Down regulation of the Sonic Hedgehog signalling pathway during neuronal differentiation of human teratocarcinoma (NT2) cells

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Introduction

Neural stem cells (NSCs) in the central nervous system has the capacity of self-renewal and differentiation into neurons (1). These cells are of interest for understanding of the basic developmental biology of the brain as well as the development of brain tumours. It is believed that NSCs may serve as potential therapeutic reagents for neurodegenerative disorders (2). Little is known about the biology of NSCs, thus investigations of the signalling pathways regulating the survival and differentiation of these cells are needed.

The Hedgehog (Hh) signalling pathway (Fig. 1) is involved in regulation of left-right asymmetry, development of many organs including the central nervous system, limbs, lungs, heart and eyes (3), as well as regulation of the proliferation of neural progenitors in the adult brain (4).

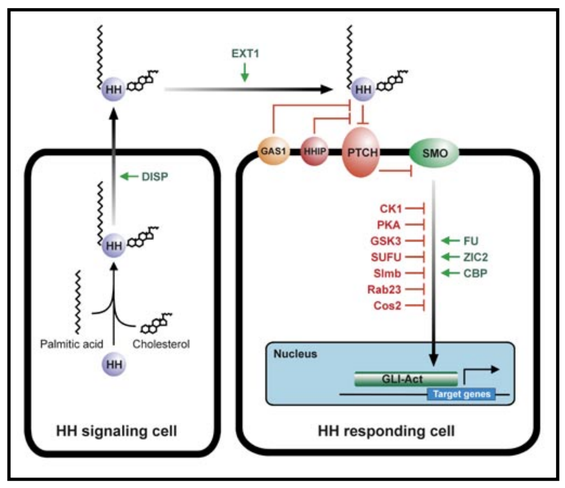

Figure1. *Simplified overview of the hedgehog (Hh) signaling pathway.*

Human teratocarcinoma NT2 (Ntera/D1) cells can be induced by retinoic acid (RA) to differentiate into postmitotic central nervous system neurons (Fig. 2). We have used RT-PCR experiments to investigate the involvement of Hh signalling during neuronal differentiation of NT2 cells.

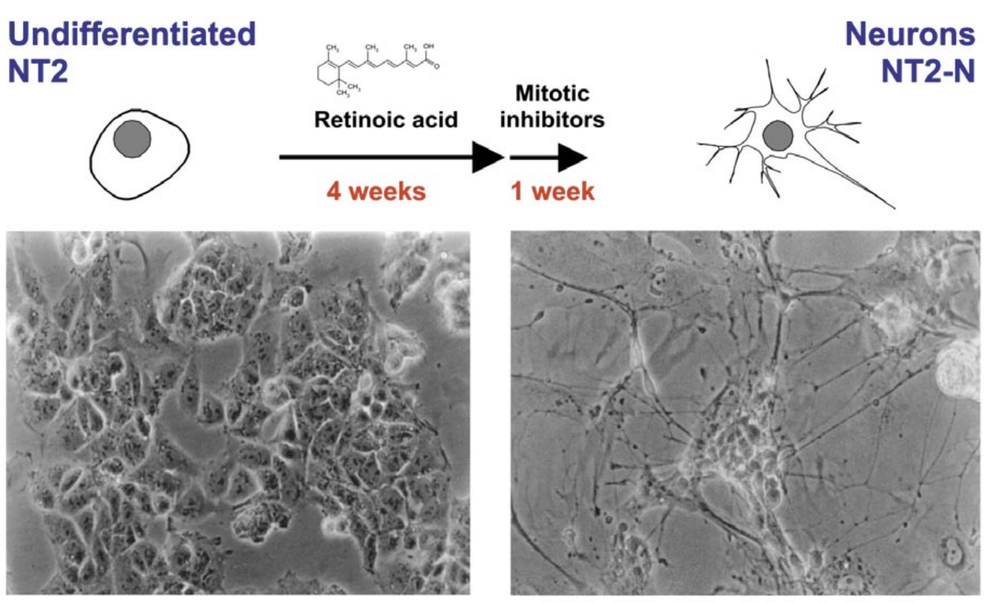

Figure 2. *Neuronal differentiation of NT2 (Ntera2/D1) teratocarcinoma cells. NT2 precursor cells (left) were treated with 10 μM retinoic acid for 4 weeks followed by treatment with mitotic inhibitors for one week. During the second period the neurons were enriched by differential trypsinisation.*

Materials & Methods

NT2 precursor cells (Stratagene) were cultured and differentiated according to the supplier's instructions. In brief, NT2 cells were grown in D-MEM/F-12, supplemented with 10% FBS and 2 mM L-glutamine. RA induction was performed in D-MEM/F-12, 10% FBS and 10 μM RA for four weeks. Subsequently, the cells were grown for one week in D-MEM/F-12, 5% FBS supplemented with 10 μM Uridine, 10 μM 5-fluoro-2'-deoxyuridine and 1 μM Cytosine β-D-Arabinofuranoside. All growth media was supplemented with 1% penicillin-streptomycin. Enrichment of neurons was performed by differential trypsinisation.

