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Brain Research Bulletin 68 (2005) 62–75

BRAIN RESEARCH BULLETIN

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Neural differentiation of mouse embryonic stem cells in chemically defined medium☆

Isabelle A. Bouhon[a], Hidemasa Kato[a], Sidharthan Chandran[b], Nicholas D. Allen[a,c,*]

[a] *Neurobiology Programme, The Babraham Institute, Babraham, Cambridge CB2 4AT, UK*
[b] *Department of Neurology, University of Cambridge, Cambridge CB2 2PY, UK*
[c] *School of Biosciences, Cardiff University, Museum Avenue, PO Box 911, Cardiff CF10 3US, UK*

Available online 29 September 2005

Abstract

Directed differentiation of embryonic stem (ES) cells has enormous potential to derive a wide variety of defined cell populations of therapeutic value. To achieve this, it is necessary to use protocols that promote cell differentiation under defined culture conditions. Furthermore, understanding the mechanisms of cell differentiation in vitro will allow the development of rationale approaches to systematically manipulate cell fates. Here we have analysed the differentiation of mouse ES cells to the neural lineage under serum and feeder cell-free conditions, using a previously described chemically defined medium (CDM). In CDM, ES cell differentiation is highly neurogenic. Cell differentiation was monitored by analysis of a gene expression array (Clontech–Atlas) and by semi-quantitative RT-PCR for a panel of genes involved in cell lineage specification and patterning of the epiblast. In addition to expression of neural markers, data identified a transient expression of several genes associated with the organising activities of the embryonic node and visceral endoderm, including regulators of WNT, BMP, Hedgehog and FGF signaling pathways. Neural differentiation in CDM does not occur by a simple default mechanism, but was dependent on endogenous FGF signaling, and could be blocked by adding BMP4, and LiCl to simulate WNT activation. Neural differentiation was also inhibited by antagonising endogenous hedgehog activity. Taken together the profile of gene expression changes seen in CDM cultures recapitulates those seen in the early embryo, and is suggestive of common developmental mechanisms.

Keywords: Mouse embryonic stem cell; Neurogenesis; Chemically defined medium; Gene expression; Development

1. Background

Neural stem cells (NSC) have considerable therapeutic potential for the treatment of neurological disorders. Currently, clinical studies have depended on the collection of human fetal tissue. However, practical, as well as ethical, constraints severely limit the number of patients who could benefit from therapies using primary donor tissue, and this emphasises the imperative that alternative sources of cells are identified [7]. Alternative cell sources under active consideration include expanded fetal or adult neural stem cell populations, and neural derivatives of in vitro differentiated embryonic stem (ES) cells. Despite extensive effort, progress using cultured fetal or adult neural stem cells has proved disappointing. Problems largely reflect the limited developmental plasticity of in vitro expanded neural stem cell populations. Expanded stem cell cultures show three overriding problems: firstly cultures lose the differentiation potential of primary precursor cells [56], secondly cells respond poorly to strategies designed to direct differentiation towards specific (and diverse) neural phenotypes, and thirdly there is a significant loss of neurogenicity in expanded cultures with the majority of progenitor cells differentiating to form glia [8,21,46,60]. As an alternative source of donor tissue neural precursors have been derived in vitro from cultured embryonic stem cells (ES-NPC). Unlike adult and fetal NSCs ES-NPCs are developmentally naïve raising the possibility that neural cell fates of ES-NPCs

☆ We dedicate this manuscript to Isabelle Bouhon who died tragically in an accident on 6th October 2005. Isa will be remembered as a loyal caring friend with wonderful spirit and a dedicated scientist. Isa's contribution of presenting the first data on the neural differentiation of ES cells under defined, serum-free, conditions, at the international Federation of European Neuroscience Societies and Society for Neuroscience meetings in 2000 and 2001 will have lasting impact. Some of that data is presented here.

* Corresponding author.
E-mail addresses: iab24@cam.ac.uk (I.A. Bouhon), katoh@tubero.tohoku.ac.jp (H. Kato), sc222@cam.ac.uk (S. Chandran), allennd@cf.ac.uk (N.D. Allen).

0361-9230/$ – see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.brainresbull.2005.08.022

may be manipulated more systematically than more mature, pre-programmed, NSC populations [25,39,67].

Systematic manipulation of cell cultures to direct differentiation to alternate neural fates requires the development of protocols that maximise control over the extrinsic signaling environment. Ideally this would involve the manipulation of cultures with high degrees of cell homogeneity, and in which all extrinsic and intrinsic signal are known. Strategies most commonly used to derive neural progenitor cells from ES cells are initiated by spontaneous differentiation that occurs following embryoid body (EB) formation, often with the addition of non-physiological neural inducing agents such as retinoic acid (RA). EBs are cell aggregates that form in non-adherent culture dishes in the presence of serum and following LIF withdrawal. EBs differentiate and grow with outer layers of visceral and parietal endoderm that surround a core of ectoderm, mesoderm and endoderm cell derivatives. Although ordered signaling from the visceral endoderm to the core has been demonstrated in EBs [36], differentiation of the ectoderm, mesoderm and endoderm lineages is not known to be organised, and the origin and fate of neuroectoderm in this system is also not clear [66]. Subsequent to EB formation ES-NPCs can be enriched from the larger population by culture in serum-free media designed for the selective survival and proliferation of neural precursor cells [6,43], or by lineage selection using selectable transgene markers expressed from neural lineage restricted promoters [30]. Given the complexity of cell populations within EBs, and the presence of serum, it is very difficult to evaluate and control the range of factors that may influence differentiation of ES cells to a neural fate. This issue can be addressed by differentiating cells from the outset in serum-free medium. Efficient neural differentiation of ES cells has been reported by co-culture with the stromal cell line PA6 in serum-free medium, however the mechanisms of PA6 induced neurogenesis are not known [24]. Serum-free culture has also been used to demonstrate neural differentiation of single ES cells, but at very low efficiencies (∼0.2% of plated cells) [62].

In this study we have analysed the progress of neural differentiation of ES cells plated in a previously described chemically defined medium (CDM) that is devoid of serum, feeder cells, or neural inducers such as retinoic acid [22]. Differentiation of mouse ES cells in CDM, has previously been shown to be refractory to mesoderm induction. Instead, ES cells differentiated to express the neural marker Pax6 and failed to express the mesodermal marker brachyury, however brachyury together with the mesoderm markers goosecoid and nodal could be induced by the addition of the mesoderm inducing factors activin or BMP4 [22]. In a separate study mesoderm induction by addition of BMP4 to ES cells in CDM was shown to regulate Msx and Id gene expression [19]. We show that ES cells plated in CDM alone generate highly neurogenic cell aggregates, or 'neurogenic embryoid bodies (NEBs)' that are distinct in structure to serum-derived EBs. We have used gene profiling to study the ontogeny of cell differentiation and to gain insight into developmental pathways that may be engaged during the differentiation process. Based on gene expression data and cell heterogeneity that exists in the cultures we propose that neuroectoderm differentiation in NEBs occurs in the context of cell interactions and a signaling environment that is present during early embryo development.

2. Results

2.1. ES cell differentiation in chemically defined serum-free medium

Classic studies in *Xenopus* established that, in the absence of mesoderm, neuroectoderm will develop from ectoderm through a default differentiation pathway [17]. Using this premise of default neural differentiation we cultured ES cells in a previously described chemically defined medium (CDM), in which mesoderm development is prevented [22]. ES cells were washed free of serum and plated directly in CDM at low density (1×10^5 ml^{-1}). After overnight culture, small cell aggregates formed as small free-floating spheres or NEBs, which compacted and grew over the next 4 to 8 days. NEBs appeared distinct from serum-derived EBs in that they did not develop overt outer layers of parietal and visceral endoderm, neither did they undergo the regulated processes of cavitation to form cystic EBs (Fig. 1) [36]. NEB formation also occurred when cells were plated at 5×10^4 cells/ml, however at lower cell densities NEB formation was markedly reduced, and none were seen when cells were plated at limiting dilutions.

To assess their neurogenic potential, plated NEBs were analysed by immunocytochemistry for expression of nestin, sox1 and RC2 to identify neural precursor cells, β-tubulin III, glial fibrillary protein (GFAP) and NG2 to identify neurons, glia and oligodendrocytes, respectively (Fig. 1). Extensive expression of nestin, sox1 and RC2 was seen between day 4 (D4) and D8, with a few cells expressing MAP2 or β-tubulinIII. Following ES-NPC differentiation the majority of cells adopted neuronal phenotypes with elaborate neurites that stained for β-tubulinIII (Fig. 1) or MAP2 (not shown). No GFAP or NG2 expression was seen in D4 or D8 cultures, however both markers could be detected in a minority of cells upon prolonged culture. Importantly we failed to detect cells immunoreactive for markers of non-neural lineages such as the muscle marker desmin, or pan-cytokeratin, that identifies epidermal derivatives. The development of cells expressing markers for neurons, glia and oligodendrocytes suggests the ES cell derived neural progenitor pool, in serum-free conditions, to be multipotent but with a very strong bias towards the generation of neurons.

