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MANIPULATION OF STEM CELL FATE IN VITRO USING PLASMID-BASED TRANSCRIPTION FACTOR OVER-EXPRESSION SYSTEMS

PM Athauda arachchi[1,2] and S Chandran[2,3]

General Sir John Kotelawala Defence University, Ratmalana, Colombo, Sri Lanka[1]
University of Cambridge-BRC,Forvie Site,Addenbrookes Hospital, Cambridge, University of Cambridge, UK[2]
University of Edinburgh, Edinburgh, UK[3]

ABSTRACT

Introduction: The ability to switch stem cell differentiation fate in-vitro, is a powerful tool that may allow the generation of large numbers of cells that may be required in order to develop biologically engineered tissues and cells required for therapeutic applications such as pharmacological testing of new medications. The transcription factor ("master switch") Olig2, alone or in conjunction with Nkx2.2, has been implicated as a key cell fate decider for emerging neuro-glial precursors derived from both embryonic stem (ES) cells and from foetal neural stem (FNS) cells.

Methods: The in-vitro system of stem cells devoid of exogenous signaling was developed. Stem cells were manipulated by pIRES plasmid vector driven, constitutively expressed Olig-2 or Olig-2/Nkx2.2 transcription factor system introduced into proliferating embryonic or foetal neural stem cells, following a similar embryological temporal patterning sequence seen in-vivo.

Findings: Successful stem cell fate modification could be achieved in-vitro using the transcription factor overexpression system. Substantially different cell fates were noted in the presence of Olig-2 alone and in combination with Nkx2.2, with the achievement of premature glial differentiation.

Conclusion: This method, therefore, may be useful to generate rare live human cells (such as Oligodendroglia or specialised myocardial cells) in-vitro.

KEYWORDS— *Stem cell re-programming, fate modulation, Transcription factors*

Corresponding Author: PM Athauda arachchi, Email:*athaudarachchipm@kdu.ac.lk, pma29@ca*

https://orcid.org/0000-0001-5401-5548

1. INTRODUCTION

Provision of rare cells such as human cardiomyocytes, neural precursors and oligodendroglia, for in-vitro studies on effects of various drugs and for disease models is a much sought after goal of many research groups.

Stem cells that are thought to mediate the replacement of many cell types of the body following trauma or disease processes have been investigated for their regenerative potential. However, achieving the exact internal milieu required for this process is difficult to recreate in-vitro. Therefore, genetic manipulation such as selective, transcription factor modulation has been sought as an attractive method to achieve large numbers of desired cells in-vitro for experiments.

The process of neuroglial specification from multipotent stem cells provides valuable insight into the importance of transcriptional factors in regulating cell fate decisions during the process of embryological development. This presents an opportunity for us to develop tools and systems to gain in-vitro mimicry of this process using the over-expression of key transcription factors for stem cell fate modulation.

Olig2, described initially as a transcription factor important in the specification of oligodendrocytes (Zhou et al., 2000), (Lu et al., 2000) has since then been demonstrated to be linked to the specification of motoneurons and oligodendrocytes (Takebayashi et al., 2000), (Zhou and Anderson, 2002), (Lu et al., 2002) in the ventral spinal cord, as markers in neoplastic disease (Lu et al., 2001), in the early stages of specification of astroglia in the neural precursors in the subventricular zone (SVZ) (Marshall et al., 2005) and ependymal cells (Masahira et al., 2006).

Several groups have attempted the strategy of overexpression of Olig2 to specify oligodendroglia from stem cell populations such as olfactory ensheathing cells (Zhang et al., 2005), and mouse neural stem cells (Copray et al., 2006). Human foetal neural stem cells/precursors present an attractive prospect of a precursor cell type that has a better safety profile with their lineage restriction compared with ES cells, but have sufficient immaturity to attempt directed differentiation to generate human oligodendroglia. However, these stem cells/precursors have not been investigated for their full potential for the generation of oligodendroglia.

We have revisited the role of Olig2 in the specification of glia from neural stem cells, derived from embryonic stem cells from a system devoid of morphogens that could otherwise over or underestimate the perceived effect and also from cultures of human foetal neural stem cells, in order to understand some of the multitudinous roles for Olig2 in operation in such systems. We discuss the use of the gene over-expression strategies for generating human oligodendroglia, using a plasmid vector-driven, constitutionally expressed transcription factor system.

2. MATERIALS & METHODS

A. Cell Culture

Mouse ESCs were cultured as previously described and differentiated in COM (Wiles and Johansson, 1999), (Bouhon et al., 2005), (Bouhon et al., 2006). ESCs were maintained by routine feeder-free culture on 0.1% gelatin (Stem Cell Inc, Temecula) in lscove's Modified Dulbecco's Medium (IMOM)/ GlutaMax I (lnvitrogen, Carlsbad, CA, http://www.invitrogen.com) supplemented with 15% foetal calf serum (Bio Sera, Ringmer, East Sussex, U.K., http://www.biosera.com), penicillin/streptomycin, nonessential amino acids (lnvitrogen), 2-mercaptoethanol (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com), and 10 ng/ml leukaemia inhibitory factor (Chemicon, Temecula, CA, http://www.chemicon.com). ESC lines E14, and W9.5 (with feeders for maintenance, followed by feeder free passaging) were used, and they showed similar differentiation properties. For terminal differentiation, enzymatically dissociated ESCs were resuspended in COM and plated at $4*10^6$ cells per 16 ml in 10-cm plastic culture dishes (Grainer Biochem). The technique for the successful culture of

embryonic stem cells in a morphogenic signal free environment in-vitro has been previously described by our group (Joannides et al, 2008) and (Athauda-arachchi, 2009).

Human foetal neural stem cells were cultured as previously described (Svendsen et al., 1998). Human foetal neural tissue was acquired, governed by local ethical guidelines, from terminated foetuses between 8 and 12 weeks in gestation. Automated tissue chopping with Mcllwain tissue chopper (Mickle Engineering, UK) was used for establishment of primary cultures and passaging. The cultures were established in T75 flasks (lwaki), with Dulbecco's Modified Eagles Medium (DMEM) and Ham's F-12 medium 2:1 with GlutaMax I, 2% B27 supplement and 1x penicillin/ streptomycin (all from Invitrogen). Growth factors were used as described previously. Cell cultures were fed every 3rd day and passaged between 3 and 6 passages before terminal differentiation. For terminal differentiation, single cell suspensions were made by enzymatic dissociation of neural precursors thus cultured and plated on poly-ornithine (POrn, Sigma) and laminin-1 (Sigma) at 10 µg/ml coated coverslips at 250,000 viable cells per coverslip and cultured in Dulbecco's modified Eagle's medium/2% B-27 (Invitrogen), 1% N2(Invitrogen). Following trophic factors were used (post nucleofection) to support terminally differentiating progeny: Glial Derived Neurotrophic Factor (GDNF) at 10ng/ml and Brain-Derived Neurotrophic Factor (BDNF) and recombinant Platelet Derived Growth Factor-a (PDGF- a) at 10ng/ml (all from R&D).

