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DECIDE

Development of differentiation assay in neuroblastoma to elucidate the role of MYCN in differentiation

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Background

Neuroblastoma is the most common extracranial solid tumour in children; patients with undifferentiated neuroblastoma subtypes have a poor prognosis with 5-year overall survival of only 50%. 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 aim of this project was to develop such assay. Using a well-established differentiation agent, retinoic acid, we developed a reliable differentiation assay by combining analysis of neurite outgrowth and expression of numerous neuronal markers.

MYCN plays an important role in stem cell homeostasis and cell proliferation. Several contradictory studies have shown that MYCN either decreases upon retinoic acid treatment (Amatruda, Sidell et al. 1985, Thiele, Reynolds et al. 1985) or is necessary for the onset of differentiation (Guglielmi, Cinnella et al. 2014). We used our differentiation assay to study the role of MYCN in differentiation.

Results

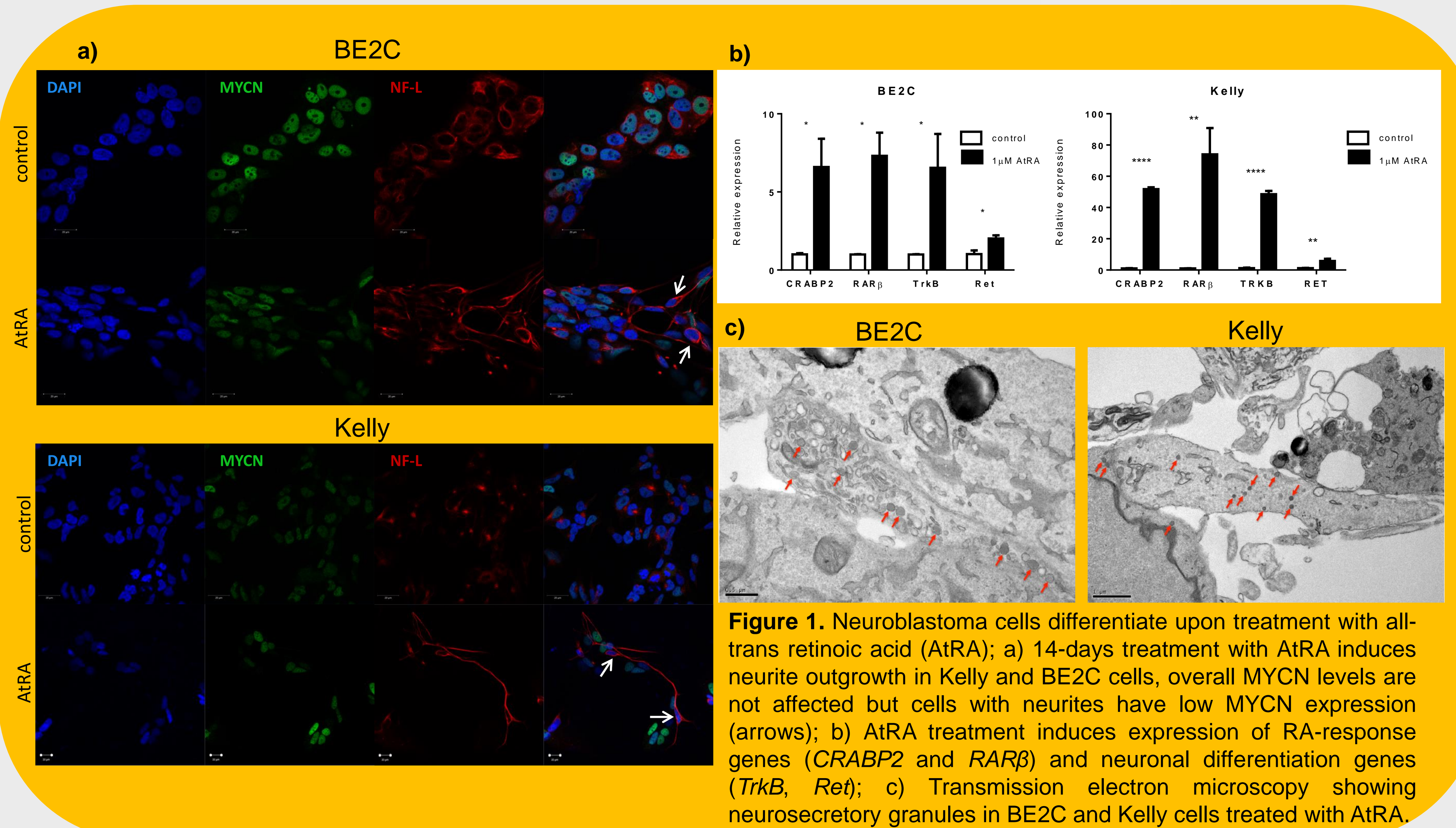

Figure 1. Neuroblastoma cells differentiate upon treatment with all-trans retinoic acid (AtRA); a) 14-days treatment with AtRA induces neurite outgrowth in Kelly and BE2C cells, overall MYCN levels are not affected but cells with neurites have low MYCN expression (arrows); b) AtRA treatment induces expression of RA-response genes (*CRABP2* and *RARβ*) and neuronal differentiation genes (*TrkB*, *Ret*); c) Transmission electron microscopy showing neurosecretory granules in BE2C and Kelly cells treated with AtRA.