2.2. Exogenous FGF2 maintains ES derived neural progenitor cell cultures

To further investigate the development and heterogeneity of the neural precursor population within CDM NEBs, flow cytometry was used to determine the number and character of nestin positive cells within the population (Fig. 2). FACS plots displaying forward scatter (FSC), a measure of cell size, against side scatter (SSC), a measure of cell granulosity, were used to define a region R1 for detailed analysis, that excluded populations comprising cell debris or cell clumps. Plots of FSC against

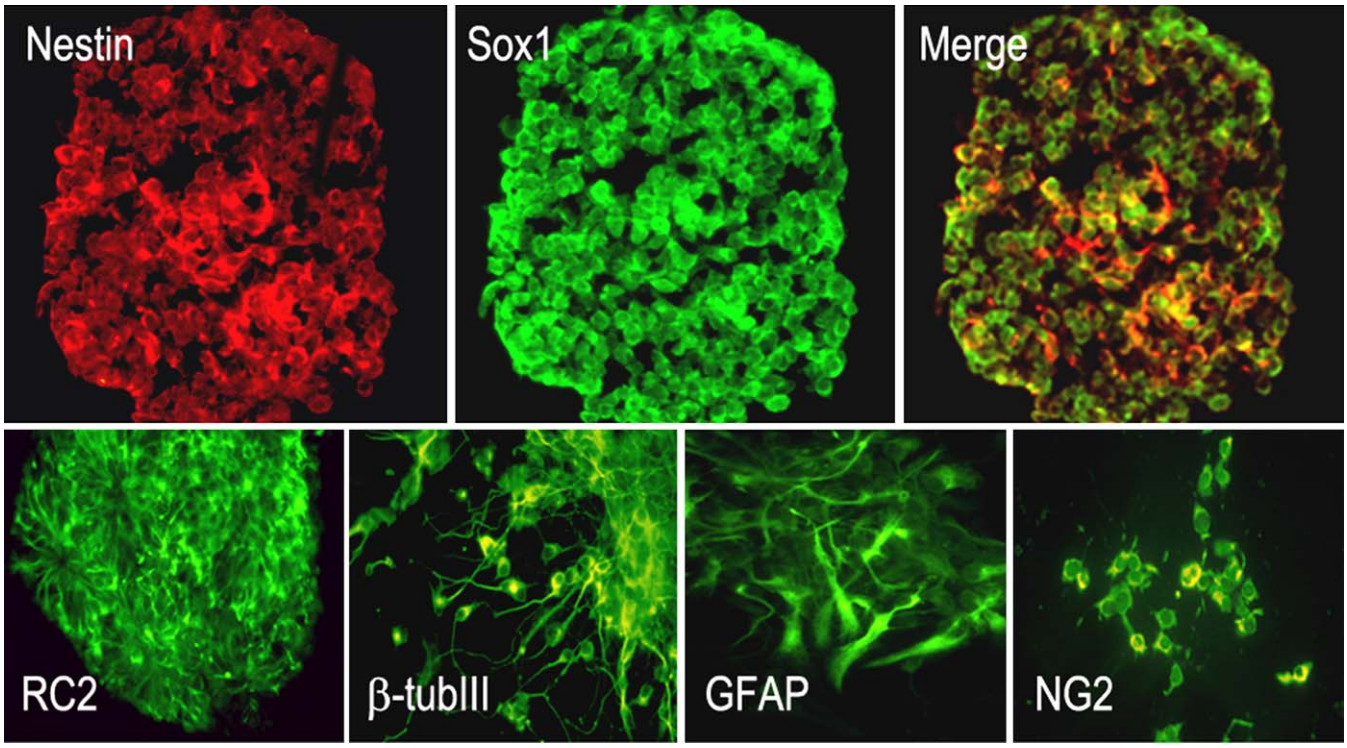

Fig. 1. Neural differentiation of ES cells in CDM serum-free medium. Nestin and Sox1 immunostaining, together with a merged image, on cryosections of D8 NEBs. RC2 staining is shown for a sphere plated in CDM at D4 on a laminin coated coverslip. NEBs plated at D8 and allowed to differentiate for a further 4 days showed elaborate neurite outgrowth and stained for β-tubulin III. Rare cell clusters immunoreactive to NG2 and GFAP could also be detected but only after more extended periods of culture (>14 days).

FL1 (fluorescence intensity) revealed two distinct cell subpopulations, R2 and R3, within R1, seen by the presence of two small peaks in nestin immunofluorescence relative to cell size. Interestingly, with development to day 8, the population delimited by R3 became more prominent, relative to R2. The distribution of cells with different granulosities seen in the SSC/FL1 dot plots showed that the R2 population had a higher granulosity and also remained fairly homogeneous with time. In contrast, the distribution of the R3 population showed it to be more heterogeneous. As development proceeded the overall granulosity of the cultures tended to decrease, reflected by a decrease in the ratio of all cells present in the upper and lower quadrants in Fig. 2D ((UL + UR):(LL + LR)).

An estimate of the total number of nestin positive cells could be made from the sum of the cells in the right hand quadrants (UR + LR) in the SSC/FL1 plots. In the plots shown 83% of cells expressed nestin on day 2 and this number decreased to 67% in day 8 cultures. The percentage of nestin positive cells present in the R2 and R3 sub-populations were calculated from FL1 histograms shown in Fig. 2E and F. The marker M1 was set on negative controls for each sample, sorted following omission of the primary antibody. The percentage of nestin positive cells within the R2 subpopulation decreased slightly from 62% on day 2 to 52% on day 8. This contrasted with the R3 sub-population that maintained a constant, albeit slightly lower, percentage of nestin positive cells at 47%.

Cell proliferation and development in CDM takes place in the absence of exogenous mitogen. Since it is established that FGF2 can support the development of early embryonic neural precursors in vitro [63], and the expansion of ES-NPCs [29,43,62], we determined the effects of adding FGF2 to cultures on D4 on the nestin population analysed by FACS on D8. For all parameters measured FGF2 appeared to maintain the characteristics of younger (D2) CDM cultures (Fig. 2). Thus, FGF2 maintained the percentage of nestin positive cells, and at the same time prevented the increase in cell heterogeneity seen in D8 cultures, in CDM alone. The predominant nestin population seen at D2 and in the FGF2 treated cultures were larger and more granular in phenotype compared to the more heterogeneous sub-population that developed by D8 in CDM alone, characteristic of neural stem cells previously analysed by FACS [37,50].

Further immunocytochemical comparison of D8 with D12 cultures maintained in CDM alone, showed a marked reduction in the level of nestin staining (not shown), suggesting differentiation and consequent loss of the NPC population. To determine whether this reflected a requirement of the NPCs for exogenous growth factor we measured three parameters of FGF responsiveness in passaged cultures; proliferation, sphere growth, and sphere formation. This showed that FGF2 was required to successfully passage ES-NPCs from D8 NEBs. FGF2 supported increased proliferation, seen by BrdU incorporation, an increase in sphere growth, and also had a major effect on the number of new NEBs formed. Together these results show that development of ES-NPCs initially involves intrinsic growth factor regulation, however later passage and maintenance of the cultures becomes dependent on exogenous FGF2 (Fig. 3).