Human Embryonic Kidney (HEK) cells were cultured in T25 flasks (lwaki) in DMEM and 10% foetal Calf serum and passaged as per standard procedures.

B. Construction of a plasmid vector system with constitutive Olig2/Olig-Nkx2.2 or Nkx2.2 expression

plRES plasmid vector with cytomegalovirus promoter (pCMV) was used to construct a gene expression system. The following cDNA (original cDNA clones: gift from Dr. Toru Kondo) was used to construct two types of plasmid vectors: mouse Olig2 cDNA in multicloning site A alone or along with rat Nkx2.2 cDNA cloned into multicloning site B. A plasmid construct with only Nkx2.2 in multicloning site B was also constructed. Control vectors included pl RES vector with no cDNA inserts and those with enhanced green fluorescent protein (eGFP). Standard molecular biology procedures were followed and accuracy of cloned sequences and their up and downstream sites were checked by gene sequencing. Mass scale plasmid production was performed and plasmid DNA was purified using an endotoxin free preparation methods (Quiagen, Endofree Maxi kit).

C. Transfection of HEK cells, mouse ES and human foetal neural stem cells

Delivery of the plasmid vectors into cultured HEK cells, mES and hFNS cells were performed by nucleofection using standard procedures recommended by the manufacturer (Amaxa, GmBH). These cell lines were transfected with a variety of programmes and the presence of transgene expression was confirmed by immunohistochemistry and western blotting and the most efficient transfection parameters were deduced for mES and hFNS cells. In the case of mES cells, transfected cells were selected for G418 resistance for 2 weeks and cell lines with stable transgene expression were amplified prior to differentiation. They were also checked for continued expression of pluripotency markers prior to differentiation. In the case of human foetal neural stem cells, transient transfection followed by terminal differentiation was performed without the use of G418.

D. lmmuno-cytochemistry

lmmuno-cytochemistry was performed on either free-floating whole-sphere (whole-mount) cryostat sections or plated cells as described. Primary antibodies used were the following: Anti- Oct4(1: 100, Santa Cruz) Anti-Olig2 (1:20,000; gift of Dr.David Rowitch); anti-Nkx2.2(1:50 from Developmental Studies Hybridoma Bank, Iowa City, IA, http://www.uiowa.edu/_dshbwww); anti- Human nestin (1:400 Chemicon); anti- Rat nestin (1:400; BD Pharmingen, San Diego,

http://www.bdbiosciences.com/pharmingen); anti-Beta-Ill- tubulin (1:200; Sigma-Aldrich); anti- EGFR (1:50; Affinity Bioreagents, Golden, CO, http://www.bioreagents.com); anti-glial fibrillary acidic protein (anti-GFAP) (1:200; DAKO, Glostrup, Denmark,http://www.dako.com);Anti-04, anti-A2B5 and anti-GalC supernatants (1:5 live staining, (Sommer and Schachner, 1981));. Secondary antibodies (lnvitrogen) were used at concentrations of 1:1,000.

Glass coverslips thus prepared were mounted in Vectafluor mounting medium (Vector Laboratories; Burlingame, CA, USA) and viewed a Leitz microscope with appropriate filters for cell identification and counting. For each coverslip, three consecutive random fields were counted using a grid, and all cells on each cryostat tissue section were counted. The number of positive cells was expressed as a mean± S.E.M. from 3 slides in each experiment. Each experiment was repeated at least 3 times.

E. Western Blotting

Transfected HEK cells and mES cells after long term selection were grown to 70% confluency were used to prepare protein lysates by the following washing in PBS and suspension in RIPA buffer (PBS, SOS 1%, Triton-x100 1%, Na deoxycholate 0.5%) with Mini-complete protease inhibitor (Roche) and lysed using Lysing Matrix C Ribolyser beads and a Ribolyser. The lysate was then centrifuged at 14000 rpm for 20 minutes at 4°C and the supernatant was collected for freezing at -20^{o} C or for use in experiments.

Protein quantification was done by copper sulphate and bicinchonic acid method (SCA assay (Pierce Kit). Lysate containing 20 ug of protein was used for 15% Sodium Dodecyl Sulphate- Polyacrilamide gel electrophoresis (SDS-PAGE) with PVDL blot transfer and detection. SDS- PAGE was performed for approximately 3 hours using a constant voltage of 40V. The transfer was done at constant direct current at 300 mA for 1 hr. Primary antibodies used were Olig2 (1:40,000; gift of Dr. David Rowitch); anti-Nkx2.2 (1:100 from Developmental Studies Hybridoma Bank, Iowa City) and secondary antibodies used were Horseradish peroxidase conjugated secondary antibodies.

F. Co-immuno-precipitation

Co-immuno-precipitation was performed to demonstrate the presence of interaction between mouse Olig2 and rat Nkx2.2 in HEK cells using immunoprecipitation starter pack (GE Healthcare Inc), using Sepharose H beads soaked in RIPA lysis buffer to adsorb complexes saturated with Anti-Nkx2.2(DSHB 1:100) overnight and detecting the complexes with western blotting using Anti-Olig2(1:40,000 gift from Dr. D. Rowitch).

G. RT-PCR analysis of gene expression

Day 8 early neurospheres derived from the mES cells with transfected plRES-Control (referred to as the Ctrl) and plRES-Olig2 (referred to as the test) plasmids, were snap frozen in -80^{o}C or added with RNAse later (Quiagen) prior to storage in -80°C.These were then used for extraction of mRNA. This involved the use of RNAeasy RNA extraction kit (Quiagen), according to the manufacturer's instructions.

The mRNA thus obtained was assessed for quantity and purity, using a spectrophotometer (GeneQuant Pro). cDNA was synthesized using Superscript Ill (lnvitrogen) and random hexamers according to manufacturers' instructions. One microlitre each of cDNA was used to analyze for the presence of the transcripts.

3. RESULTS

We established the accuracy of the gene cloning by independent sequencing (Lark Technologies, Takerly, UK) and checked for expression of Olig2 at protein level in human & mouse cells by western blotting and demonstrated that the Olig2 and Nkx2.2 expressed by this system form a physical complex by PCR, co-immunoprecipitation and imunnohistochemical evidence of expression (Fig 1,2 ,3,4,5).

