregates/spheres of nondifferentiated cells in serum-free DMEM/F12, 10 ng/ml LIF, 10 ng/ml EGF, 10 ng/ml bFGF (**a**), heterogeneous culture of nonadherent, rounded, nondifferentiated cells (arrow) and adherent, committed neural progenitors in DMEM/F12, 2% FBS supplemented with insulin, transferrin/selenium solution (**b**), differentiating for 2 weeks in the presence of dBcAMP cells that attain neuronal phenotype (**c**; arrow). Scale bars: 100 μm.

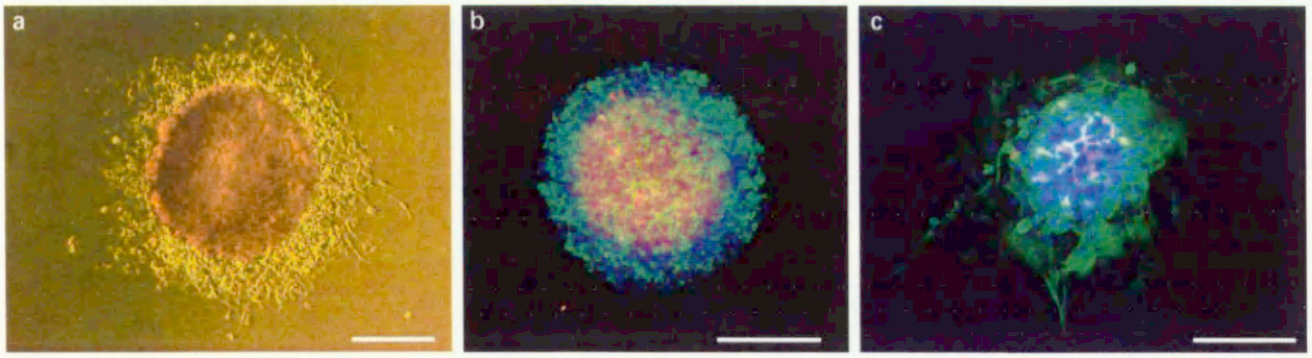

Fig. 4. a Neurospheres derived from HUCB attach to the bottom of culture dish in the presence of serum. **a**, **c** Cells from the surface region of the attached neurosphere spread out and differentiate mainly toward neurons positive for β-tubulin III (**c**; green); however, some glial cells positive for GFAP are present as well (**c**; red). Neurospheres derived from HUCB before adhesion are cultured and propagated as floating cell clusters in SF medium supplemented with EGF. **b** Undifferentiated floating neurospheres comprise at least two regions of neural stem/progenitors: inner cells positive for Nestin (red) and outer cells positive for GFAP (green). **b**, **c** Cell nuclei are stained with Hoechst (blue). Scale bar: 100 μm.

HUCB-NSC Can Be Maintained in Culture at Different Developmental Stages

Freshly isolated mononuclear fraction of HUCB was shown to be a source of neural progenitors differentiating toward the cells expressing neural marker genes and proteins but hardly expand and survive for longer than 3 months [4, 5]. However, from the same neural progenitors we succeeded to establish clonogenic HUCB neural stem cell (HUCB-NSC) line by prolonged exposure to serum and mitogens and further selection of nonattached progeny [9, 10]. These cells have been expanded as undifferentiated, proliferating cell line in continuous culture for more than 3 years. The cells retain normal chromosomal pattern and unchanged capacity to proliferate and self-renew with clone formation [9]. The conditions have been worked out to maintain HUCB-NSC in culture at different developmental stages: (1) as nondifferentiated, nonattached 3D culture of cells forming floating spheres/aggregates (fig. 3a), (2) proliferating at a high rate culture of mixed, floating, nondifferentiated and adherent, already committed neural progenitor cells in LS (2%) condition (fig. 3b) and (3) cells predifferentiated into neuronal, astrocytic and oligodendroglial lineages in high-serum medium or LS medium supplemented with neuromorphogens (fig. 3c) [9, 10].