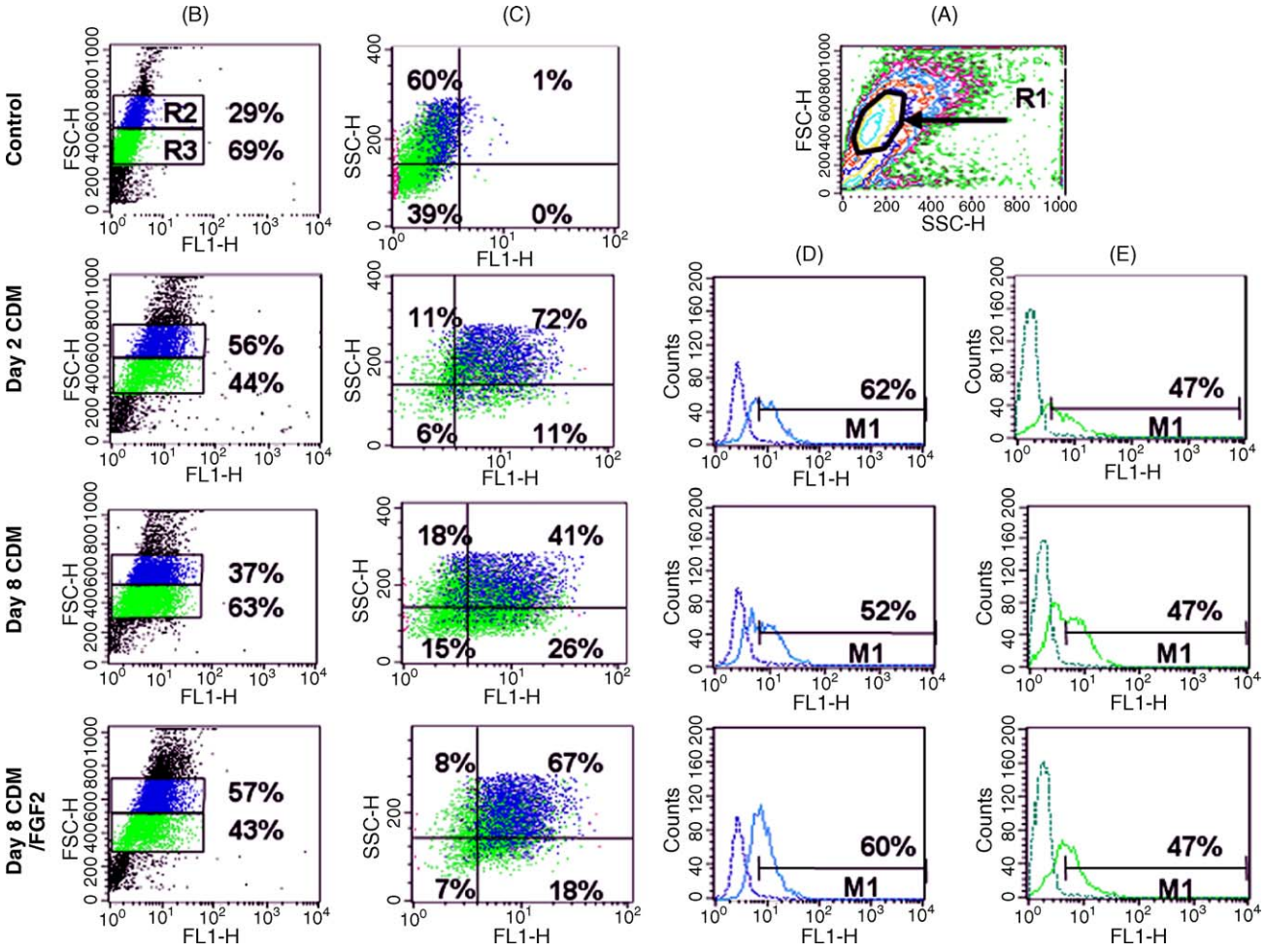

Fig. 2. FACS analyses showing developmental profile and heterogeneity of cells immunoreactive for nestin. NEBs cultured in CDM for 2 and 8 days, and supplemented with FGF2 (10 ng/ml) between D4 and D8, were dissociated to a single cell suspension and analysed by FACS for nestin immunoreactivity. (A) FACS plot displaying forward scatter (FSC), a measure of cell size, against side scatter (SSC), a measure of cell granulosity, defined a region R1 for detailed analysis (B–E), that excludes populations comprising cell debris or cell clumps. (B) Plots of FSC against FL1 (fluorescence intensity) showed a major shift in the population to the right (increase in fluorescence), indicating extensive nestin expression in D2 cultures, persisting to D8. Analysis also revealed two cell sub-populations (R2 and R3) within R1, seen by the presence of two small peaks in nestin immunofluorescence relative to cell size. R2 and R3 are coloured blue and green, respectively. With development, R3 became more prominent, relative to R2. (C) SSC/FL1 dot plots showed the R2 sub-population to have a higher granulosity and also remained fairly homogeneous with time. In contrast, the distribution of cells within R3 appeared more heterogeneous. The percentage of cells in each quadrant is indicated. (D) and (E) FL1 histograms with stringent placement of the marker M1, on negative controls for each sample, to calculate the minimum percentage of nestin positive cells R2 (D) and R3 (E) sub-populations. Addition of FGF2 to the cultures maintained a nestin profile characteristic of younger CDM cultures, similar to D2 (B–E). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.3. Gene regulation upon ES cell differentiation

Culture of ES cells in CDM clearly restricted the majority of cells to differentiate down a neuroectodermal pathway. To further determine the neurogenicity of cultures and to address the mechanisms by which the lineage restriction occurs we determined changes in the expression profile of a set of 1176 known genes in ES cell, days 4 and 8 CDM cultures using a nylon broad coverage cDNA expression array (Clontech, mouse 1.1 Atlas). This array included representatives of several gene families involved in development. An example of hybridisations is shown in Fig. 4A. The data provided a semi-quantitative indication of gene expression levels for the higher and moderately expressed genes. Several genes expressed at low levels could be detected following long exposure of autoradiographs however we would not exclude that genes scoring negative by hybridisation were not expressed at low levels and would not be detectable by more sensitive RT-PCR techniques. Table 1 summarises the number of genes expressed in ES cells and in D8 cultures, and the proportion of genes that were up or down-regulated upon differentiation and the list of up and down-regulated genes is given in Table 2.

Although of gene expression in ES cells is often described as ‘relaxed’, non-specific, or ectopic, gene expression is likely to be of low abundance since the profile of genes detected here was highly restricted. Of the 1176 genes analysed expression of 335 (28.3%) were detected by filter hybridisation. Genes expressed at moderate to high levels in ES cells included the ES cell specific marker *Pou5f1* (formerly *Oct 4*) and those involved in maintaining pluripotency such as the LIF recep-

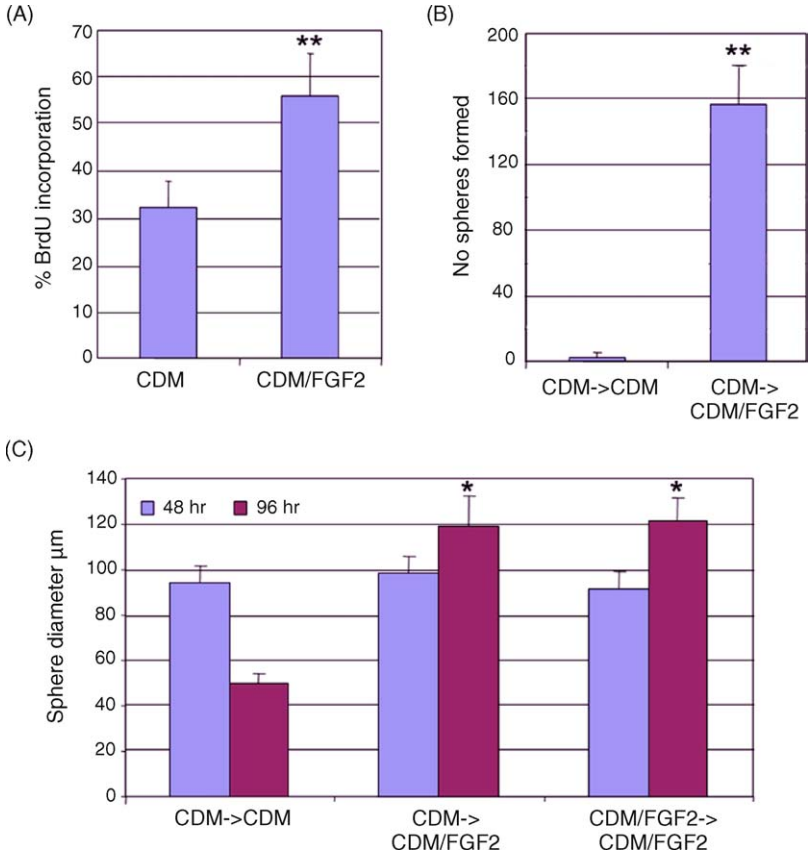

Fig. 3. Responsiveness of ES derived neural progenitors to FGF2. (A) CDM cultures supplemented with FGF2 (20 ng/ml) between D4 and D8 showed increased BrdU incorporation. (B) FGF2 supported the development of new NEBs from passaged D8 cultures, (C) FGF2 increased the growth of individual NEBs passaged from either CDM or CDM/FGF2 cultures ($^{*}p > 0.01$, $^{**}p > 0.001$).

Table 1
Summary of gene expression changes analysed

	Total (1176)	Off	On	Down-regulated (≥2)	Up-regulated (≥2)
ES cells	335	66 (19.7%)			
Day 8 CDM	402		133 (33%)		
ES + day 8 CDM	269			52 (19.5%)	64 (23.8%)

Table 2
Genes up and down regulated by >2× upon ES cell differentiation in CDM for 8 days

	Genes up-regulated	Genes down-regulated
Transcription factors	*Myt1, Dlx1, Dlx5, Arnt2, Zhfx1a, Nkx6.2, Ndn, Pou3f3, Ascl1, Dbx1, Gsh1, Gsh2, Nkx2.2, Tbr1, Mrg2, Eya1, Lhx1, Lhx5, Shox2, Lef, Foxa1, Foxa2, Bhlhb2, Irx3, Pou6f1, Hes5, Tclf1, Sox3, Pax5, Pax6, Hoxa7, Nmyc, Lmyc1, Dlgh4, Cabp2, Nab1, Ezh2, Cbx4*	*Zfp36, Tead4, Snai1, Tcf15, Pem, Cdx2, Tbx3, Prrx2, Egr1, Eps8, Elk4, Gata4, Klf2, Klf4, Ifi1, Edr1, Pou5f1, Tcea3, Irf1, Stat1, Stat6, Rarg, Myc*
Intracellualar signaling	*Mfng, Rfng, Smad3, Smad5, Irs1, Rasgrf1, Fyn, Shc3 Ikbkg, Mapkpk2, Ptpn13, Gnao, Cited1, Pea15, Vamp1, Apc, Mapre1, Tsg101, Hras1, Nras, Bcl2l, Wsb1, Sgne1*	*Mybl2, Mdm2, Il6st, Cish1, Cish2, Syk, Camk2b, Map2k1, S100a10, Dab2, Nfkbia, Jub, Eng*
Intercellular signaling	*Dcc, Dll1, Notch1, Notch2, Notch3, Efna2, Efnb1, Ephb2, Sema4a*	*Efna3, Epha2, Sema4b, Notch4*
Growth factors/receptors	*Cspg3, Gdf1, Gdf9, Bmp1, Bmp7, Fgf3, Fgf15, Fgfr1, Cst3 Ednrb1, Ldlr, Insr, Igf2, Igf1ra, Igfbp5, Ptn, Vegfa, Wnt3, Wnt7a, Wnt7b, Dkk1, Sfrp2, Sfrp3, Fzd3, Fzd6, Ntrk3, Grin1, Thra*	*Cmklr1, Pdgfra, Lifr, Tnfrsf1b, Acvr1b, Tgfbr3 Essrb, Igfbp6, Fgf4, Tgfb1, Nodal, Ptch2*
Cell adhesion/cytoskeleton/extra cellular matrix	*Cdh2, Cdh4, Cdh6, Catnd2, Kif3a, Kif3b, Kif3c, Ina, Tubb4, Kif1b, Kif5c, Vtn, Sdc3, Cspg3, Lamb2, Tmsb4x*	*Cdh1, Cd31, Cd44, Spp1, Itga3, Lamb1-1, Lama5, Lamc1, Fn1, Nid*
Other	*Hap1, Fapbp7, Kl, Cnil, 168*	*Hsp25, Gpx3, Sqstm1, Gadd45a, St14, Timp2, Timp3, Pole2, Pms2, Rad51, Mre11a, Laptm5, Cobl, Rga*