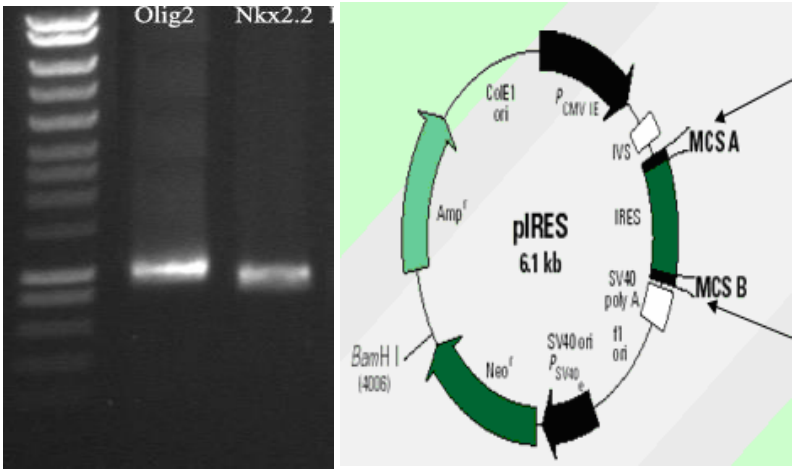


Figure 1: Cloned PCR amplicons Olig 2 and Nkx2.2, from cDNA library, and intended multicloning sites (MCS) A or B of plamid vector pIRES

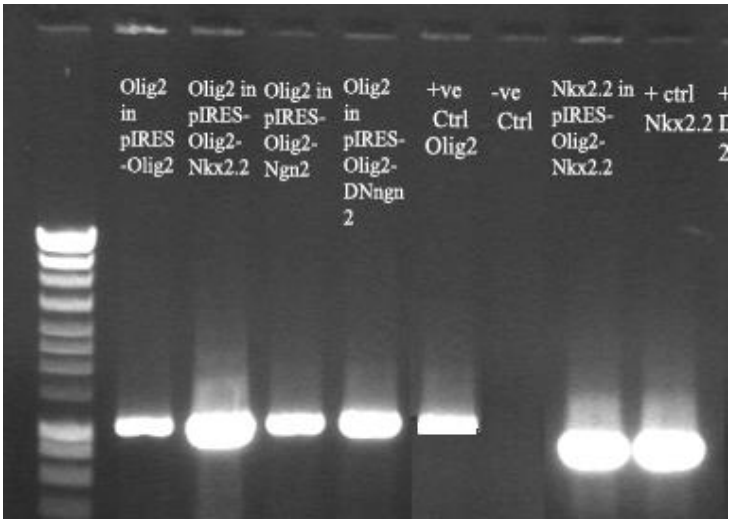


Figure 2: PCR detection of over expression of cDNA Amplicons at MCS A or MCS B in various plamid contructs tested in mouse ES cells

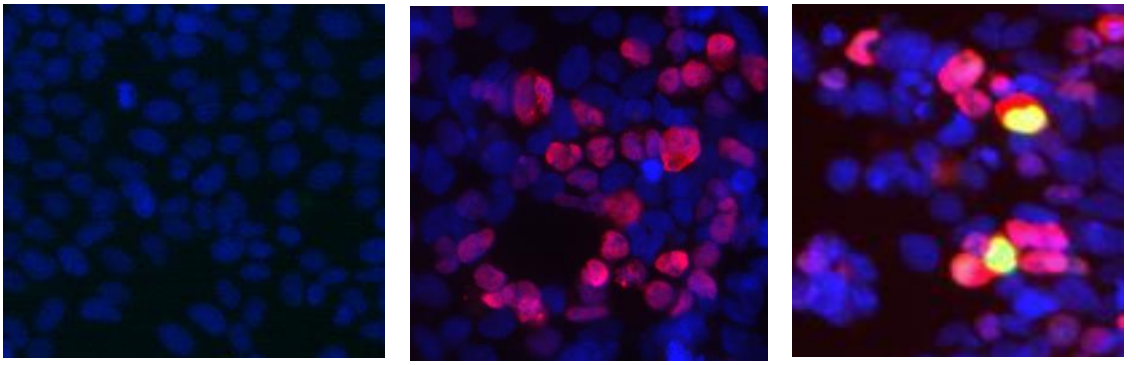


Figure3: Proof of Expression of plasmid based Olig2 and Nkx2.2 in tranfected HEK (Human Embryonic kidney cells) assessed by immuno-histochemistry.A-Control pIRES plasmid transfected cells-no expression of either Olig2 or Nkx2.2. B-Olig2 cDNA containing pIRES plasmid transfected cells-Olig2 expression.C-Olig2-Nkx2.2 double cDNA containing pIRES plasmid transfected cells- express both Olig2 & Nkx2.2

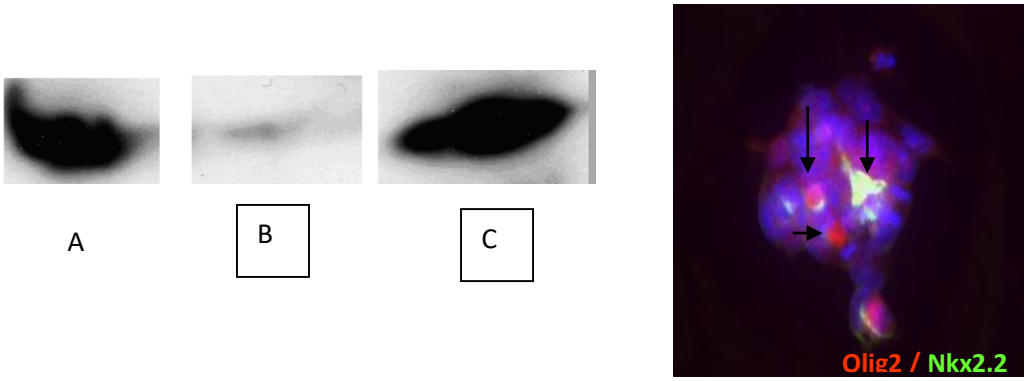


Fig 4: Immunoprecipitation using protein lysates from mouse embryonic Stem cells transfected with Olig2 and rat Nkx2.2 in pIRES-Olig2-Nkx2.2 transfected cell collections:
A- western blot confirmation of olig2 expression of 32 kDa protein band
B- control for non-specific binding: beads only
C- antigen pull-down from lysates with anti-rat Nkx2.2 and recognition of complex with anti-mouse-olig2 during co-immunoprecipitation

