Development of differentiation assay for neuroblastoma

Zuzanna Urban[1], Kevin Petrie[1], Louis Chesler[1,2]

Introduction

Neuroblastoma is the most common extracranial solid tumour in children with the incidence of around 1 in 7000 live births. Long term survival of children with high-risk neuroblastoma is less than 40% and therefore there's a huge need to develop new treatment strategies. Amplification of *MYCN* and overexpression of its coded protein is associated with rapid tumour progression and poor outcome.

Induction of terminal differentiation is a very promising approach to neuroblastoma treatment. However, there's no golden standard to asses neuroblastoma differentiation and therefore the first aim of this project was to develop such assay. In my project several methods have been employed to measure differentiation level in order to develop a reliable differentiation assay. Combined analysis of neurite outgrowth and expression of numerous neuronal markers can give an overview of neuroblastoma cells' differentiation status.

MYCN plays an important role in maintaining undifferentiated phenotype and is negatively regulated by retinoic acid. To improve our ability to target MYCN and to further understand the interplay between MYCN and retinoic acid further goal of this work is to conduct a siRNA screen in isogenic cell lines with/without MYCN upon AtRA treatment in search of synthetic lethality.

The final aim of this project is to investigate impact of novel differentiation-inducing agents such as vitamin D analogues and epigenetic drugs on neuroblastoma cell lines, to further enhance level of differentiation.

Results

Morphological differentiation

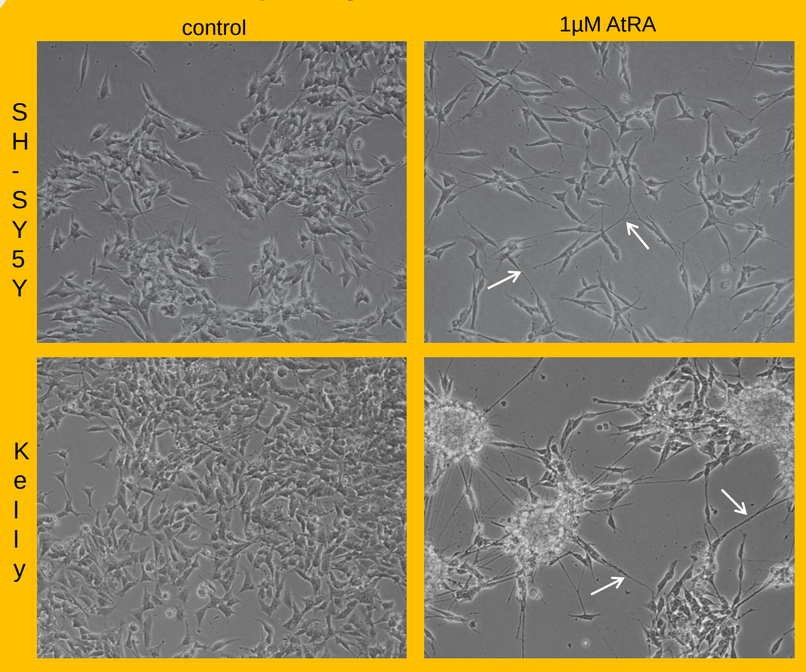


Figure 1. **Morphological differentiation**
SH-SY5Y cells (not expressing *MYCN*) and Kelly (with *MYCN* amplification and overexpression) were treated with 1µM all-trans retinoic acid (AtRA) for 7 and 14 days, respectively, along with controls (DMSO and ethanol). Even after 1 day of treatment SH-SY5Y cells started to change morphology, cells becoming longer and growing neurites, reaching its maximum on 7th day. It took much longer for *MYCN* amplified Kelly cells to undergo morphological differentiation with the most striking morphological change on day 14th. Such result suggests that MYCN may play a role in inhibiting neuronal differentiation in neuroblastoma cells.

Neuronal markers expression (protein)

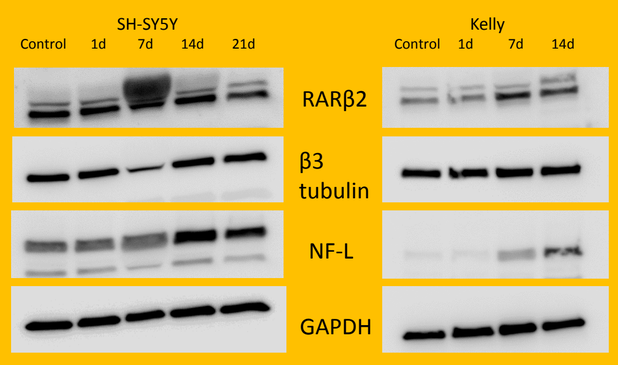


Figure 2**. Neuronal markers expression**
SH-SY5Y and Kelly cells were treated with 1µM AtRA for 21 and 14 days respectively. Morphological differentiation shown in Figure 1 is followed by increase in neurofilament-L protein expression in both cell lines.