Thus, we can monitor NSC behavior at different developmental stages. HUCB-NSC can form the neurosphere-like structures in vitro, reassembling NSC residing in the tissue-specific niche [13]. Undifferentiated HUCB-NSC may remain 'dormant' in these structures for months, then after stimulation with mitogens give rise to the cohorts of proliferating intermediate precursors. Undifferentiated, nonadherent spheres contain cells expressing Nestin and GFAP but not β-tubulin III or other markers of advanced neuronal (MAP2) or astroglial (S100β) differentiation. The same is true for the undifferentiated single floating cells. Upon attachment and in the presence of serum, such spheres/aggregates spread out, and cells located at the edges began to migrate away from the center of the sphere (fig. 4a). Immunostaining of such spheres after plating revealed the presence of proteins characteristic for differentiating neurons (β-tubulin III – red) and astrocytes (GFAP – green) on the surface of the sphere, while the core retained immunonegative cells with the blue, Hoechst-stained nuclei (fig. 4c). These results are consistent with the widely investigated pattern of differentiation in neurospheres derived from the human CNS [14].

In LS, mitogen-free medium, HUCB-NSC proliferate continuously as self-renewing, undifferentiated floating cells loosely attached to a more differentiated monolayer. Apart from undifferentiated morphology, the 'floaters' express specific genes (revealed by microarray analysis) implicated in Wnt, Notch and Lif/Jak/Stat signaling pathways important for the maintenance of self-renewal capacity of stem cells [9]. The adherent cell monolayer contains cells expressing early markers of neuronal differentiation, including NF-200, and β-tubulin III, but not advanced neuronal marker proteins. Among these neurally committed cells, there are also some cells of ameboidal or egg-shaped morphology and still poorly defined phenotypes with some similarity to CB-derived stromal-like feeder layer described for proliferating hematopoietic progenitors [15]. In nondifferentiated population of HUCB-NSC, as revealed by RT-PCR, germ layer marker genes for the mesoderm (T gene) and endoderm (FoxaA1) are not present, indicating neural commitment; however, mRNA for Oct4 – pluripotent stem cell marker – is expressed [16]. This expression is lost after stimulation of HUCB-NSC with neuromorphogens.

Neurogenic Potential of HUCB-NSC

Another approach was to evaluate the neurogenic potential of HUCB-NSC cultured in different in vitro settings: serum-free (SF; fig. 3a) or LS (fig. 3b) conditions [16]. The proportion of proliferating cells, as shown by Ki67 immunocytochemistry, was similar for both cultures. Semiquantitative RT-PCR analyses for candidate genes specific to neural markers, i.e. Nestin, GFAP, NF-200 and MAP-2 revealed similar expression of GFAP and NF-200 in HUCB-NSC cultured in SF and LS media and a slight decrease in Nestin transcript accompanied by increase in MAP2 for cells growing in LS medium. This result clearly indicates that nondifferentiated HUCB-NSC is already neurally committed. However, the differences between these two cultures were much more evident on the level of protein expression. This was demonstrated by both: significantly lower expression of Nestin, immunopositive cell number (44.6 ± 2.11% for LS and 96.5 ± 1.5% for SF), and appearance of type III β-tubulin, a neuronal marker not expressed in SF cultures, in LS culture. GFAP was expressed on a similar level (about 40%) in both SF and LS cultures. In undifferentiated HUCB-NSC and to a lower extent in differentiated cultures, concomitantly to expression of Nestin, GFAP was detected in the same cells [16]. This is consistent with the

common view that GFAP can be a marker of NSC in neurogenic zones, based on the observation of Doetsch [17] and Wurmser et al. [18] that NSC present in the neurogenic zones of the CNS express GFAP.

GFAP was also occasionally coexpressed with β-tubulin III in the attached fraction of HUCB-NSC grown in LS medium, suggesting still early stage of neural commitment of nondifferentiated culture grown in LS medium. Such coexpression of GFAP and β-tubulin III was observed before (but only in 2% of the population) in control cells growing in 10% FBS [5]. This observation seems to match a finding that newborn neurons appearing in the subventricular zone [19], as well as those differentiating from fetal stem cells in vitro [20], coexpress GFAP and probably originate from a certain type of common, neuroastroglia progenitor.

The potential to differentiate HUCB-NSC growing either in LS or in SF medium was further evaluated in three different in vitro experimental settings: (1) in the presence of RA and BDNF; (2) in co-culture with neonatal rat astrocytes, and (3) after seeding on the surface of rat organotypic hippocampal slices.