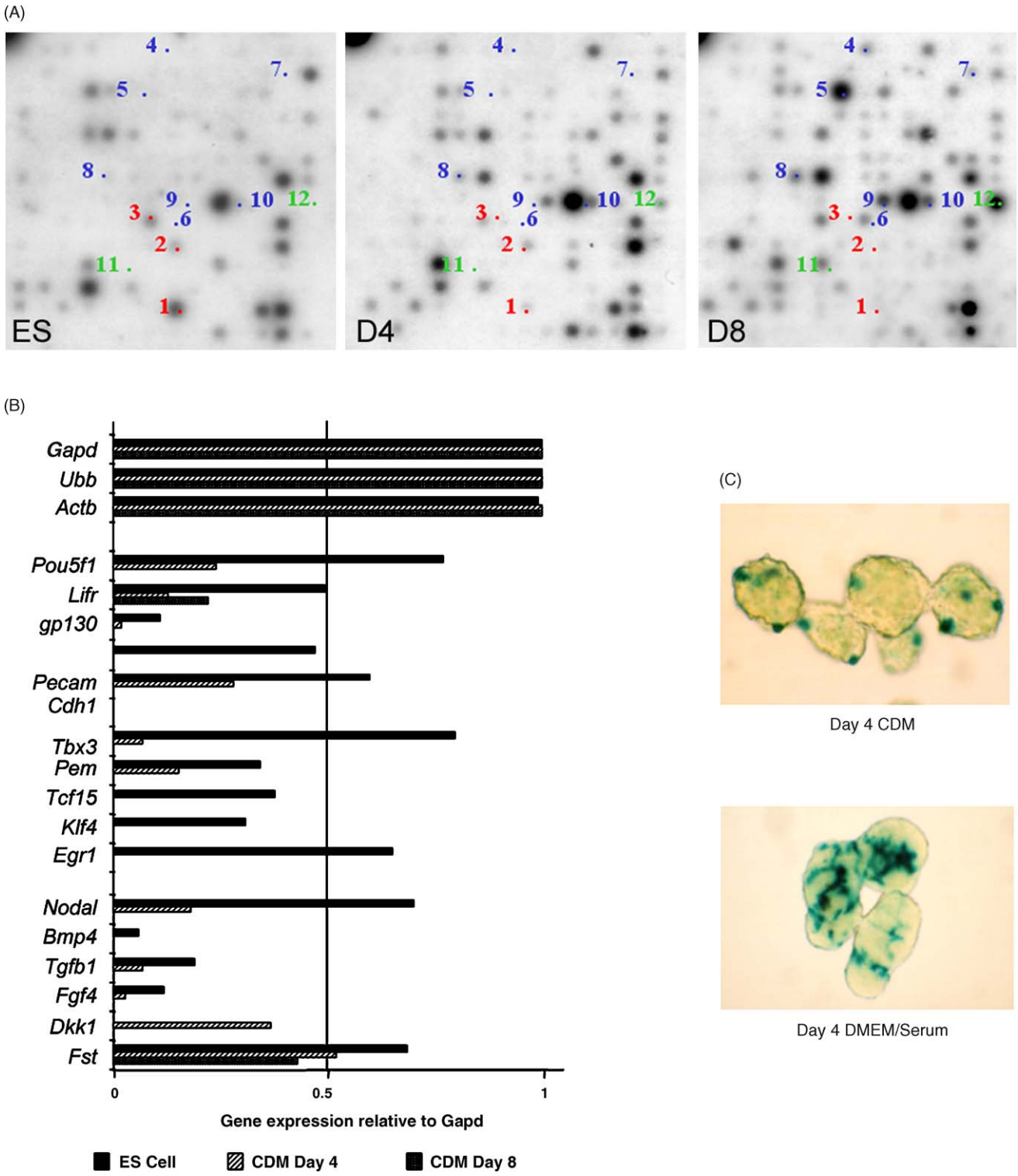

Fig. 4. Gene expression changes during ES cell differentiation in CDM serum-free medium. Gene expression profiling was performed using RNA prepared from ES cell, D4 and D8 CDM cultures, and screening Clontech mouse 1.1 Atlas nylon gene arrays. Example hybridisations are shown for ES, D4 and D8 samples in (A). Genes identified in (A) show down-regulation of ES cell specific genes ((1) Tbx3, (2) Pem, (3) Tcf15), and up-regulation of fate determining ((4) Nkx2.2, (5) Dbx1, (6) Tbr1, (7) Irx3, (8) Dlx1, (9) Lhx2, (10) Foxa2) and neurogenic genes ((11) Ascl1, (12) Hes5). (B) genes down-regulated upon cell differentiation. Levels of gene expression were determined from autoradiographs by densitometry and analysis using Atlas Image software (Clontech), and expressed in arbitrary units relative to the levels of Gapd expression. (C) Distinct spatial restriction of *Pou5f1* expressing cells in D4 CDM NEBs compared to D4 embryoid bodies grown in 10% serum. LacZ expression marks Pou5f1 positive cells in CDM NEBs and embryoid bodies derived from the ES cell clone 710 [65].

tor (*Lifr*) and LIF signal transducer *Gp130* (*Il6st*) [41]. Several genes known to be expressed in ES cells, the blastocyst inner cell mass (ICM), primitive endoderm, and the pre-gastrula epiblast were also detected (Fig. 4A, Table 1), including the transcription factors *Tbx3*, *Gata4*, *Tcf15*, *Pem*, *Cdx2*, *Klf4*, and *Egr1* [11,31,47], growth factors such as *nodal*, *Fgf4*, *Bmp4*, *Tgfβ1* and *follistatin* [3,9,13,74], and surface markers such as *E-cadherin* (*Cdh1*), *CD44* and *CD31* (*Pecam*) [51,64]. Upon differentiation, genes expressed in the ICM and also involved in posterior primitive streak and paraxial mesoderm development were rapidly down-regulated, including *Nodal*, *Tbx3* and *Tcf15*. Since low expression of *Pou5f1* persisted for some time after LIF withdrawal, we determined the pattern of *Pou5f1* expression within D4 NEBs using an ES cell line that expressed *lacZ* from an *Pou5f1* promoter [65]. Interestingly, the *lacZ* expressing cells clustered, in a polar manner, at the edge of each sphere suggesting that the residual ES cells were being selectively excluded, possibly through differential cell adhesion (Fig. 4C). In contrast, in conventional EBs formed in the presence of serum and without LIF, a much larger ES cell population persisted and was located, and maybe protected from differentiation, within the middle of the NEBs.

2.4. Proneural and neurogenic gene expression

Neural differentiation of ES cells in CDM was associated with an up-regulation of proneural and neurogenic gene expression (Fig. 5). Significantly, *Notch1*, *2* and *3* but not *Notch4* were expressed upon differentiation [20,28]. The Notch ligand Delta-like 1 (*Dlk1*) was markedly up-regulated, and two neural effectors of Notch signaling, the bHLH genes *Hes1* and *Hes5* were expressed. *Hes1* was expressed in ES cells; however the level of expression decreased approximately two-fold over the 8 days of differentiation. In contrast *Hes5* was not expressed before D4 and became one of the most abundantly expressed genes in the cultures at D8. Although Notch signaling is used extensively to regulate cell fate throughout the embryo, it is significant that *Hes5* is expressed exclusively in the developing CNS, and is required to sustain a NPC population in E11.5 telencephalon derived neurosphere cultures in vitro [42]. Together the patterns of Notch and Hes gene expression are in good agreement with the differentiation of ES cells to a neural progenitor cell population in CDM.

Targets for transcriptional repression by Hes genes includes the bHLH neurogenic genes. Of four genes present on the array (*Neurog1*, *Neurog2*, *NeuroD* and *Ascl1*) only *Ascl1* was abundantly expressed in D8 cultures, although expression of the other genes could be detected by RT-PCR (Fig. 4B). Despite high levels of *Hes5* expression, *Ascl1* expression could be explained by the down-regulation of *Hes1* since *Hes1* is a transcriptional repressor that directly binds the *Ascl1* promoter [10].