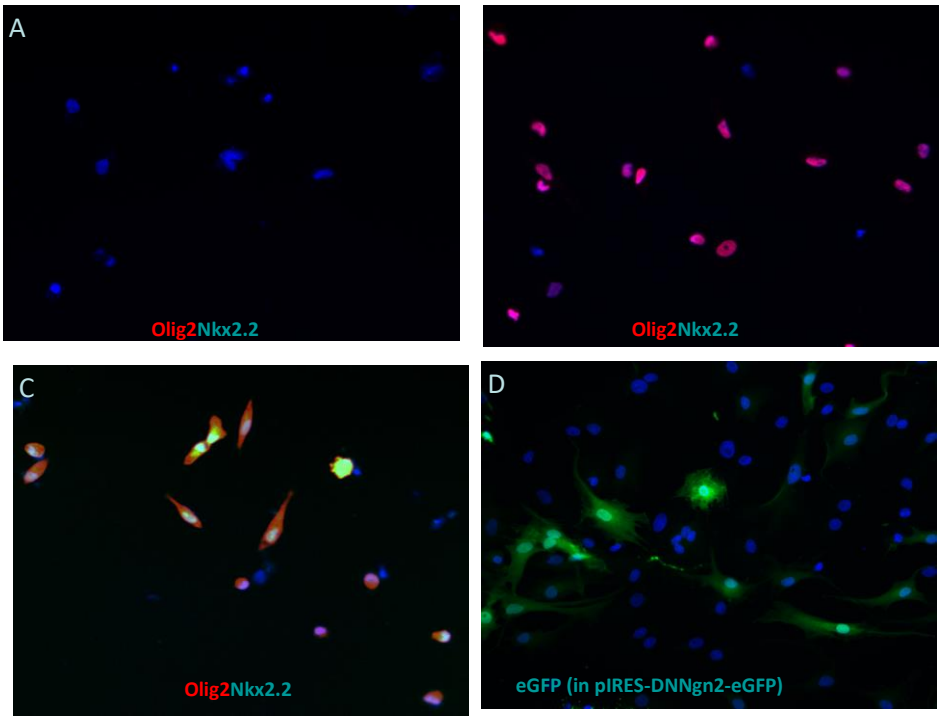


Figure 5: Expression of Olig2, Olig2 with Nkx2.2, GFP (in B locus of pIRES with dominant negative Ngn2 in A locus) in human foetal neural stem cells 48 hours post transfection with pIRES constructs:
A-pIRES control, B-pIRES-Olig2,C-pIRES-Olig2-Nkx2.2, D-pIRES-ΔNgn-GFP

A. Mouse ES cells fate regulation

At the commencement of the experiment, the cellular morphology and expression of pleuripotency marker Oct-4 were comparable in both the controls and Olig2 over-expressing mES lines. The only difference detected was the expression of Olig2.

Terminal differentiation of early (day8) neural precursors for 10 days demonstrates that in the presence of Olig2 over-expression, premature gliogenesis occurs when compared to the controls which generate a predominant neuronal phenotype (Fig 6). Quantification reveals that 10 days post-plating, GFAP positive cells arise (52.82% +/-1.75% n=3) with a reduction of ß -I11-tubulin positive cells (40.48%+/-2.89% n=3) in the presence of Olig2 misexpression, in sharp contrast to the controls where a predominance of ß -11I-tubulin positive cells (91.88%+/-2.01%, n=3) were present, without any cells expressing GFAP (0%, n=3) (Fig 7). The GFAP positive cells with Olig2 misexpression also co-stained for S100f3 but do not stain for nestin (figure 8). Olig2 over expression also leads to the presence of bipolar A2B5 positive, nestin negative cells at 5 days' post plating (Fig 9), but does not generate 04 positive cells at day 10 (0%, n=3) under these conditions.

In contrast to the above, terminal differentiation of early (day8) neural precursors for 10 days in the presence of over-expression of both Olig2 and Nkx2.2, 04 positive cells were also analysed (6.083%+-/0.57% n=3) (figure 11). Some of these 04 positive cells also stained for GalC (1.86%+/-0.15% (n=3)) and appeared morphologically larger and more arborised; whilst the remainder were only expressing 04 and were comparatively smaller, reminicient of early oligodendroglia (fig 10).

In the presence of Nkx2.2 and Olig2 expression, the total number of glial cells does not differ significantly from the situation when only Olig2 is over-expressed (48.68%+/- 4.34% n=3 and 52.82%+/-1.75% n =3 respectively, figure 10). Further, there is no significant difference in the beta-11I- tubulin positive cell numbers in the two situations above (39.45%+/-2.92% n=3 and 40.48%+/- 2.89% n=3 respectively). This implies that the presence of Nkx2.2 is immaterial to the neuronal-glial fate decision making which therefore is entirely dependent on the presence or absence of Olig2 misexpression in this system. In contrast, Nkx2.2 in the presence of Olig2 is likely to affect an oligodendroglial-astroglial fate decision after the neuronal-glial fate decision has occurred.

B. Human neural stem cells fate regulation & demonstration of specification of human oligodendroglia in-vitro

We examined the fate of human foetal neural stem cells subjected to transient over-expression of Olig2 with or without Nkx2.2(Fig 5). The transfection efficiencies for these cells were low (27.41%+/-1.36%) even under optimal conditions. However, this was still sufficient to specify 04 expressing cells after 14 days of terminal differentiation, when both Olig2 and Nkx2.2 were over-expressed (3.087%+/-0.49%, n=3). There were also 04 and GalC double positive arborized cells with typical human oligodendroglial morphology at day 14 (1.75%+/-0.226%, n=3), (figures 12,13). Olig2 alone was not sufficient to specify 04 positive cells, neither were the controls.

4. DISCUSSION

A. Premature acquisition of gliogenic competency during the generation of mouse ES derived early neural precursors an human neural precursors triggered by over-expression of key transcription factors

Directed differentiation of neuronal and glial precursors needs a thorough understanding of how cell fate decisions occur during development can be studied using ES neuralisation. The radial glial specification and their transition from an early neuronal to late glial competency in the context of ES differentiation is a much discussed phenomenon (Liour et al., 2006), (Pollard and Conti, 2007). Further, tissue derived neural precursors seem to exhibit a similar pattern in development through radial glial stages in-vivo (Gotz and Huttner, 2005; Misson et al., 1988). Transcription factors have been described to affect the progeny specified from radial glia in culture (Hack et al., 2004).

