Molecular Analysis of Pax3 in the Development of Neural Crest Cells

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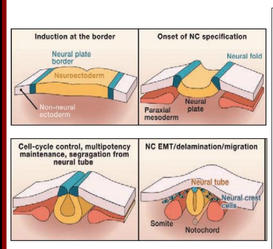
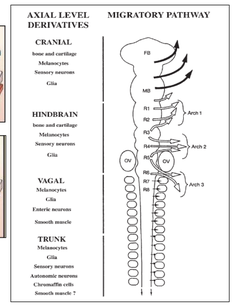
The Big Question: Which Cofactors Work with Pax3 in NCC Development?



Adapted from: (Sauka-Spengler and Bronner, 2010) and (LaBonne and Bronner-Fraser, 1998).



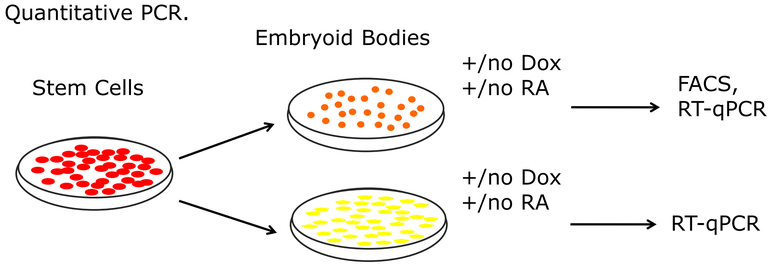
- Neural crest cells are a transient cell population developing near the neural tube that migrate throughout the embryo and differentiate into many different cells types.
- Defects in the development, migration, or differentiation of NCCs can lead to a variety of pathologies, including Waardenburg syndrome.

- Pax3 is a transcriptional activator and is essential in both the myogenesis and neural crest developmental pathways.
- Relaix et. al. (2003) showed that while Pax3 works as a transcriptional activator in the myogenesis pathway, it is not effective on its own.
- Thus Pax3 likely requires the binding of cofactors to help increase its efficiency.
- The only Pax3 cofactors that have been identified are HIRA, DAXX, and Rb. All inhibit Pax3's activity.
- This project aims to determine the cofactors of Pax3 in the NCC development pathway.
- First step in this project: optimizing culture conditions for growth of neural crest cells from mouse embryonic stem cells

Materials and Methods

Optimize the culture conditions for NCC growth:

Embryonic stem cells were cultured and differentiated into either embryoid bodies or into a monolayer. Combinations of 800ng/mL Doxycycline (dox), 10^{-7}M Retinoic Acid (RA) was added to the cells at different time points and then cells were analyzed by Real-Time Quantitative PCR.



References

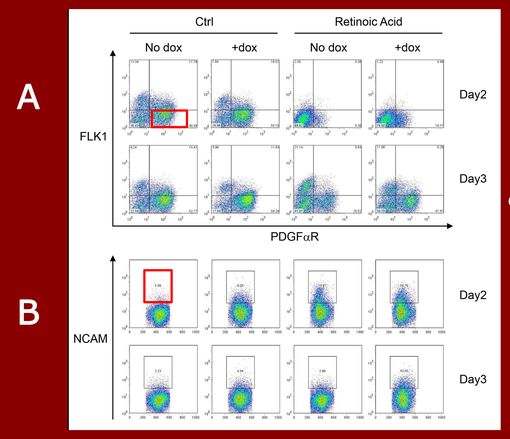
•LaBonne, C., and Bronner-Fraser, M. (1998). Induction and patterning of the neural crest, a stem cell-like precursor population. Journal of Neurobiology *36*, 175–189.

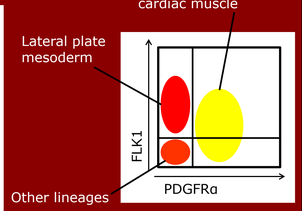
•Relaix, F., Polimeni, M., Rancourt, D., and et al. (2003). The transcriptional activator PAX3-FKHR rescues the defects of Pax3 mutant mice but induces a myogenic gain-of-function phenotype with ligand-independent activation of Met signaling in vivo. Genes Dev. *17*, 2950–2965.

•Sakurai, H., Era, T., Jakt, L.M., Okada, M., Nakai, S.N., and Nishikawa, S.-I. (2005). In Vitro Modeling of Paraxial and Lateral Mesoderm Differentiation Reveals Early Reversibility. Stem Cells *24*, 575–586.

•Sauka-Spengler, T., and Bronner, M. (2010). Snapshot: Neural Crest. Cell *143*, 486–486.e1.