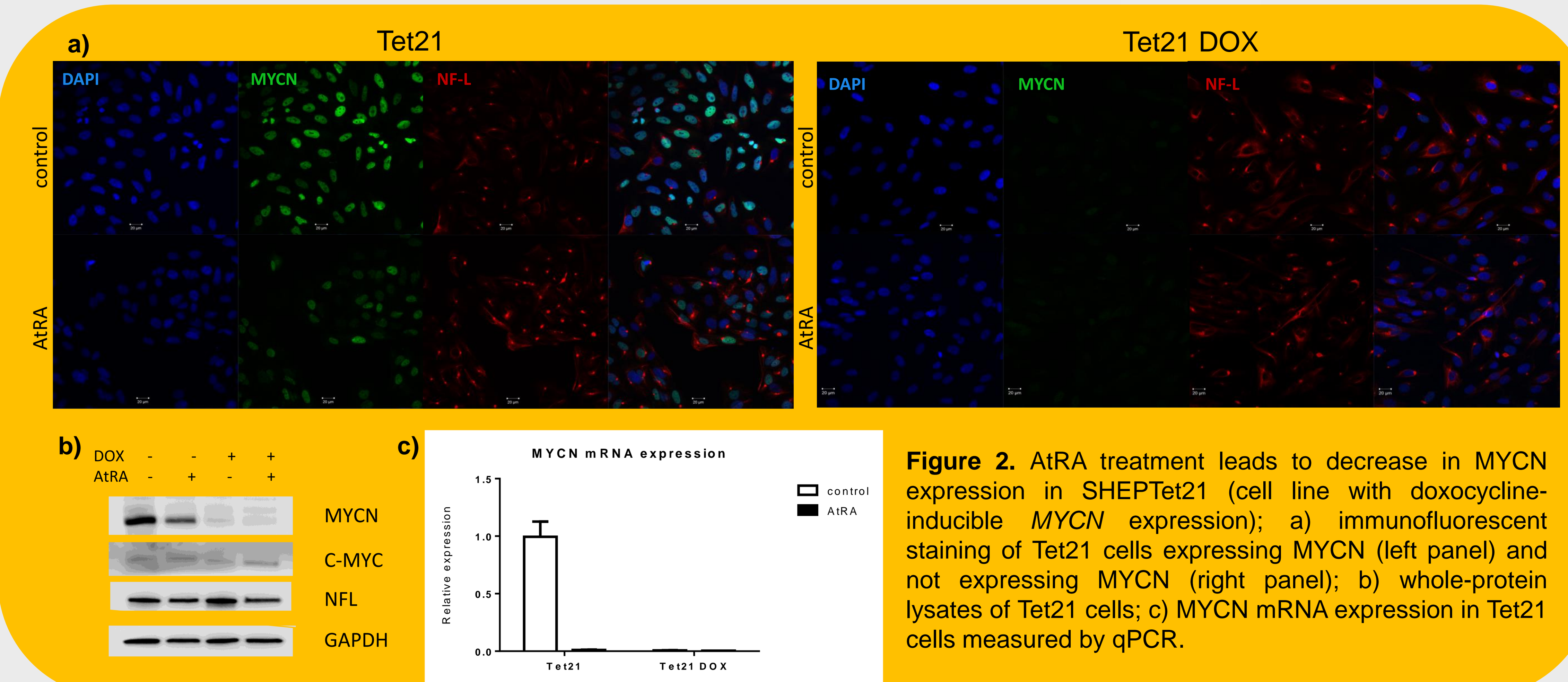

Figure 2. AtRA treatment leads to decrease in MYCN expression in SHEPTet21 (cell line with doxocycline-inducible *MYCN* expression); a) immunofluorescent staining of Tet21 cells expressing MYCN (left panel) and not expressing MYCN (right panel); b) whole-protein lysates of Tet21 cells; c) MYCN mRNA expression in Tet21 cells measured by qPCR.