Comparison of D8 with D4 gene expression profiles showed the induction or up-regulation of several genes expressed in developing neural tissue, indicating continued differentiation of the neural precursor cell population (Fig. 5A, Table 1). Neural specific genes included neural adhesion molecules, neurofilament and cytoskeletal components, and a change in the expression profile of extracellular matrix and receptor genes (Table 1). Expression of markers of more differentiated neuroblasts and neurons may in part be associated with the onset of *Ascl1* expression, as it functions in postmitotic neurons, and is consistent with the limited ability to passage cultures without the addition of exogenous mitogen to maintain a proliferating progenitor cell pool. In this regard it is noteworthy that addition of FGF2 to maintain NSC proliferation in the cultures acted to limit expression of the neurogenic genes (Fig. 5B).

Perhaps the most significant change associated with differentiation was seen in the profile of expressed transcription factors. Strikingly, several of the genes up-regulated serve as markers of neuroectoderm with restricted positional identities. Notably, markers used to predict anterior versus posterior fate indicated that CDM NEBs developed with anterior fate characteristics (Fig. 5). Hence, markers normally restricted to the prosencephalon such as *Foxg1*, *Dlx5*, *Dlx1*, *Lhx5* and *Tbr1* were expressed [45,73] whereas markers for the midbrain, hindbrain and spinal cord, including *En2*, *Gbx2*, *Mafb*, *Erg2* and several Hox genes were not detected [32]. Several genes which are expressed in longitudinal domains, which traverse different rostrocaudal boundaries in the embryo were also expressed, for example *Pax6*, *Dbx1*, *Gsh2* and *Gsh1* (Fig. 5) [45,55].

2.5. Cell heterogeneity in CDM cultures

Although the majority of cells in the CDM cultures adopt a neural fate, evidence from the array analysis identified markers of primitive endoderm that were present in the initial ES cell population (e.g. *Cdx2*, *Pem*) which down-regulated with differentiation (Fig. 4). Additionally we observed a transient peak in the expression of markers for visceral endoderm (e.g. *Ihh*, *Lamb1*, *Hesx*, *Dkk1*, *Foxa2*). The initial presence of primitive endoderm derivatives in CDM cultures is of potential importance since in vivo, it is the anterior visceral endoderm (AVE), in conjunction with the node, that signals to specify anterior epiblast fates and participates in neural induction [59,61]. To further investigate the expression of genes associated with the AVE organiser and also the node we performed additional semi-quantitative RT-PCR analysis on cell samples taken after 2, 4, 6 and 8 days of differentiation (Fig. 6). Since FACS data suggested that FGF2 maintained a more homogeneous nestin population we also analyzed cultures supplemented with FGF2 between D4 and D8. RT-PCR detected expression of the transcription factors *Hesx1*, *Otx2*, *Hex*, *Gata6* and *Ihh*, that serve as markers for AVE, anterior definitive endoderm and mesendoderm (ADE/AME) [33,34,36]. In vivo *Goosecoid* (*Gsc*) and *Foxa2* genetically interact to regulate neural patterning and at the earliest stages of development serve as markers for cells of the AVE and the anterior node [14]. Expression of *Gsc* and *Foxa2* peaked between D4 and D6 of differentiation. The population of cells expressing *Foxa2*, a marker for cells characteristic of both the AVE and the node, was determined by FACS analysis and estimated to be 7–10% on day 8. Interestingly immunohistochemistry of whole NEBs showed that Foxa2 (Hnf3β) positive cells tended to cluster in a polarised manner within the NEBs (Fig. 6). Addition of FGF2 had a major effect, causing a marked

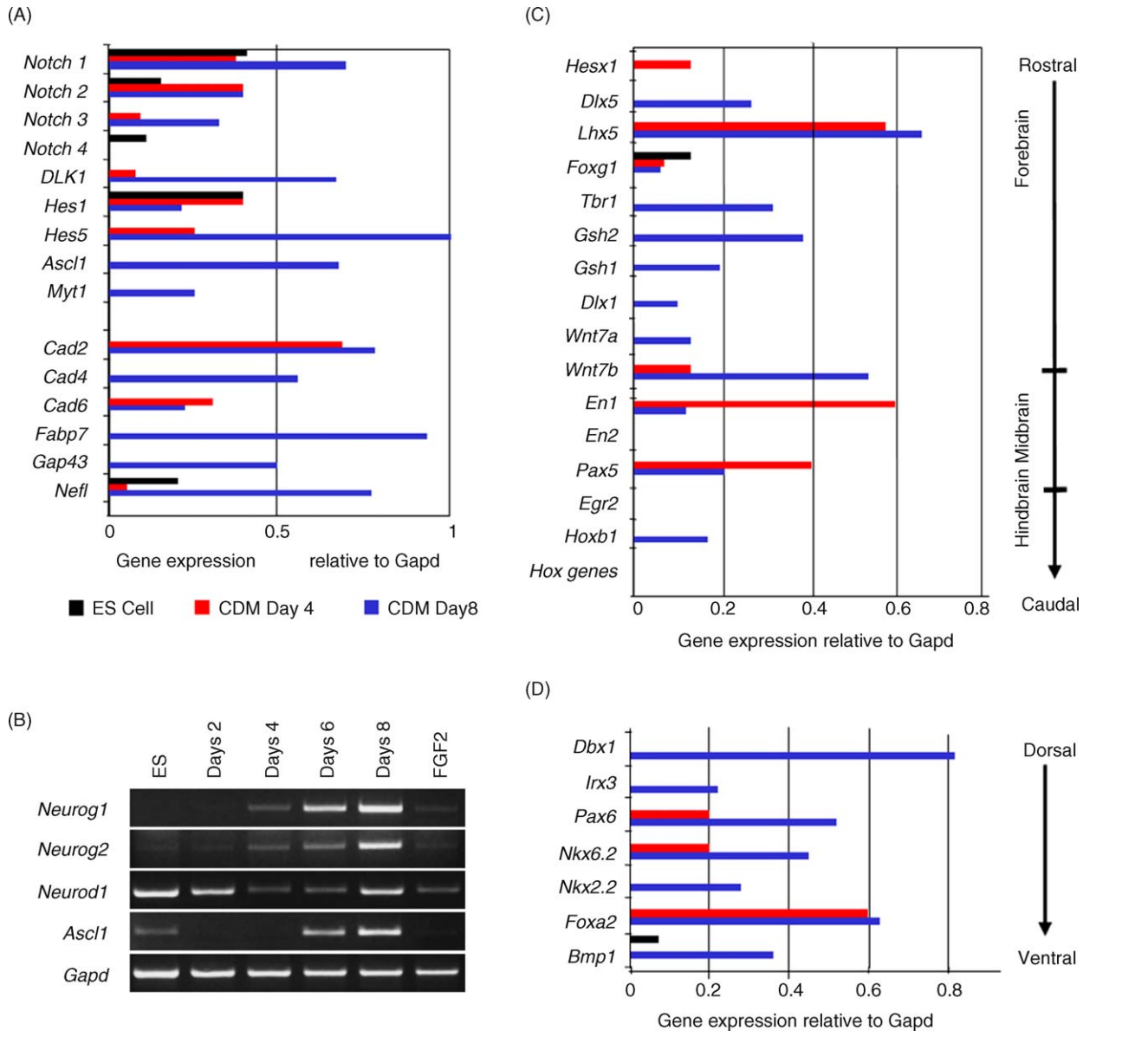

Fig. 5. Expression profile of proneural and neurogenic genes, and markers of cell differentiation and positional identity. Expression profile of genes present on the Clontech mouse 1.1 Atlas array for ES cell, D4 and D8 CDM cultures (A, C and D). (A) Proneural and neurogenic genes together with the levels of neural cadherin gene expression and neural and glial lineage restricted markers. (B) developmental profile of neurogenic gene expression determined by semiquantitative RT-PCR on normalised cDNAs prepared from ES cells and CDM cultures harvested at D2, D4, D6 and D8 of differentiation and parallel CDM cultures supplemented with FGF2 (10 ng/ml) between D4 and D8, and harvested at D8. Note the effect of FGF2 to attenuate the onset of neurogenic gene expression seen in the untreated cultures. (C) expression profile of rostrocaudal markers of positional identity. (D) expression profile of dorsoventral marker of positional identity. In (C) and (D), genes are ordered according to their approximate rostrocaudal and dorsoventral expression domains, respectively. Gene expression is shown relative to hybridisation to *Gapd*.

reduction in the detection of AVE and node markers (with the exception of *Hex*).

Although our initial premise for using serum-free culture for neural differentiation of ES cells was based on the model, established using *Xenopus* animal cap assays, that differentiation of dissociated primitive ectoderm will default to neuroectoderm in the absence of BMP/activin signaling [18], the gene expression data suggests that more complex mechanisms of differentiation can not be excluded, possibly involving cell–cell signaling that more closely recapitulates mechanisms of neural induction in vivo [70]. Recent in vivo data has highlighted a requirement to antagonise not only BMP, but also WNT signals for neural development [72]. Our current analysis identified up-regulated expression of both WNT (Dkk1, Sfrp2, Sfrp3 and Frzb1) and BMP (follistatin, noggin, cerberus and chordin) antagonists early in the differentiation process (Fig. 6). The function of these antagonistic signals in this system remains to be determined, however it is noteworthy that both autocrine BMP and WNT signaling contribute to the maintenance of ES cell pluripotency [53,75].