The differentiation effect of neuromorphogens or co-culture conditions was significantly higher in HUCB-NSC cultures grown in LS medium than SF cultures. As indicated by expression of advanced neuronal marker – MAP-2, incubation with RA+BDNF stimulated expression of MAP-2 only in cells growing in LS medium, while co-culture with neonatal astrocytes in both, but significantly higher in LS (63.8 ± 8.5 and 12.8 ± 3.2% for LS and SF cultures, respectively). When HUCB-NSC were co-cultured with hippocampal slices, SF or LS conditions differed in their morphological features – LS cells developed advanced neuron-like morphology and elaborated extensive cell-cell contacts with hippocampal tissue, while SF-derived cells were less differentiated.

In conclusion, co-culture conditions reassembling in vivo niche for NSC are the best to direct HUCB-NSC into neuronal phenotype. Certain level of stem/progenitor cell commitment is important for the optimal HUCB-NSC response to the signals provided by the surrounding environment in vitro.

Advanced Neuronal Differentiation of HUCB-NSC

To prove advanced neuronal differentiation, besides morphological characteristics of neurons one should look for physiological commitment that would allow cord blood-derived cells to function within a neural network and respond to neurotransmitters released from neighboring neurons.

To induce advanced neuronal differentiation, HUCB-NSC were incubated for 2–4 weeks in the presence of dBcAMP. The population of cells directed into neuronal pathway was obtained (up to 80% β-tubulin III) and referred as HUCB-NSCD. Directed HUCB-NSCD did not show expression of Nestin and relatively small amount of astrocytic, GFAP-positive cells (up to 19%) [21]. For both morphological and functional characterization of undifferentiated HUCB-NSC and directed HUCB-NSCD, the whole-cell patch-clamp technique was applied together with microarray and immunocytochemical analyses [21]. The cells of either typical undifferentiated (fig. 3b) or differentiated neuronal morphology (fig. 3c) were taken for recordings. Two types of voltage-sensitive currents were recorded: inward rectifier current (Kir) and outward potassium channel current (I_{K+}). The treatment with specific Kir antagonists like Cs^+ (5 m*M*), Ba^{2+} (5 m*M*) or Cd^{2+} (0.1 m*M*) completely but reversibly eliminated Kir. In contrast, I_{K+} antagonists TEA (15 m*M*) or 4-AP (1 m*M*) had no influence on Kir but blocked I_{K+} (fig. 5) [21] thus verifying specificity of each evaluated channel.

There is growing evidence that potassium channels play an important role in early embryonic development [22] and later stages of neuronal differentiation [23]. Kir, which is always expressed in neurons and astrocytes, plays an important role in regulating membrane potential and controlling membrane excitability [24]. Previously, Kir was identified in 30% of acutely isolated HUCB cells [25]. We find Kir current almost in 100% of our cells, what may further indicate their strong neural commitment. Moreover, expression of the Kir gene was detected in whole populations of HUCB-NSC and HUCB-NSCD.

I_{K+} – voltage-sensitive potassium current – was not detected in undifferentiated HUCB-NSC, but appeared in HUCB-NSCD after 5 days of differentiation and then increased gradually through incubation time. I_{K+} current, present in different types of excitable cells, including neurons, is important for polarization of depolarized cells and bringing the membrane back to resting potential [26].

Different neurotransmitters acting through their cognate receptors are involved in the regulation of cell proliferation and promotion of neuronal differentiation and migration in the developing nervous system [27]. To determine whether the neurotransmitter receptors identified in HUCB-NSC and HUCB-NSCD by gene arrays