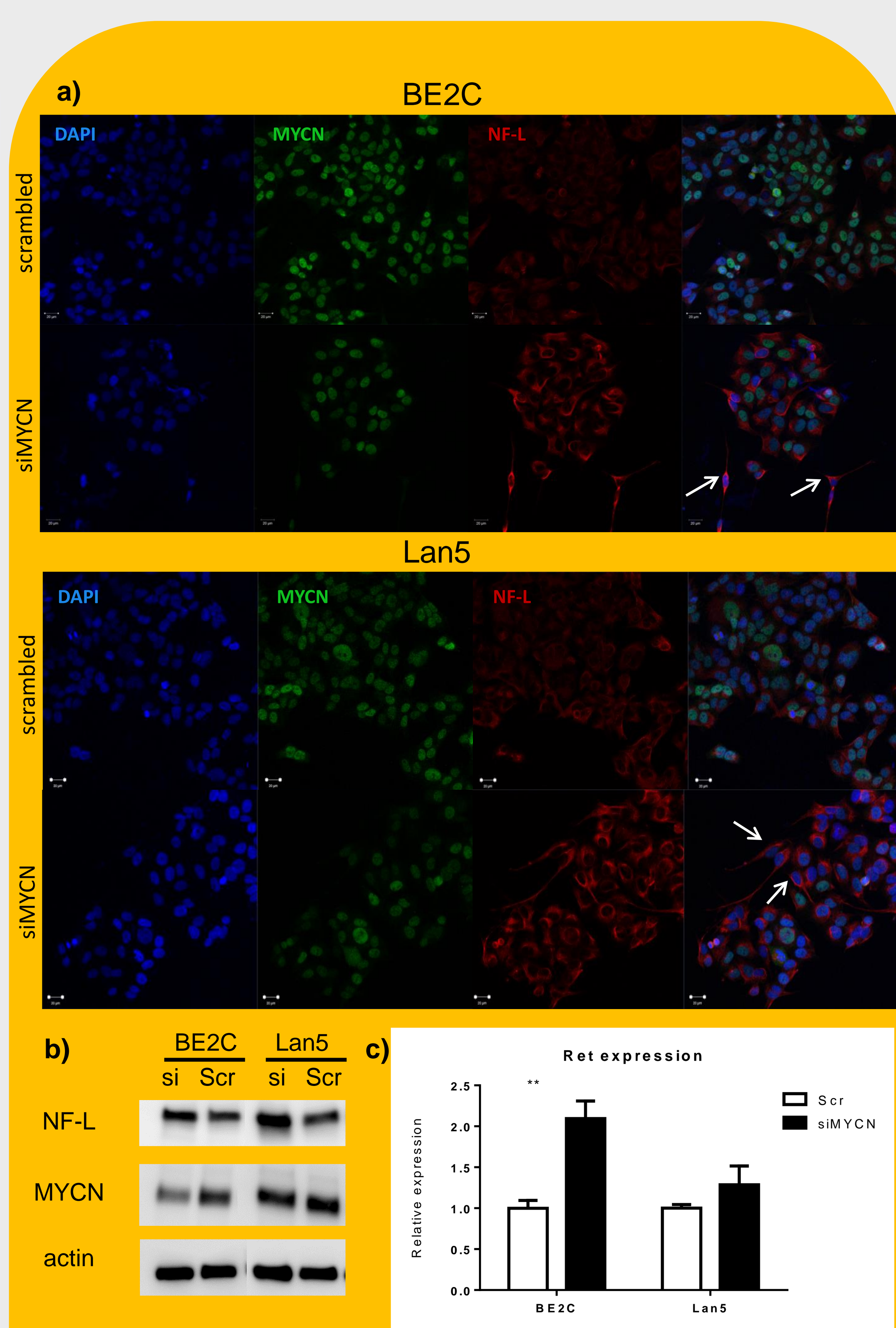

Figure 3. MYCN silencing leads to differentiation of neuroblastoma cell lines; a) transfection of BE2C and Lan5 cell lines with MYCN-directed siRNA; cells with longest neurites have low levels of MYCN (arrows); picture taken after 72h b) Western blot analysis confirms partial knock-down of MYCN and increase in NF-L expression; c) MYCN knock down leads to upregulation of Ret (neuronal differentiation marker).

Conclusions

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. Therefore we developed a reliable and robust differentiation assay to correctly distinguish differentiated cells i.e. after treatment with a drug.

We used the assay to establish a correlation between differentiation and MYCN expression. AtRA (a well known differentiation agent) leads to increase in neurofilament-light expression; we observed that cells with particularly long neurites have low MYCN expression. To study it further, we used a MYCN-inducible SHEP-Tet21 cell line in which treatment with AtRA lead to clear downregulation of MYCN protein and mRNA expression. Knock-down of MYCN in *MYCN*-amplified cell lines was not entirely efficient but again, cells with particularly long neurites had low MYCN expression.

All these results support the conclusion that MYCN inversely correlates with differentiation, which has been suggested before but has never been shown so clearly.

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