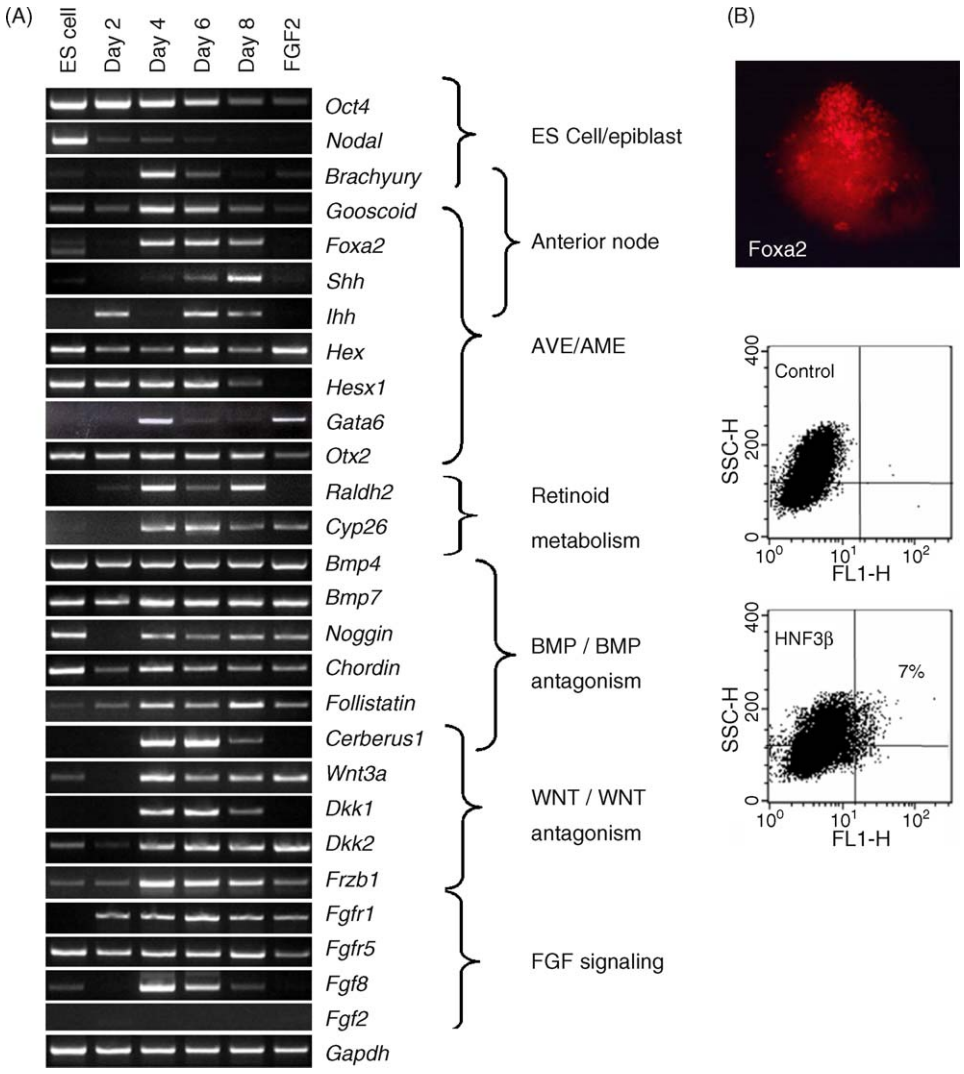

Fig. 6. Developmental expression profile of genes related to patterning and differentiation of the epiblast. (A) Genes are ordered to indicate relevant sites of expression in the embryo, and those involved in BMP, WNT and FGF signaling. Gene expression is compared from normalised cDNA samples prepared from ES cells and CDM cultures harvested at D2, D4, D6 and D8 of differentiation and parallel CDM cultures supplemented with FGF2 (10 ng/ml) between D4 and D8, and harvested at D8. (B) HNF3β expression in D8 CDM NEBs. Immunostaining for HNF3β identified positive cells that tended to cluster, demonstrating a degree of cell organisation within the growing NEBs. The proportion of HNF3β positive cells in non-manipulated D8 NEBs was estimated to be of the order of 5–10%, by FACS analysis of dissociated immunostained cells, seen by the shift in fluorescence intensity of cells in plots of SSC against FL1.

In addition to regulation of WNT and BMP signals, in vivo studies in the chick have shown that induction of neural competence of the epiblast involves FGF signaling [57,71]. Plating ES cells in CDM caused a large up-regulation of *Fgfr1* gene expression, amongst eleven FGFs screened on the array we observed a down-regulation of *Fgf4* and identified a major induction of *Fgf15* (Figs. 4B and 6). The steady expression of *Fgf15* makes it a strong candidate to be involved in maintenance of early embryonic neural stem cell proliferation. Although proliferation of embryonic neural stem cells may be maintained in vitro using exogenous FGF2 [63], endogenous FGF2 is unlikely to be relevant [62] since *Fgf2* expression could not be detected by array hybridisation or by RT-PCR (Fig. 6). In addition to *Fgf15*, RT-PCR consistently detected a transient expression of *Fgf8* which interestingly followed a similar pattern to expression of the node/AVE markers.

2.6. Neural differentiation is dependent on endogenous FGF signaling and antagonism of BMP, WNT signals

To test the effects on neural differentiation of BMP, WNT and FGF signaling ES cells were plated in CDM in the presence of BMP4, or LiCl (which activates GSK3β thereby mimicking WNT signaling), or the FGF receptor antagonist SU5402 [40]. Given the regulation of HH gene expression in the CDM cultures, and the established importance of Ihh expression in primitive endoderm in particular [36], we also determined the effect on neural differentiation of blocking HH signaling using KAAD, a derivative of cyclopamine and potent inhibitor of the HH signaling pathway [58]. The percentage of Sox1 positive cells at D4 in culture under each condition is shown in Fig. 7. This showed that the percentage of neural progenitor cells induced was significantly reduced by all treatments used, confirming antagonism

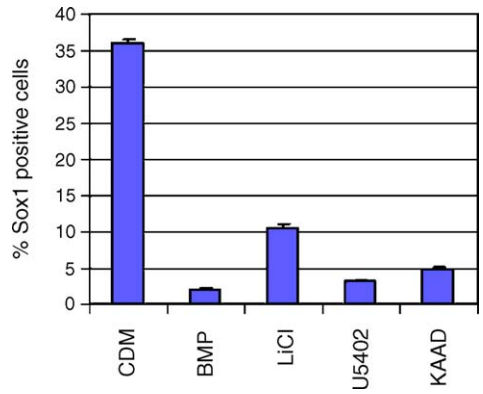

Fig. 7. Suppression of neural differentiation of ES cells by BMP4 and the WNT pathway agonist LiCl, and FGF and HH pathway antagonists SU5402 and KAAD. Drugs and growth factors were added to cultures at the time of plating ES cells in CDM. The percentage of Sox1 positive cells was determined by immunocytochemistry at D4, cultures were dissociated and cells plated onto laminin coated coverslips for 4 h to allow cell adherence, before being fixed and processed for Sox1 immunocytochemistry.

of neural differentiation by BMP and WNT signaling, and a dependency on endogenous FGF signaling (selectively through Fgfr1), and interestingly a possible role for HH signaling.

3. Discussion

Given the therapeutic potential of embryonic and adult stem cells, the in vitro differentiation of lineage restricted progenitor cells from pluripotent stem cells is an area of considerable interest. The derivation of neural progenitors from ES cells in completely defined medium (i.e. in the absence of serum, feeder cells, or conditioned medium) is particularly important as it provides ultimate control over the extrinsic signaling environment that can be systematically manipulated to influence derived cell fates. Here, we describe the differentiation profile of mouse ES cells cultured in a chemically defined medium CDM.

Gene expression analysis supports the notion that neuroectoderm differentiates in the absence of significant mesoderm formation in CDM spheres. ES cell specific genes such as *Pou5f1* were rapidly down regulated as were mes/endoderm specific genes that are also expressed in ES cells, such as *Tbx3*, *Fgf4*, *Pdgfra*, *Tcf15*, and *Nodal*. Furthermore, induction of genes involved in specification of embryonic endoderm, mesoderm, or paraxial mesoderm such as *Twist* or *Tbx6* were not observed. Although these results demonstrate that a significant mesodermal cell population is not induced, it is still possible that neural induction may be influenced by a transient mesoderm population, or from factors expressed by residual ES cells (Pou5f1 expressing cells) that we have seen polarised to at the edge of developing NEBs.

Importantly, neuroectoderm differentiation in CDM is influenced by the initial ES cell plating density. NEBs were generated with greatest efficiency by plating between 5×10^4 and 1×10^5 cells/ml (10 ml/10 cm plate). At higher cell densities (1×10^6cells/ml), larger embryoid bodies formed in the initial 24–48 h by cell aggregation and resulted in a complex pattern of cell differentiation (data not shown). This could result from mes/endoderm induction by BMP's and activins that are expressed at low levels in ES cells. At low cell densities the concentration of ES cell derived mes/endoderm inducing factors may not be sufficient to influence cell fate. In contrast at much lower cell densities (1×10^4 cells/ml) ES cell survival and differentiation was very poor. A recent study claiming 'default neurogensis' from single ES cells demonstrated a requirement for LIF when ES cells were plated at limiting dilution densities in B27 supplemented medium [62]. The function of LIF in this case was not clear, however it may have provided a cell survival function that allowed cell division and the initial formation of an aggregate of a critical cell number that is required to sustain further sphere development. The dependence of muscle precursor cells on a critical cell density for survival and differentiation has been documented as a 'community effect' and a similar phenomenon could be operating here [15]. In CDM, addition of LIF did significantly increase the number of developing NEBs, however unlike the previous report using B27 medium [62], LIF maintained high levels of Pou5f1 expression over an 8-day period (data not shown).