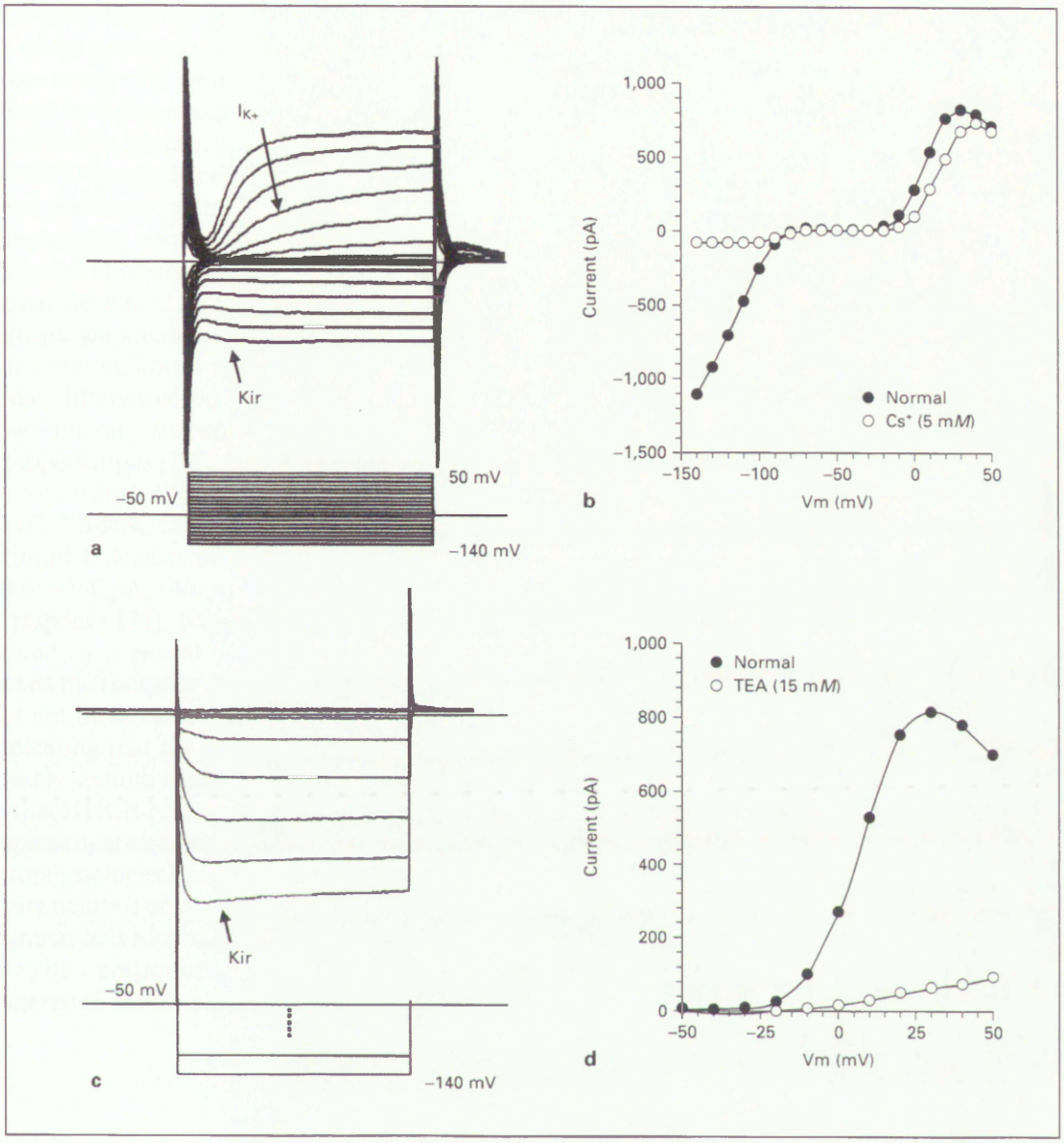

Fig. 5. Outward potassium current (I_{K+}) and Kir current present in HUCB-NSCD. **a** Kir and I_{K+} were induced in voltage clamp (holding potential −50 mV, voltage steps from −140 to +50 mV, 10-mV steps). **b** I/V curve of I_{K+} and Kir currents. Kir was blocked by Cs^+, but I_{K+} was largely unaffected. **c** Kir was induced in voltage clamp (holding potential −50 mV, voltage steps from −70 to −140 mV, 10-mV steps). **d** I_{K+} was activated around −25 mV (●) and was reversibly blocked by TEA (15 m*M*).

and immunocytochemistry were functional, whole-cell patch-clamp recordings were performed while applying receptor agonists and antagonists. To test for the presence of functional GABA, acetylcholine, glycine, and serotonin receptors, HUCB-NSCD agonists were applied to the cells: (1) maintained at a holding potential of −50 mV, to avoid activating Kir and (2) stepping the voltage from a holding potential of −50 to −140 mV, to activate Kir. Under this condition, applied agonists failed to induce a current, but greatly reduced Kir. This effect was inhibited by agonist-specific inhibitors, confirming further functionality of tested ligand-gated channels.