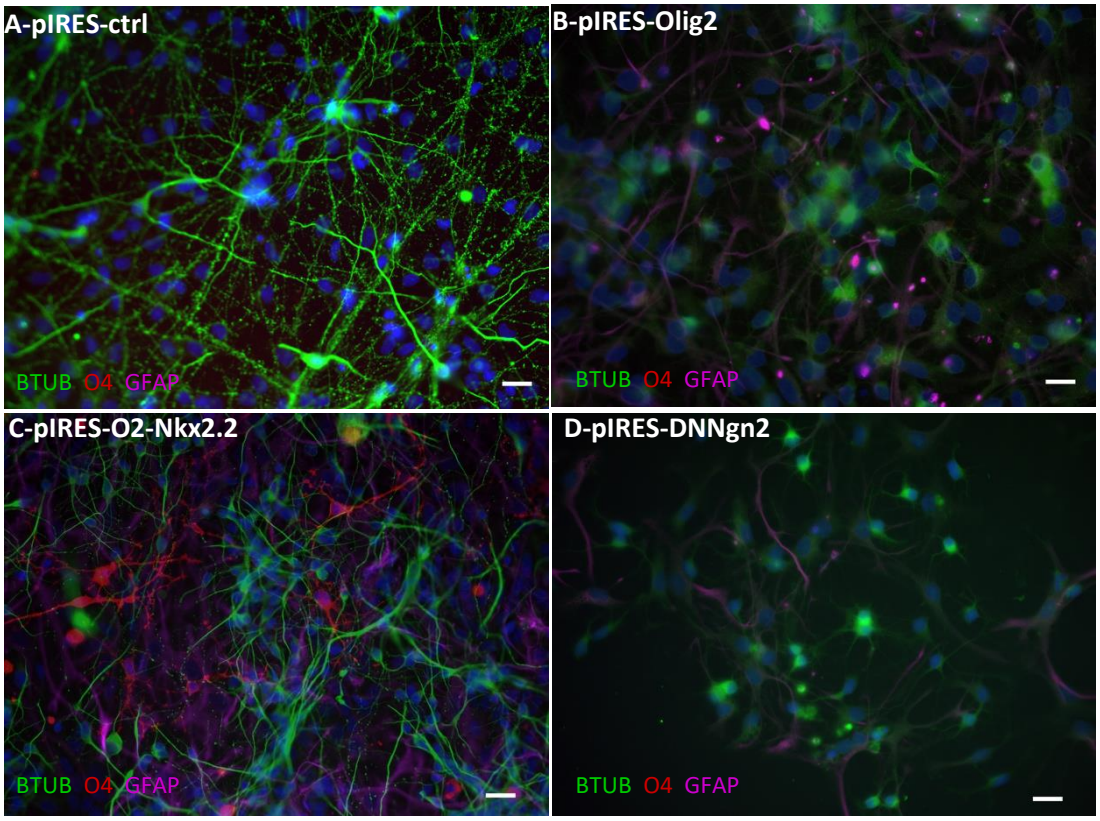


Figure 6: mES derived NPCs terminally differentiated and subjected to immuno-cytochemical analysis for lineage markers: GFAP (astroglial) ß-III-Tubulin (neural) lineages

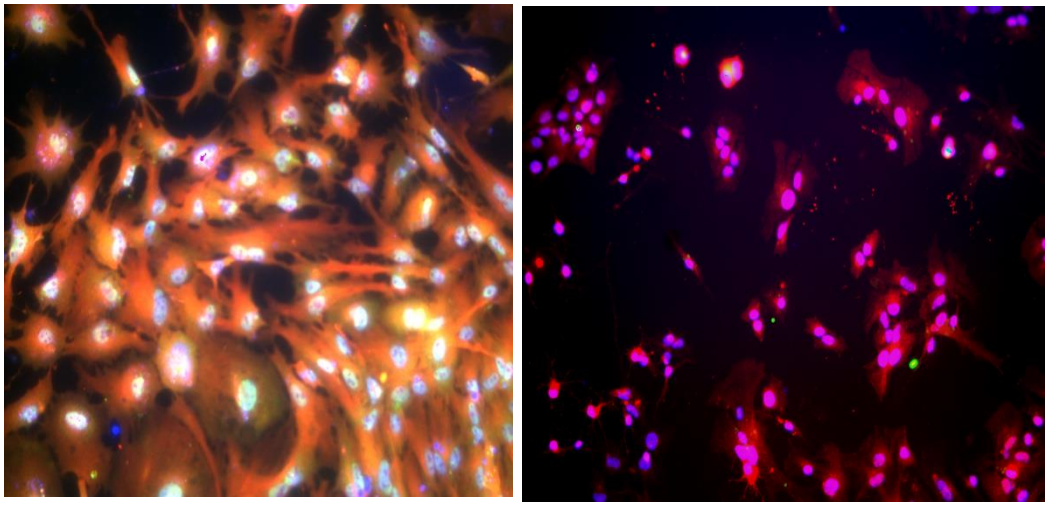
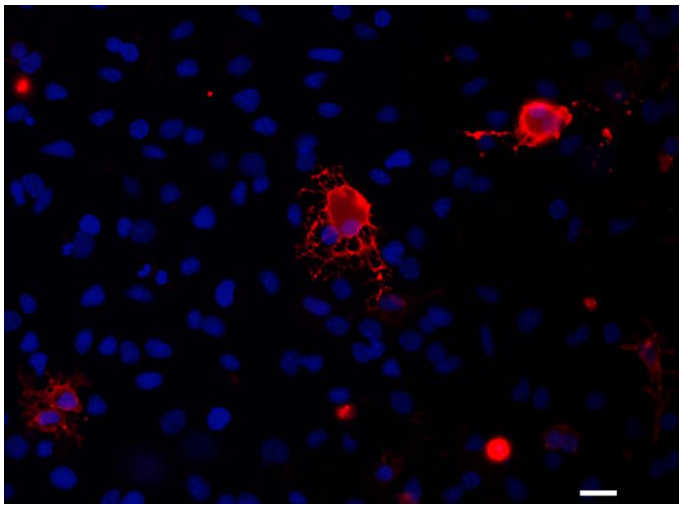
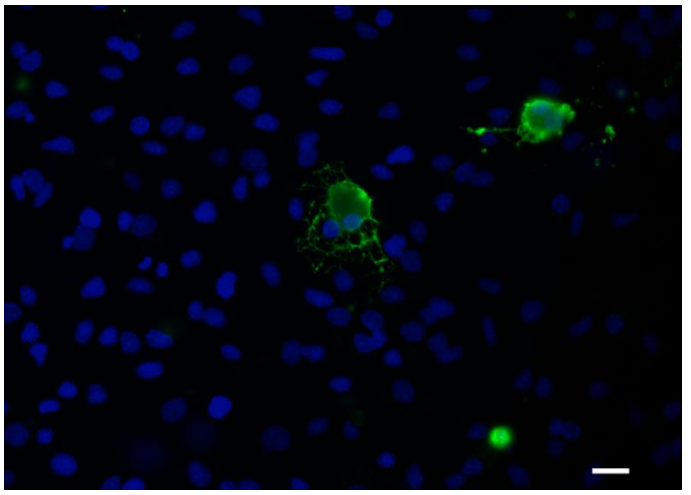


Figure 7: mES derived NPCs terminally differentiated and subjected to immuno-cytochemical analysis - additional immunocytochemical markers present in GFAP positive cells



Demonstration of the presence of oligodendroglial markers O4 and GalC amongst terminally differentiated progeny 10 days after plate down.