RNA was extracted using TRIzol reagent (Invitrogen). Synthesis of cDNA was performed by extension of an anchored oligo d(T) primer using superscript II reverse transcriptase (Invitrogen). Real-time RT-PCR experiments were performed in a DNA engine Opticon™ instrument (MJ Research) using a Faststart DNA master SYBR Green I kit (Roche Diagnostics). Relative mRNA concentrations were calculated using a standard-curve based on ten-fold (10^{-1} to 10^{-12}) serial dilutions of RT-PCR products. Quantitation of the "housekeeping gene" glucose-6-phosphate dehydrogenase (*G6PD*) was used to adjust for differences in total mRNA/cDNA concentration between samples. Semi-quantitative RT-PCR was performed using *Taq* DNA polymerase with reaction conditions as recommended by the manufacturer (Amersham-Pharmacia Biotech). Detection of PCR products was performed in 2% agarose gels using EtBr staining. All PCR primers were designed so that PCR fragments generated from genomic DNA would span at least one intron. Primer sequences can be obtained by request to the first author.

References

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Results and Discussion

Analysis of the expression profile of GLI1, GLI2, GLI3 (Hh mediators) and PTCH (Hh target gene) at different time-points during neuronal differentiation of NT2/D1 cells show that Hh signalling is active in NT2/D1 precursor cells and down regulated 10-100 fold during the differentiation process as indicated by the expression levels of GLI1 and PTCH (Fig. 3). Interestingly, the data show a drop in Hh signalling in a 5-10 days period immediately following initiation of RA treatment. However, the precursor cells seem to compensate for RA inhibition suggesting involvement of other yet unknown factors. The slow drop in expression level of GLI1 and PTCH from day 39-46 probably reflect the slowly increasing proportion of neurons during the treatment with mitotic inhibitors and selective trypsinisation. The expression level of GLI2 and GLI3 seems unaffected by RA treatment. However, presence of GLI2 and GLI3 mRNA does not necessarily indicate active Hh signalling. In contrast to GLI1, inactivated forms of GLI2 and GLI3 proteins function as repressors of Hh signalling (5).

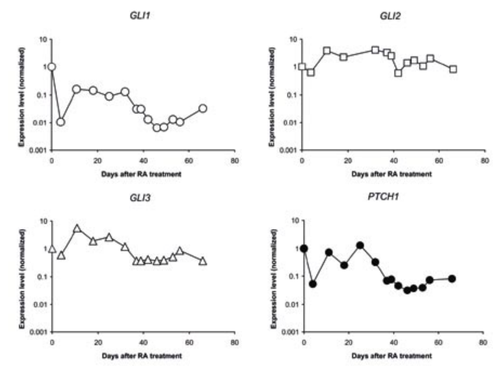

Figure 3. *Real-time RT-PCR analysis of Hh signaling. Quantitative Analysis of the expression profile of genes involved in Hh signalling. GLI1, GLI2 and GLI3 are mediators of Hh signalling and PTCH are a target gene. Gene expression was measured at different time-point during neuronal differentiation of NT2 cells.*

Investigation of the expression level of three Hh signalling modulators (HHIP, FU, SUFU) and two possible Hh target genes (WNT5A and WNT7A) revealed an upregulation of FU and WNT7A in neurons (Fig. 4A). The biological function of Fused is largely unknown, thus the observed up-regulation of FU expression in neurons is especially interesting.

It has recently been demonstrated that Hh signalling can induce G_1 cyclin expression in murine cerebellar granule neuron precursors (5). Thus it is tempting to speculate that Hh signalling regulates stem cell proliferation through control of cyclin gene expression. RT-PCR experiments suggest that CCNB1 and CCNE1 encoding cyclin B1 (M-phase cyclin) and cyclin E1 (G_1 phase cyclin), respectively, is down regulated in neurons compared to NT2 precursor cells (Fig. 4B). Thus, the preliminary data suggest that the neurons are withdrawn from the cell cycle but the apparently unchanged expression of cyclin D1-D3 contradict a model in which G_1 cyclin expression is dependent of Hh signalling.

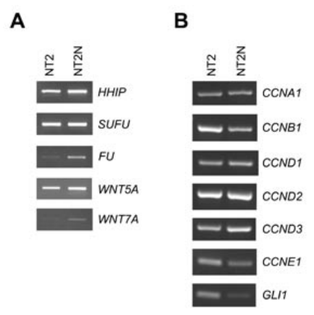

Figure 4. *RT-PCR analysis of Hh signaling. Semi-quantitative analysis of gene expression in NT2 precursor cells and neurons (NT2-N). Real-time quantitative RT-PCR analysis of the "housekeeping gene" G6PD revealed a 1:3 ratio of total RNA/cDNA concentration between NT2 and NT2-N, respectively (data not shown). A. Hh mediators and potential target genes. B. Genes encoding cyclin A1, B1, D1, D2, D3 and E1.*

Conclusion

Hh signalling is down regulated during neural differentiation of NT2 cells, which is in agreement with recent results showing that Hh signalling is involved in regulating proliferation of neural progenitors (4). Furthermore, the data suggest that NT2 cells may serve as a human model for investigation of signalling transduction in NSCs.