Kainate (KA) (0.5 m*M*) induced an inward current from HUBC-NSCD, which was totally blocked by CNQX, a non-NMDA receptor antagonist. Moreover, KA partly blocked the Kir induced by voltage step. Thus, functional, KA-sensitive, non-NMDA glutamate receptors were revealed on HUCB-NSCD by whole-cell patch-clamp recordings, and this was consistent with both: microarray analysis and immunolabeling results. NMDA-induced currents were not detected in whole-cell recordings, consistent with our microarray data [21]. These data suggest that functional ionotropic glutamate receptors play a crucial role not only in rapid neurotransmission, but also during early neuronal differentiation of HUCB-NSC, which was also shown for rat neuroepithelial cells [28] and NSC from the hippocampus [29].

To prove terminal neuronal differentiation, it is important to show that HUCB-NSC can fire action potential under appropriate stimuli. Indeed in some HUCB-NSCD, current pulses (–600 to +250 pA, 140 ms) induced an 'action potential-like' response [21], consisting of a rapid depolarization followed by a partial hyperpolarization, similar to that reported by Tourneur [30]. However, the depolarization could not be blocked by TTX, a sodium channel blocker, indicating that the real mechanism of this response is probably sodium channel independent.

We can conclude that HUCB-NSC in vitro, in the presence of dBcAMP, express neurotransmitter receptors, ion channels, and electrophysiological properties similar to those seen in immature neurons or glia; still, terminal differentiation into neuronal cells according to the definition that the neuron has to be a postmitotic polarized cell capable of firing voltage-gated action potential [31] has to be proven.

Perspectives

It is shown that neural stem-like cells derived from the nonhematopoietic fraction of HUCB and expanded as a stable, clonogenic line over a long time, retain their capacity to differentiate into neuron-like cells that express specific cytoskeletal markers, neurotransmitter receptors, and electrophysiological characteristics of immature neurons.

However, the absence of voltage-gated sodium channels clearly indicates that the process of differentiation of HUCB-NSC into neurons in the presence of a neuromorphogen (dBcAMP) is still incomplete and that other factors or conditions are required. The approach of co-culture with astrocytes or organotypic brain slices to induce more advanced differentiation of HUCB-NSC toward mature neurons has already been undertaken by our group. Now further physiological analysis is required to trace the formation of functional connections of HUCB-NSC with the host network and responses to the environmental signals. The alternative, also already undertaken by our group, is to transfect HUCB-NSC with neurogenic transcription factors. Such molecular modification together with the neuromorphogen treatment may result in further differentiation of HUCB-NSC into mature neurons of different specification.

Acknowledgments

We thank Dr. R. Salvi and Wei Sun for help in preparing figure 5. The work was supported by the State Committee for Scientific Research Grants (K 045/P05/2002 and 28/E-32/SPUB/JRC/P-05/DIE 62/2005).

References

▶1 Conti L, Pollard SM, Gorba T, Reitano E, Toselli M, Biella G, Sun Y, Sanzone S, Ying Q, Cattano E, Smith A: Niche-independent symmetrical self-renewal of mammalian tissue stem cell. PLOS Biol 2005;3:1–12.

▶2 Toma JG, Akhavan M, Fernandes KJ, Barnabe-Heider F, Sadikot A, Kaplan DR, Miller FD: Isolation of multipotent adult stem cells from the dermis of mammalian skin. Nat Cell Biol 2001;3:778–784.

▶3 Woodbury D, Schwarz EJ, Prockop DJ, Black IB: Adult rat and human bone marrow stromal cells differentiate into neurons. J Neurosci Res 2000;61:364–370.

▶4 Sanchez-Ramos JR, Song S, Kamath SG, Zigova T, Willing A, Cardozo-Pelaez F, Stedeford T, Chopp M, Sanberg PR: Expression of neural markers in human umbilical cord blood. Exp Neurol 2001;171:109–115.

▶5 Bużańska L, Machaj ED, Zabłocka B, Pojda Z, Domańska-Janik K: Human cord blood-derived cells attain neuronal and glial features in vitro. J Cell Sci 2002;115:2131–2138.

▶6 Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du J, Aldrich S, Lisberg A, Low WC, Largaespada DA, Verfaillie CM: Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 2002;418:41–49.

▶7 Zigova T, Song S, Willing AE, Hudson JE, Newman MB, Saporta S, Sanchez-Ramos J, Sanberg PR: Human umbilical blood cells express neural antigens after transplantation into the developing rat brain. Cell Transpl 2002;11:265–274.