Note the presence of some O4 +ve cells with GalC (larger, more arborised cells) or without GalC (smaller cells) expression.

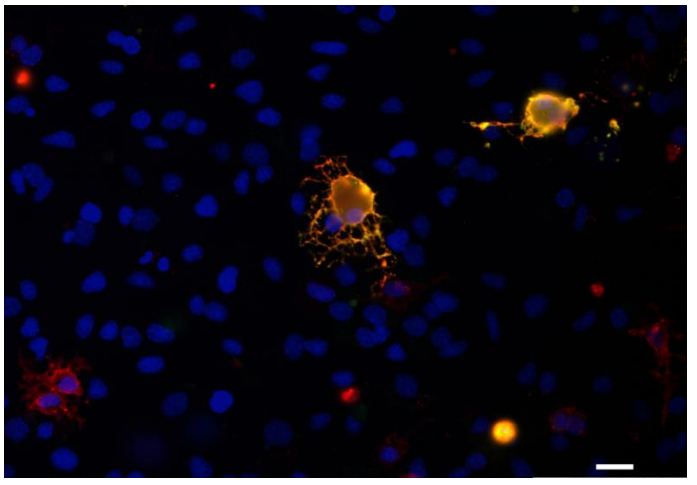


Figure 8: terminal differentiation of mouse ES cells, transfected with pIRES-Olig 2 and Nkx 2.2 constructs.

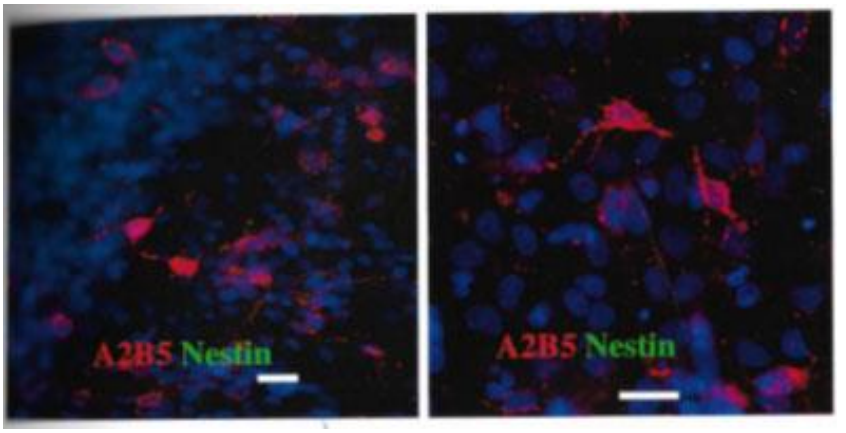
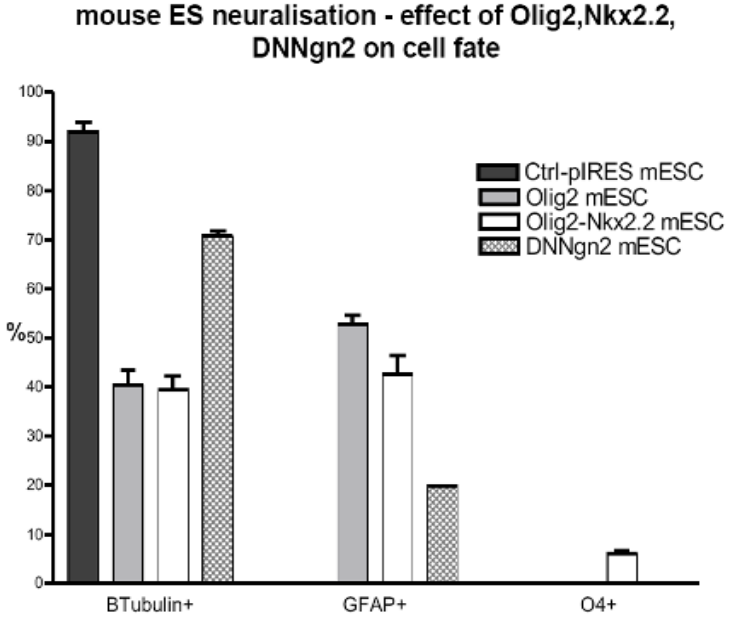


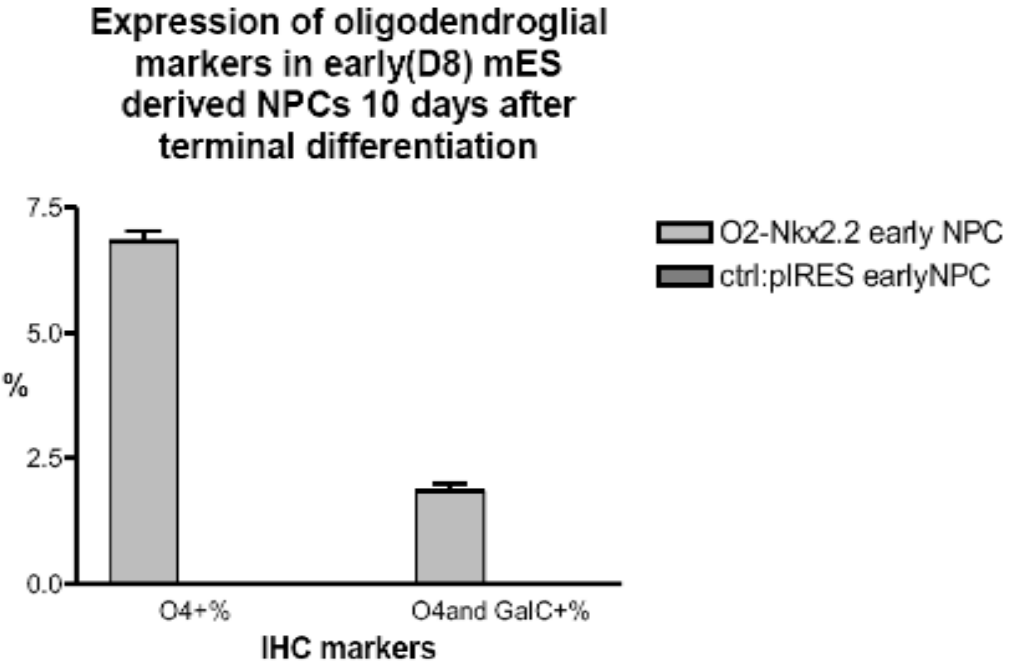
Figure 9: Further characterization of some progeny of pIRES-Olig2 transfected mES NPC (D8) terminally differentiated for 5 days after plate down. Immunohistochemical analysis with primary antibodies mouse lgM supernatants with antiA2B5 and mouse anti-rodent-Nestin. [Occasional A2B5 positive cells (which are mostly bipolar in morphology) and negative for nestin are seen, suggestive of a phenotype of late OPCs. (higher magnification on right-Scale bars 25 µm].



