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Histone deacetylase inhibitor Trichostatin A induces neural tube defects and promotes neural crest specification in the chicken neural tube

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ABSTRACT

Epigenetic mechanisms serve as key regulatory elements during vertebrate embryogenesis. Histone acetylation levels, controlled by the opposing action of histone acetyl transferases (HATs) and histone deacetylases (HDACs), influence the accessibility of DNA to transcription factors and thereby dynamically regulate transcriptional programs. HDACs execute important functions in the control of proliferation, differentiation, and the establishment of cell identities during embryonic development. To investigate the global role of the HDAC family during neural tube development, we employed Trichostatin A (TSA) to locally block enzymatic HDAC activity in chick embryos *in ovo*. We found that TSA treatment induces neural tube defects at the level of the posterior neuropore, ranging from slight undulations to a complete failure of neural tube closure. This phenotype is accompanied by morphological changes in neuroepithelial cells and induction of apoptosis. As a molecular consequence of HDAC inhibition, we observed a timely deregulated cadherin switching in the dorsal neural tube, illustrated by induction of *Cadherin 6B* as well as reciprocal downregulation of N-Cadherin expression. Concomitantly, several neural crest specific markers, including *Bmp4*, *Pax3*, *Sox9* and *Sox10* are induced, causing a premature loss of epithelial characteristics. Our findings provide evidence that HDAC function is crucial to control the regulatory circuits operating during trunk neural crest development and neural tube closure.

1. Introduction

Eukaryotic transcription takes place in a chromatin context, and is, beyond other mechanisms, tightly regulated by the action of chromatin modifying enzymes (Strahl and Allis, 2000). Reversible acetylation of lysine residues is controlled by the antagonistic action of histone acetyl transferases (HATs) and histone deacetylases (HDACs). Histone acetylation is correlated with local chromatin opening and transcriptional activity. HDACs remove acetyl moieties, resulting in chromatin condensation, but recent findings have shown that HDACs also participate in transcriptional activation (Zupkovitz et al., 2006; Wang et al., 2009). Furthermore, numerous non-histone proteins such as transcription factors (e.g., p53) or cytoskeletal proteins (e.g., Tubulin) are substrates of HDAC dependent deacetylation. The HDAC family is highly conserved throughout eukaryotic and prokaryotic species and is comprised of 18 members in higher vertebrates. Based on sequence similarity to yeast proteins and domain organization, HDACs have been divided into four classes: classic HDACs include class I (HDAC1, 2, 3, 8), class II (HDAC4, 5, 6, 7, 9, 10) and class IV (HDAC11) enzymes, whereas the NAD^+-dependent Sirtuins comprise the functionally unrelated class III (Gregoretti et al., 2004; Yang and Seto, 2008).

All class I HDACs are co-expressed in most cells and tissues, and exhibit deacetylase activity towards common substrates, suggesting functional redundancy among these enzymes *in vivo*. Specifically, HDAC1 and 2 are highly homologous, and loss of function of one paralogue is associated with upregulation of the other family member. Furthermore, HDAC1, 2 and 3 can function as catalytic subunits of multiprotein chromatin remodeling complexes such as the SIN3, CoREST, NuRD or nuclear hormone dependent corepressor complex NCoR/SMRT (reviewed in Yang and Seto (2008), Brunmeir et al. (2009)).

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Despite redundant functions of individual HDACs *in vitro* or in adult tissues, deletion of each member of the HDAC family in mice leads to specific and often lethal phenotypes, demonstrating the unique roles of each HDAC during embryonic development (reviewed in Brunmeir et al. (2009), Haberland et al. (2009a), Reichert et al. (2012)). Furthermore, we have recently shown that class I HDACs show a distinct spatio-temporal expression pattern during mouse and chick development revealing exclusive and overlapping expression areas of each HDAC family member. The most notable observation within this study was the fact that all class I HDACs are highly expressed in the developing CNS; in particular *Hdac1* shows a hot spot-like expression in the open posterior neural tube in HH12 chicken embryos, indicating that HDACs have essential roles in regulating embryonic neural development (Murko et al., 2010).

In recent years, HDACs have become promising targets for therapeutic interventions in the treatment of cancer and neurodegenerative disorders. A large number of HDAC inhibitors have been isolated from natural sources, and considerable effort has been taken into the development of new synthesized inhibitors (for a recent review see Miller et al. (2011)). One of the most widely used HDAC inhibitors is Trichostatin A (TSA, Yoshida et al., 1990), a hydroxamic acid with strong inhibitory potential against class I and class II HDACs, though the effectivity is highest against HDAC1-3 (Bantscheff et al., 2011). TSA shows beneficial effects in a variety of neurodegenerative disease models, including Alzheimer's disease, spinal muscular atrophy, Huntington's disease, Parkinson and stroke (reviewed in Ververis and Karagiannis (2011)). Despite their therapeutic potential, HDAC inhibitors can cause severe teratogenic effects on the developing CNS, in particular, the closing neural tube, when applied during embryogenesis in various model systems (Nau et al., 1991; Damjanovski et al., 2000; Menegola et al., 2005; Di Renzo et al., 2007).