FACS analysis shows Retinoic Acid treatment during EB differentiation affects mesoderm formation



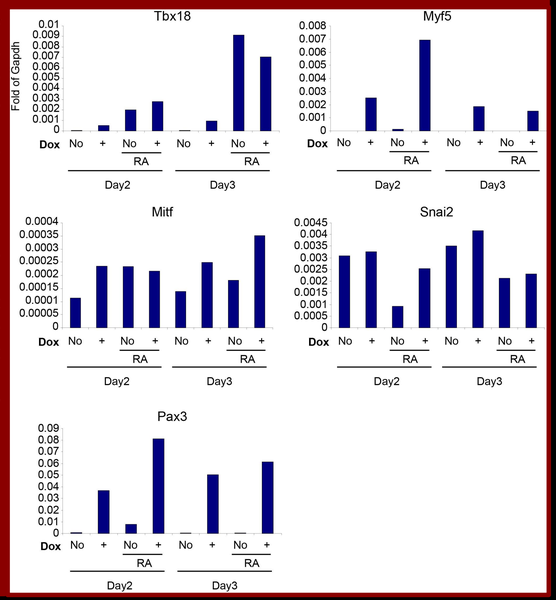


Developed based on Sakurai et al. (2005) where FLK1+ cells represent lateral plate mesoderm (red), Flk1+/PDGFRα+ and PDGFRα+ cells represent cardiac muscle and presumably paraxial mesoderm (yellow), and cells not expressing either marker represent other lineages (orange).

Fig. 1 FACS analysis of the effects of retinoic acid (RA) on the fate of Pax3 ES cells differentiating in an EB system. Doxycycline (dox) and RA were added on either day 2 or day 3 of differentiation, or not added, and all cultures were analyzed on day 6. (A) Representative FACS profile of FLK1 and PDGFRα expression, with fluorescence intensity on the y-axis indicating FLK1 expression and on the x-axis for PDGFRα expression. (B) Representative FACS profile of NCAM expression, indicated by fluorescence on the y-axis.

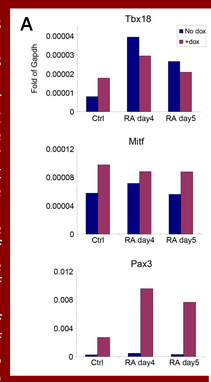
RA treatment during EB differentiation does not increase NCC formation

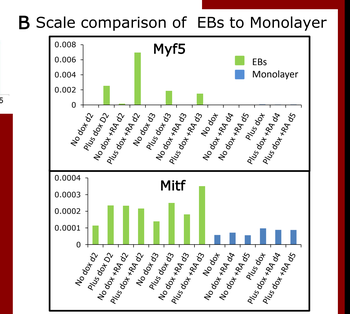
Fig. 2: qRT-PCR analysis of Pax3 ES cells grown in an EB system showing the expression levels of genes as relative to the expression levels of GAPDH. Doxycycline (dox) and retinoic acid (RA) were added on either day 2 or day 3 of differentiation, or not added, and all cultures were analyzed on day 6. Myf5 and Tbx18 represent mesodermal markers and are increased when RA and dox are added. Mitf marks neural crest cells, and increases only slightly with dox and RA. Snai2 marks cells in an epithelial-to-mesenchymal transition, and increase when dox and RA were added compared to RA only from day2 of EB differentiation.



ES differentiation as a monolayer reveals a reduction in mesoderm formation but no increase in NCC formation

Fig. 3: (A) qRT-PCR analysis of Pax3 ES cells grown in a monolayer system showing the expression levels of genes as relative to the expression levels of GAPDH. Controls were given doxycycline (dox) on day 4 of differentiation. For experimental treatments, dox and retinoic acid (RA) were added on either day 4 or day 5 of differentiation, or not added, and all cultures were analyzed on day 6. Myf5 and Tbx18 were reduced with the addition of dox and RA. Mitf and Snai2 had no appreciable change with the addition of dox and RA.
(B) Scale comparison of expression of Myf5 and Mitf between monolayer and EB conditions. It is important to note that while there was a reduction in Myf5 expression in the monolayer, Mitf was expressed at comparable levels in both culture conditions. This means that the monolayer condition affects the formation of mesoderm more than the formation of ectoderm.





Conclusion

RA alone is not effective inducer of NCCs from cells grown as EBs or in a monolayer.

Future Directions

Other potential tests for optimizing culture:
- Use a range of RA concentrations
- Change the concentration of serum in the media
- Use a reporter to isolate NCC (e.g. Sox10-GFP)
- Add different neural-inducing supplements (e.g. N2, B27)
- Add growth factors (e.g. Shh, FGF)

Next step: Perform a time course experiment to analyze all phases of NCC development.
- Induce cells differentiating in a monolayer with dox and RA
- Harvest cells at different time points for analysis (3, 4, 5, 6, 7, days after differentiation, etc.)
- Analyze by qRT-PCR with markers representing all phases of NCC development (early stage: Zic1, Msx1; mid-stage: FoxD3, Sox9; late-stage: Sox10, Mitf, Snai2).

Overall Goal:
- Using optimized culture conditions, generate NCC from Flag-tagged Pax3 embryonic stem cells.
- Perform a co-immunoprecipitation experiment to precipitate Pax3 and the cofactors it is bound to.
- Characterize cofactors via mass spectrometry.