3.1. Potential mechanism of neural differentiation in CDM

The extensive neurogenicity seen in CDM cultures is achieved as a consequence of omitting RA, or other exogenous neural inducers or mitogens, from the culture medium. Although RA can induce neurogenesis through the activation of genes such as *Neurog1* [54] it also acts to restrict the fate potential of multipotent neural progenitors [67]. In vivo, RA does not have a major function in the neural induction of pluripotent epiblast tissue. Indeed to the contrary, neural induction is associated with anterior specification of the epiblast and it has been found that active repression of RAR signaling is required to specify anterior fate characteristics of the embryo [1,26,52].

Specification of neural competence of pluripotent cells is known to involve FGF signaling and antagonism of WNT and TGFβ signals [70]. In the chick ongoing FGF signaling, provided by *Fgf3* expression, is required for neural induction [71]. In mice, although a number of Fgf and Fgfr genes are expressed early in post-implantation development [4,12,13,16,35,77], their relative contributions to neural induction are not clear. In CDM cultures, blocking FGFR signaling with the antagonist SU5402 [40] caused a significant reduction in the number of sox1 positive cells. This observation has been made independently by Ying et al. [76], and is consistent with a previous observation of reduced neural induction of Fgfr1−/− mutant ES cells in B27 supplemented serum-free medium [62].

We found significant expression of *Fgf15* and a limited expression of *Fgf8*. It is also established that *Fgf4* and *Fgf5* are expressed in ES cells [48] and have significant roles to play in epiblast development. *Fgf15* represents a candidate for the induction of neural competence. *Fgf15* was induced early, and in vivo it has recently been identified as a downstream target gene of *Otx2* [77], a transcription factor essential for the specification of anterior head structures and neuroectoderm [2,49]. *Fgf15* is also a strong candidate for an endogenous autocrine/paracrine factor to maintain early neural stem cell proliferation since its

expression was maintained at steady levels in all cultures studied, and is broadly expressed in the neural tube of D10 mouse embryos [38].

Neural specification of competent cells involves antagonism of TGFβ and WNT signals that would otherwise divert differentiation to alternative cell fates. Despite early expression of *Bmp4*, *Bmp7* and *Wnt3a* the expression of several BMP and WNT antagonists were also developmentally regulated in CDM cultures (Figs. 4 and 6). Although neural induction can occur as a default process [17], neural differentiation of ES cells in CDM is likely to involve active BMP and WNT antagonism [72,70]. The origin of these signals may be from sub-populations of cells with 'anterior organiser' activities, suggested by early expression of markers of the node and AVE, including *Gsc*, *Foxa2*, *Hex1*, *Hesx1*, *Lhx1*, *Gata4*, *Gata6* and *Otx2*. It will be of particular interest in future studies to further determine the spatial organisation of neural and non-neural cell populations in developing NEBs, and to determine the requirement for the node and visceral endoderm-like cell populations in the CDM cultures for differentiation of cells to neuroectoderm. A recent study found that HEPG2 conditioned medium restricted the pluripotency of ES cells to early primitive ectoderm (EPL cells), but was non-permissive for visceral endoderm differentiation [48]. Interestingly, EPL cells and EBs derived from EPL cells were defective in neural development. However, neural development could be rescued in mixed ES cell/EPL cell embryoid bodies in which visceral endoderm development was restored, suggesting a direct role for the visceral endoderm in neural induction [27].

Negative effects on neural differentiation of the BMP and WNT pathways were confirmed by adding BMP4 and LiCl to cultures, and confirm observations also reported by Aubert et al. [5]. Surprisingly, inhibiting the HH signaling pathway by addition of KAAD, a cyclopamine derivative that blocks smoothened activity with high efficiency [58], also reduced the percentage of Sox1 positive cells induced in CDM cultures. The mechanism for this is not clear since analysis of Smo−/− mutant embryos does not suggest a function of HH signaling in neural induction per se, but rather affects neural patterning [68,78]. The action of KAAD and the function of HH signaling in neural differentiation in vitro will require further study.

4. Methods

4.1. Cell culture

ES cells were maintained following standard techniques [23] by routine culture on mitotically arrested primary embryonic fibroblasts. ES cell culture medium used was DMEM:F12 with glutamax (Gibco) supplemented with 10% fetal calf serum, penicillin/streptomycin, β-2-mercaptoethanol, non-essential amino acid (Sigma) and 10 ng/ml LIF. Studies focussed on use of the ES cell line E14 (129/Ola derived), however similar differentiation was observed and confirmed using additional ES cell lines including CGR8.8 (129Ola derived), TgTP6.3 (derived from E14) [44], 710 (HM1 (129Ola) derived [65]), and IMT11 (129Sv derived), all of which showed similar differentiation properties. Prior to differentiation of ES cells, feeder cell contamination was minimised by passaging cultures twice onto culture dishes coated with 0.1% gelatin (in the presence of LIF). In the case of cell lines CGR8.8 and TgTP6.3 similar differentiation was observed after growing ES cells in feeder-free conditions. For differentiation ES cells were trypsinised to a single cell suspension and washed three times in PBS to remove serum and LIF. ES cells were resuspended in CDM medium (Iscove's modified Dulbecco's medium)/Hams F12 1:1 (Gibco Invitrogen), 1× lipid concentrate (Gibco Invitrogen, Cat. No. 11905-031), penicillin/streptomycin (Gibco Invitrogen), transferrin 150 μg/ml final (Sigma), insulin 7 μg/ml final (Sigma), monothioglycerol 450 μM (Sigma), bovine serum albumin (Sigma, Cat. No. A-3311) [69] and plated at between 5×10^4 and 1×10^5 cell/ml on 10 cm bacteriological grade culture dishes (Sterilin). Medium was changed every 2 days by first harvesting growing cell aggregates, or 'NEBs', by gravity in conical tubes. Cultures were passaged by dissociating NEBs using enzyme-free cell dissociation buffer (Gibco-BRL) or by trypsinisation and stopping reactions with soy bean trypsin inhibitor, washing in culture medium and replating. Growth factors and drugs were used at the following concentrations unless otherwise stated, FGF2 10 ng/ml, BMP4 5 ng/ml (R&D System) KAAD 1 μM (Toronto Research Chemicals), 10 μM SU5402 (Calbiochem), 10 mM LiCl (Sigma).

BrdU incorporation was determined in D8 cultures following a 24 h pulse-label with 10 μM BrdU. After labeling, NEBs were dissociated to a single cell suspension, plated onto laminin coated coverslips for 4 h to allow cell adherence, fixed in 4% PFA and processed for BrdU immunocytochemistry. BrdU positive cells were counted and expressed as a percentage of DAPI. NEB formation was assessed by dissociating D8 NEBs and plating single cells at a density of 1×10^4 cell/ml in CDM or CDM/FGF2. NEB diameters were determined as an index of NEB growth. D4 NEBs were transferred individually to drops (10 μl) of medium and cultured under liquid paraffin oil to prevent evaporation, and NEB diameters were measured using a graticule. Diameters of the same NEBs were then measured at days 6 and 8. This procedure allowed the growth of individual NEBs to be followed with time and was necessary due to the high tendency of NEBs to aggregate to each other in bulk culture.

4.2. Immunocytochemistry

Immunocytochemistry was performed on either free-floating whole NEBs (wholemount), cryostat sections, or plated cells. For wholemount staining NEBs were collected and treated in V-bottomed tubes. Cells were fixed in ice cold 4% paraformaldehyde (PFA) in PBS for 20–30 min, and then washed three times in PBS. For intracellular antigens, cells were permeabilised for 20 min in 0.1% Triton X-100/PBS (Sigma). Non-specific antibody binding was blocked by pre-incubation with 5% normal goat serum. Primary antibody incubations were performed at room temperature for 2–4 h or overnight at 4 °C, in 1% serum and 0.1% Triton in PBS. After washing three times, cells were incubated with secondary antibodies for 1–2 h at room temperature in PBS and finally washed three times in PBS. Cells were mounted in Vectafluor mounting medium (Vector) and visualised by fluorescence microscopy (Zeiss Axiophot). For cryostat sections, NEBs were harvested, fixed in fresh 4% PFA for 2 h, and dehydrated in 30% sucrose (in PBS) overnight. Pelleted NEBs were equilibrated with OCT cryo-compound (BDH) and frozen on dry ice. Nine micrometers frozen sections were cut onto slides (Bright), dried and processed for immunostaining as described above. For plated cells, cells were fixed with 4% PFA for 10 min and permeabilised with absolute methanol at −20 °C for 15 min and processed as described above. DAPI nuclear stain (1:5000) was included in the final antibody application. Cell counting was performed using a grid and five consecutive fields were counted for each coverslip. The number of positive cells was expressed as a mean ± SEM from two to four coverslips and from three separate experiments.