Ascl1 expression (qPCR)

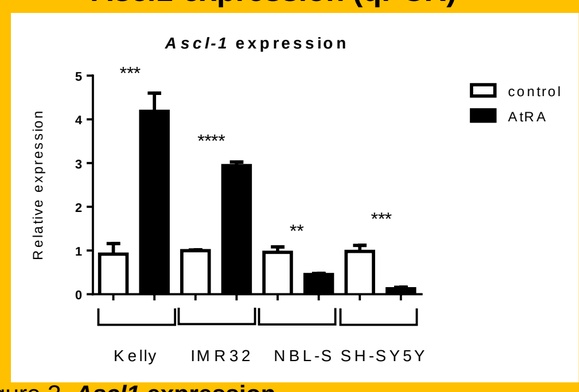
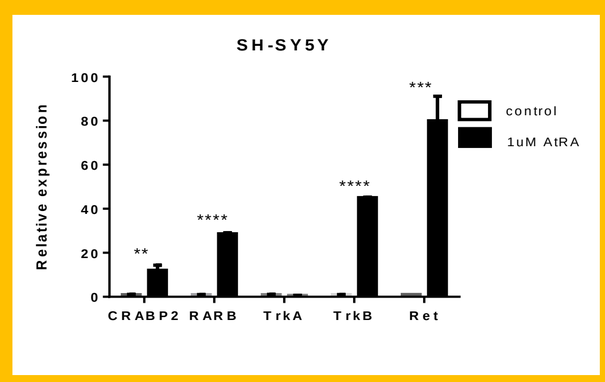


Figure 3**. *Ascl1* expression**
Neuroblastoma cell lines were treated with 1µM AtRA for 7 days. *Ascl1* expression seems to be elevated in *MYCN*-amplified cell lines (Kelly, IMR32) and reduced in non-*MYCN* amplified cell lines (SH-SY5Y, NBL-S).

Neuronal markers expression (mRNA)



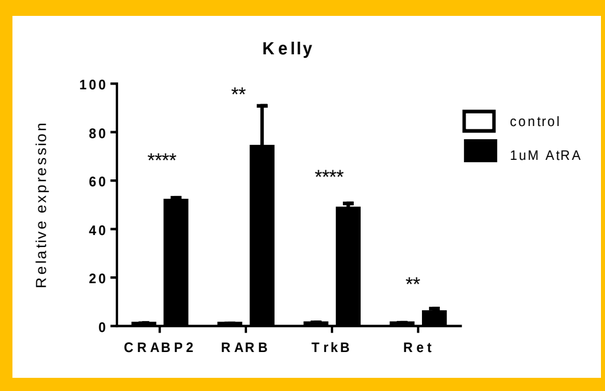


Figure 4**. Neuronal markers expression (qPCR)**
SH-SY5Y and Kelly cells were treated with 1µM AtRA for 7 days. mRNA was harvested and expression of retinoic acid response genes (*CRABP2* and *RARβ*) as well as neuronal differentiation genes (*TrkA*, *TrkB*, *Ret*) has been analysed. Expression of all genes, except for TrkA, is elevated in AtRA-treated cells therefore they may serve as differentiation markers.

Discussion

Poorly differentiated cancers present an aggressive phenotype – they grow quickly, have higher metastatic potential and are correlated with unfavourable outcome. The idea of differentiation therapy is therefore well understood. However, there's no standard method to assess differentiation in neuroblastoma cells. A reliable and robust differentiation assay is therefore necessary to correctly distinguish differentiated cells i.e. after treatment with a drug.

Change of morphology is the most often used method to asses differentiation. However, it's rather subjective and not easily quantifiable. To simplify measurement of neurite outgrowth it is planned to create cell lines expressing GFP (GFP expression would enable more automatized measurement of neurite outgrowth with the use of confocal microscope and appropriate software). Analysis of both morphological change and neuronal markers expression can give a reliable assay to assess differentiation.

Presented differentiation assay will be used in further steps of my PhD project. These will include siRNA screen (in search of genes which block AtRA-induced differentiation) and combination of AtRA with several agents that may enhance differentiation (such as epigenetic drugs, Hsp90 inhibitors, novel vitamin D analogues). Conclusions of these studies will be drawn on the basis of differentiation assay outcome and therefore it has to be accurate, robust and reliable.

Contact

Zuzanna.Urban@icr.ac.uk

[1] Institute of Cancer Research, 15 Cotswold Road, SM2 5NG, Sutton, Surrey
[2] The Royal Marsden NHS Trust, Downs Road, SM2 5PT Sutton, Surrey, UK

Acknowledgments

The research leading to these results has received funding from the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme FP7/2007-2013/ under REA grant agreement n° 315902.