▶8 Mezey E, Chandross KJ, Harta G, Maki RA, McKercher SR: Turning blood into brain: cells bearing neuronal antigens generated in vivo from bone marrow. Science 2000;290:1779–1782.

9 Bużańska L, Stachowiak E, Stachowiak MK, Domańska-Janik K: Neural stem cell line derived from nonhematopoietic population of human umbilical cord blood. Stem Cells Dev, in press.

▶10 Bużańska L, Habich A, Jurga M, Sypecka J, Domańska-Janik K: Human cord blood – derived neural stem cell line – possible implementation in studying neurotoxicity. Toxicol In Vitro 2005;19:991–999.

▶11 Carpenter MK, Cui X, Hu Z, Jackson J, Sherman S, Seiger A, Wahlberg LU: In vitro expansion of a multipotent population of human neural progenitor cells. Exp Neurol 1999;58: 265–278.

▶12 Svendsen CN, Caldwell MA, Ostenfeld T: Human neural stem cells: isolation, expansion and transplantation. Brain Pathol 1999;9:499–513.

13 Jurga M, Domańska-Janik K: Neurospheres of human umbilical cord blood derived neural stem cells – in vitro model of stem cell niche. FENS Forum Abstracts 2 2004;A040.16.

▶14 Svendsen CN, Caldwell MA: Neural stem cells in the developing central nervous system: implication for cell therapy through transplantation. Prog Brain Res 2000;127:13–34.

▶15 Ye ZQ, Burkholder JK, Qiu P, et al: Establishment of an adherent cell feeder layer from human umbilical cord blood for support of long-term hematopoietic progenitor cell growth. Proc Natl Acad Sci USA 1994;91:12140–12144.

▶16 Jurga M, Markiewicz I, Sarnowska A, Bużańska L, Łukomska B, Domańska-Janik K: Preconditioning of human neural stem cells in low serum medium increases their differentiation into neurons. J Neurosci Res 2006;83:627–631.

▶17 Doetsch F: A niche for adult neural stem cells. Cur Opin Genet Dev 2003;13:543–550.

▶18 Wurmser AE, Palmer TD, Gage FH: Cellular interactions in the stem cell niche. Science 2004;304:1253–1254.

19 Barres BA: A new role for glia: generation of neurons! Cell 1999;97:667–670.

▶20 Rosser AE, Tyers P, TerBorg M, Dunnett SB, Svendsen CN: Co-expression of MAP-2 and GFAP in cells developing from rat EGF responsive precursor cells. Brain Res Dev Brain Res 1997;98:291–295.

▶21 Sun W, Bużańska L, Salvi R, et al: Voltage-sensitive and ligand-gated channels in differentiating neural stem-like cells isolated from the non-hematopoietic fraction of human umbilical cord blood. Stem Cells 2005;23:931–945.

▶22 Day ML, Pickering SJ, Johnson MH, Cook DI: Cell-cycle control of a large-conductance K+ channel in mouse early embryos. Nature 1993; 365:560–562.

▶23 Neusch C, Weishaupt JH, Bahr M: Kir channels in the CNS: emerging new roles and implications for neurological diseases. Cell Tissue Res 2003;311:131–138.

24 Hille B: Ionic Channels of Excitable Membrane. Sunderland, Sinauer, 1992, p 607.

▶25 Shirihai O, Attali B, Dagan D, Merchav S: Expression of two inward rectifier potassium channels is essential for differentiation of primitive human hematopoietic progenitor cells. J Cell Physiol 1998;177:197–205.

▶26 Rudy B: Diversity and ubiquity of K channels. Neuroscience 1988;25:729–749.

▶27 Nguyen L, Rigo JM, Rocher V, et al: Neurotransmitters as early signals for central nervous system development. Cell Tissue Res 2001;305:187–202.

▶28 Maric D, Liu QY, Grant GM, et al: Functional ionotropic glutamate receptors emerge during terminal cell division and early neuronal differentiation of rat neuroepithelial cells. J Neurosci Res 2000;61:652–662.

▶29 Song HJ, Stevens CF, Gage FH: Neural stem cells from adult hippocampus develop essential properties of functional CNS neurons. Nat Neurosci 2002;5:438–445.

▶30 Tourneur Y: Action potential-like responses due to the inward rectifying potassium channel. J Membr Biol 1986;90:115–122.

▶31 Reh TA: Neural stem cells: form and function. Nat Neurosci 2002;5:392–394.