X Labels	Ctrl-pIRES mESC			Olig2 mESC			Olig2-Nkx2.2 mESC			DNNgn2 mESC		
	Y1	Y2	Y3	Y1	Y2	Y3	Y1	Y2	Y3	Y1	Y2	Y3
BTubulin+	93.92	93.87	87.86	41.44	44.94	36.06	42.51	42.22	33.61	71.29	68.85	72.03
GFAP+	0.00	0.00	0.00	50.36	51.90	56.21	45.17	47.48	35.17	19.91	19.86	19.60
O4+	0.00	0.00	0.00	0.00	0.00	0.00	6.17	7.03	5.05	0.00	0.00	0.00

Quantification of terminally differentiated mES derived from day 8 NPC, 10 days after plate down. Data for the final triplicates of experiments with fully dissociated precursors shown in table.

Figuree: 10- progeny emerging from differentiating mES neural precursors by Immuno-cytochemistry



IHC markers	O2-Nkx2.2 early NPC			ctrl:pIRES earlyNPC		
	Y1	Y2	Y3	Y1	Y2	Y3
O4+%	7.2100	6.4300	6.8200	0.0000	0.0000	0.0000
O4and GalC+%	1.6100	1.8400	2.1400	0.0000	0.0000	0.0000

Quantification of O4 alone or O4 and GalC positive cells amongst the terminally differentiated NPC (day8) derived from pIRES-O2-Nkx2.2 mES cells 10 days after plate down.
(Data for triplicates of experiments is shown in table.)

Figure: 11: Oligodendroglial markers expressed by differentiating mES neural precursosr transfected with constructs

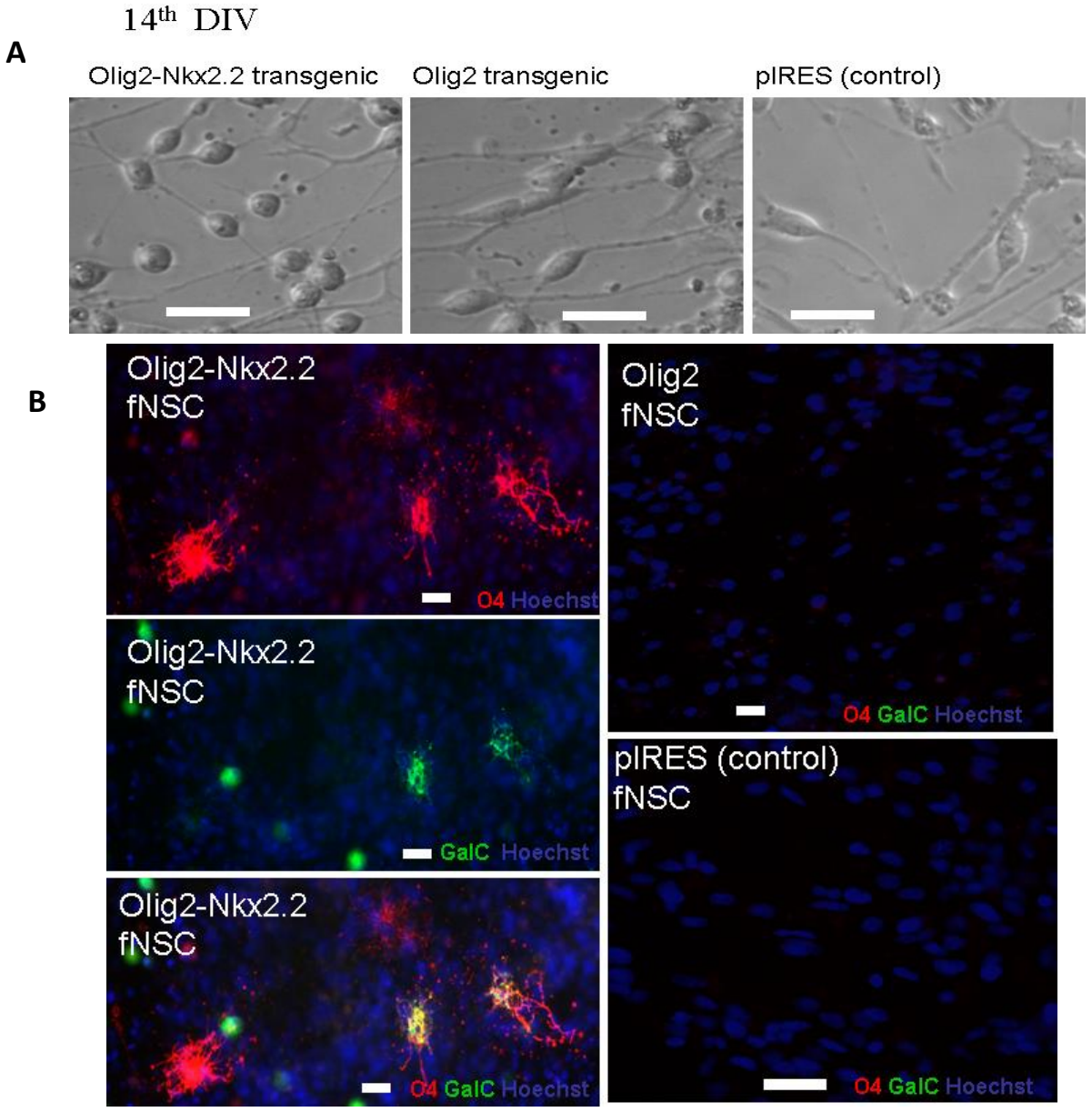


Fig: Appearance foetal NPC, after 14 days in vitro differentiation:
A-Live images.
B-Expression of oligodendroglial markers O4 and GalC, only in coverslips with the Olig2-Nkx2.2 transfected cells.
(Scalebars 25 um).