Primary antibodies used were monoclonal supernatants (used at 1:5 dilutions) 4C7 and RC2 reactive to Foxa2 (HNF3β) and radial glia, respectively (University of Iowa, Developmental Studies Hybridoma Bank), anti-BrdU (Sigma), Rat401, anti-nestin (Pharmingen 1:400), G-A-5, anti GFAP (1:400, Sigma), TuJ1, anti-βtubulin III (1:400, Sigma), NG2 reactive to oligodendrocyte precursors (1:200, Pharmingen) and rabbit polyclonal anti-Sox1 (1:500, kind gift of Robin Lovell-Badge) Secondary antibodies used were FITC-anti mouse IgG (1:200, Sigma), Cy3-anti-mouse (1:800, Chemicon) FITC-anti-rabbit IgG (1:200, Sigma).

4.3. LacZ staining

β-Galactosidase activity in NEBs derived from ES clone 710 was determined in situ in whole NEBs fixed in 4% formaldehyde in PBS (20 min) followed by

staining in PBS containing 35 mM potassium ferricyanide, 35 mM potassium ferrocyanide, 1.5 mM magnesium sulphate and 1 mg/ml X-gal overnight at 37 °C.

4.4. Flow cytometry

Prior to immunostaining, cell aggregates were dissociated by incubation for 15 min at 37 °C in Trypsin + DNAse (Clontech), the reaction was stopped in trypsin inhibitor (Sigma) or serum and a single cell suspension was obtained by gentle trituration. Cell suspensions were washed in Ca/Mg-free PBS, 2% serum, 10 mM sodium azide (PFN), fixed in ice cold 4 % PFA for 30 min, then washed in ice cold PFN. Cells were counted and aliquoted in samples of $2–5 \times 10^5$ cells per well in a 96-well U-bottomed microtitre plates (Nunc, Denmark). Cells were pelleted by centrifugation (1200 rpm). Incubation with primary antibodies was performed in PFNS (PFN + 0.14% saponin) for 30 min at 4 °C. The same antibodies and dilutions were used as described for immunocytochemistry above. Cells were then washed three times by centrifugation (1200 rpm for 2 min) in the plate with PFNS and then incubated with the secondary antibodies for 30 min at 4 °C. Finally, cells were washed three times in PFN and used for sorting.

FACS analysis was performed using a FACScalibur flow cytometer (Becton Dickinson) equipped with an argon laser emission wavelength of 488 nm. FITC was identified by using a 530 band pass filter. Data analysis was performed using CellQuest software (Beckton Dickinson). Three to ten thousand events were acquired per sample.

4.5. Gene expression profiling

Gene expression profiles for cultures were determined for 1176 independent genes gridded on the Clontech mouse Atlas 1.1 nylon array. Total RNA was prepared from cells of duplicate cultures using Tri-reagent (Sigma) or RNeasy (Qiagen) and ^{32}P-radiolabelled (Amersham) cDNA probes were synthesised using a primer set specific for the genes on the arrays (Clontech). Array filters included negative controls for genomic DNA and plasmid contamination together with positive control house keeping genes. Hybridised filters were analysed by autoradiography followed by image analysis using Atlas Image 1.5 software (Clontech).

Systematic analyses of specific gene expression patterns on CDM cultures sampled after 2, 4, 6, and 8 days in culture, and at 8 days after addition of FGF2 on day 4, were performed by RT-PCR using SMART technology (Clontech). SMART enabled reproducible, linear amplification of RNA allowing multiple PCRs to be performed from matched cDNA samples. Gene expression comparisons were always made using matched samples, normalised between each other for the level of *Gapd* expression quantified by ethidium bromide- or SYBR Green staining and band intensity measurements using FLA-3000 Imager System (Fujifilm). The basic PCR condition is: 16 mM $(NH_4)_2SO_4$, 67 mM Tris–HCl (pH 8.8 at 25 °C), 0.01% Tween-20 (provided as 10× buffer; Bioline), 0.8 mM dNTP (Sigma, Promega) each, 0.4 μM each primer and 1unit Taq DNA polymerase (Sigma, Bioline) for a 25 μl reaction. $MgCl_2$ and DMSO (in %) are added as shown in the primer table.

PCR primer specifications

PCR ID	Accession no.	Forward primer	Forward primer Seq	Reverse primer	Reverse primer Seq	Mg	DMSO	T_{ann}
Bmp4	NM_007554	Bmp4F01	tcttcaacctcagcagcatcc	Bmp4R01	ccaatcattccagcccacg	3	0	50
Bmp7	NM_007557	Bmp7F02	aagacgccaaagaaccaaga	Bmp7R02	gctcaggagaggttggtctg	3	0	50
Brachyury	NM_009309	T1F02	ccttgcataagtatgaacc	T1R02	taccattgctcacagacc	3	0	50
Cerberus1	NM_009887	Cer1F02	gcttgttctcttgcctctgg	Cer1R02	gtcttcatgggcaatggtct	3	0	50
Chordin	NM_009893	ChrdF02	ctatggctcagaggctcagg	ChrdR02	ctgcgttgtttctctggaca	3	0	50
Dkk1	NM_010051	Dkk1F01	gtccaagatctgtaaacc	Dkk1R01	gagtcaagacaatcaacc	3	10	50
Dkk2	NM_020265	Dkk2F01	atactgccacagtccccacc	Dkk2R01	gctccacattcttaccacatcc	3	0	50
Fgf15	NM_008003	Fgf15F02	catctccatatcatcttcatcc	Fgf15R02	tgctcgctaaaaccatcc	3	0	50
Fgf2	*NM*_008006	Fgf2F01	accaggccacttcaaggac	Fgf2R01	tcagctcttagcagacattgga	3	0	50
Fgf8	U18746	Fgf8F02	cacttgctggttctctgc	Fgf8R02	gcacgatctctgtgaatacg	3	0	50
Fgfr1	NM_010206	Fgfr1F01	tataaccccagccacaacc	Fgfr1R01	atgagagaagacagagtcctcc	3	10	50
Follistatin	NM_008046	FstF02	cctgtataagacagaactgagc	FstR02	gtaagtcactccatcatttcc	3	0	50
Frzb1	NM_011356	Frzb1F01	ctctgtgcaatgtacgcacc	Frzb1R01	actttcttaccaagccgatcc	3	10	50
Gapd	NM_008084	G3PDH 5′	accacagtccatgccatcac	G3PDH 3′	tccaccaccctgttgctgta	3	0	50
Goosecoid	NM_010351	GscF02	aagccctggagaacctcttc	GscR02	cagtcctgggcctgtacatt	3	0	50
Hesx1	NM_010420	HesxF01	agtaagaccccacagacc	HesxR01	ctatacaactggaagagaagc	3	0	50
Hex1	NM_008245	Hex1F01	gaaatacctctccccacc	Hex1R01	gaaagtcagtcaagatcagc	3	0	50
Foxa2	NM_010446	Foxa2F01	ggacaagggaaatgagagg	Foxa2R01	aaataaagcacgcagaaacc	3	0	55
Ihh	NM_010544	IhhF01	cagtgatgtgcttattttcc	IhhR01	tagagtcccttcagcttcc	3	0	50
Lim1	NM_008498	Lim1F02	catcccctcgactctaattgc	Lim1R02	caaaggctgccttcaacg	3	0	50
Ascl1	NM_008553	Ascl1F01	cgtcctctccggaactgat	Ascl1R01	tcctgcttccaaagtccatt	3	0	50
Nestin	NM_016701	NestinF01	agtcagagcaagtgaatgg	NestinR01	agaaacaagatctcagcagg	3	0	50
Netrin	NM_008744	Ntr1F01	gccccttgcatcaagattcc	Ntr1R01	ttgcacttgccccttcttctcc	3	0	50
NeuroD1	AK005073	NeuroD1F01	gtgcatccctactcctacc	NeuroD1R01	actgacgtgcctctaatcg	3	0	50
Neurog1	NM_010896	Neurog1F02	actctctgaccccagtagtcc	NgnR02	ctcaaaccgaattttcatgc	3	0	50
Neurog2	NM_009718	Neurog2F02	gatgccaagctcacgaagat	Neurog2R02	ggagacgagagagggagacc	3	0	50
Noggin	NM_008711	NogF02	cggccagcactatctacaca	NogR02	cttggatggcttacacacca	3	0	50
Pou5f1	NM_013633	OCT-1	cgcgttctctttggaaaggtgttc	OCT-2	ctcgaaccacatccttctct	3	0	50
Otx2	AA199520	Otx2F03	gctctgtttgccaagacc	Otx2R03	cagcatagccttgactataacc	3	0	50
Ptc1	NM_008957	Ptc1F02	ctccgcacagagtatgacc	Ptc1R02	agtcactgtcaaatgcatcc	3	0	50
Shh	X76290	ShhF01	tcacccccaattacaacc	ShhR01	acgtaagtccttcaccagc	3	10	50
Wnt3a	NM_009522	Wnt3aF01	ataccaagacctaacaaacc	Wnt3aR01	cagacagacactagagaagc	3	10	50

Acknowledgements

This work was supported by grants from the Parkinson's Disease Society UK and the Biotechnology and Biological Sciences Research Council (BBSRC) UK. We thank Jim McWhir, Tom Pratt and Susie Hunter, for the gifts of the clone 710, TgTP6.3, IMT11 ES cell lines, respectively. Robin Lovell-Badge for Sox1 antisera, the Developmental Studies Hybridoma Bank, University of Iowa for antisera.

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