Figure: 12 : Terminal differentiation of human neural precursors transfected with transcription factor expressing plasmid constructs

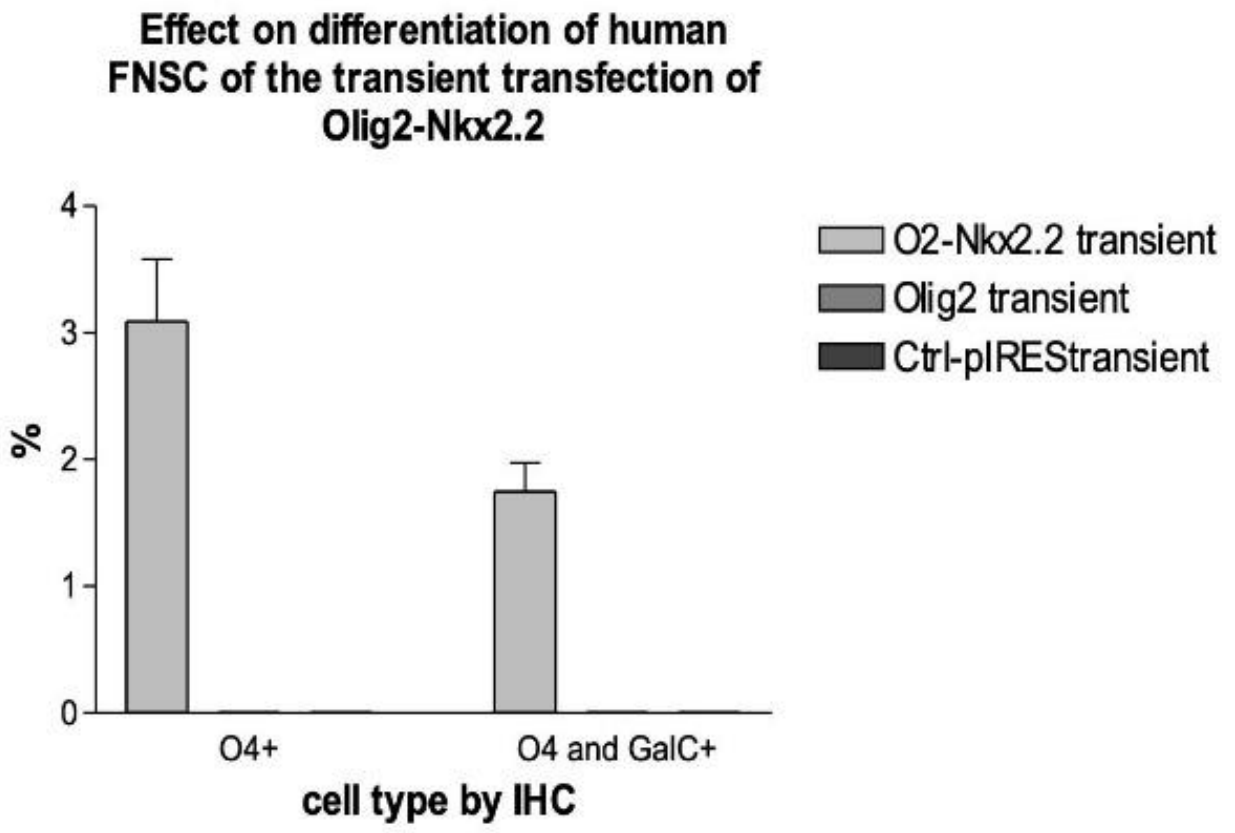


Figure: 13: Immuno-cytochemical analysis of terminal cell fate and specification of human oligodendrocytes in-vitro from human foetal neural stem cells transfected with Olig-2 & Nkx2.2 over-expression.

More recently, 3-D aggregate in-vitro culture techniques also suggested the association of high levels of these transcription factors during glial development. (Youn W. et al., 2020).

Therefore, along similar lines, we investigated whether Olig2 alone or in combination with its binding partners, can accelerate the process of acquisition of glial competency. A major practical problem in elucidating the true effect attributable to a transcription factor is the necessity in tissue culture for added morphogens, such as retinoids and sonic hedgehog, which could confound the perceived effect. Therefore, we chose to study the effect of Olig2 on neural precursors developing from embryonic stem cells in an in-vitro model of neuralisation, which does not employ the use of such morphogens. We also investigated the potential of utilising such a strategy for generating human oligodendroglia.

We have demonstrated that Olig2 can accelerate the process of achieving a premature glial competency. Given the similarity of sub-ventricular zone derived precursors/radial glia to ES derived neural precursors (Liour et al., 2006), our findings are in keeping with the observations made by two other groups for a proposed novel role for Olig2 in the process of differentiation of subventricular zonal precursors to astrocytes as well as oligodendrocytes (Marshall et al., 2005), (Masahira et al., 2006) and also indirectly supports the theory of glial restricted progenitors arising during neural development (Mayer-Proschel et al., 1997), (Rao and Mayer-Proschel, 1997). We

also demonstrate that the technique of transcriptional factor control can be applied to reveal the presence of a considerable oligodendroglial potential in human foetal neural stem cells, which have a much limited nascent potential to differentiate into many cell types, compared to ES cells. Therefore, this may be a safer and efficient method to attempt direction of differentiation to achieve human oligodendroglial cells for research.

B. Proof of concept of directed stem cell differentiation, using cDNA vectors in-vitro for diagnostic & therapeutic applications

Extrapolation of these results suggests that the co-operative action of Olig2 and Nkx2.2, which are highly conserved transcription factors in evolution, irrespective of the source of cDNA extraction, under optimal DNA transfection or transduction conditions, should be able to restore the oligodendroglial potential in a vast majority of cultured human foetal neural stem cells as well as embryonic stem cells, mirroring normal.

5. CONCLUSION

Whilst acknowledging this is a limited study of proof of concept, we conclude that directed differentiation of human foetal neural stem cells or embryonic stem cells, under relevant transcriptional regulation, may represent an attractive option for the generation of rare human cells such as oligodendroglia, illustrating potential for stem cell based diagnostic or therapeutic applications in many other fields such as neurology, cardiology or endocrinology.

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