Primary neurulation is a fundamental process during vertebrate development and defined as the time between the formation of the neural plate and the closure of the neural tube. The formation of the neural plate starts during gastrulation by the induction of a neural fate in cells at the midline. The neural plate then elongates rostro-caudally while it becomes narrowed mediolaterally by a polarized rearrangement of cells within the neural plate midline. The outer most edges of the neural plate will form the neural folds, which further elevate and converge towards the midline. Finally, the two folds come into contact with each other and fuse, thereby creating a hollow structure. Closure first starts at the level of the future midbrain and proceeds in a zipper like fashion in both directions. The anterior and posterior ends of the embryo are the final regions to close (Copp et al., 2003). Failure of closure at any of these sites leads to neural tube defects (NTDs), with the severity of the defect depending on the site where closure fails (Copp et al., 2003; Wallingford, 2005).

During the process of neural tube closure, the cells at the boundary between prospective neural tube and epidermis form the neural crest. This cell type is established as two dorso-lateral stripes along the entire length of the neural tube, from which cells delaminate and migrate away throughout the embryo body. The neural crest, sometimes called the fourth germ layer, will give rise to a large variety of different structures of the peripheral nervous system, endocrine and paraendocrine derivatives, the facial skeleton and pigment cells (LeDouarin, 1982). A complex genetic network of transcription factors coordinates the different stages of neural crest development (reviewed in Sauka-Spengler and Bronner-Fraser (2008), Betancur et al. (2010)). The first step of neural crest induction occurs during gastrulation, where signaling pathways such as wingless (WNT), bone morphogenetic protein (BMP), fibroblast growth factor (FGF) and retinoic acid (RA) define areas of neural crest competence and induce neural plate border specifiers such as *Pax3* and *Pax7* (Basch et al., 2006). During neural fold elevation, the first neural crest specifiers start to be expressed in the caudal neural tube (e.g., *Slug*/*Snail*, *FoxD3*, *Sox9* and *Sox10*), which maintain and regulate neural crest fate. Finally, neural crest specifiers induce numerous downstream effectors that regulate migration and terminal differentiation into diverse cell types (Meulemans and Bronner-Fraser, 2004; Sauka-Spengler and Bronner-Fraser, 2008; Betancur et al., 2010). To emigrate from the neural tube, neural crest cells need to undergo an epithelial to mesenchymal transition (EMT) to acquire motile properties. This process, which occurs after neural tube closure in the chick, involves changes in gene expression and morphology, including loss of epithelial junctional polarity by downregulation of *N-Cadherin* and upregulation of *Cadherin 6B* in the premigratory neural crest cell population (Park and Gumbiner, 2010).

Given the fact that neural tube closure and neural crest development are spatio-temporally connected, regulation by epigenetic components seems to be one potential way to control the dynamic changes in gene expression needed for both processes. Furthermore, neural crest cells, although first specified already during gastrulation, need to maintain stem cell properties until emigration from the neural tube. Histone modifications might play a crucial role to maintain the multipotency of neural crest cells in the intervening time.

We have previously shown that *Hdac1* expression is inversely correlated with posterior neural tube closure during chick embryogenesis (Murko et al., 2010). Here we demonstrate that HDACs play a crucial role in both closure of the neural tube and timing trunk neural crest specification. We have used the global HDAC inhibitor Trichostatin A (TSA, Yoshida et al., 1990) to interfere with HDAC function in the posterior neural tube of chick embryos. TSA treated embryos show a variety of neural tube defects and further display morphological and molecular changes in the dorsal neural tube. Strikingly, several neural crest specific marker genes are upregulated or prematurely induced in these embryos. Our study provides evidence that histone deacetylation is fundamental for regulating both neural crest development and neural tube closure.

2. Results

2.1. TSA injection into the posterior neural tube induces neural tube closure defects

To investigate the function of histone acetylation during caudal neural tube development, we applied the HDAC inhibitor Trichostatin A (TSA, Yoshida et al., 1990) to chick embryos *in ovo*. We therefore injected TSA into the open posterior neural tube of Hamburger Hamilton stage HH10-12 embryos (Hamburger and Hamilton, 1992), and subsequently reincubated the embryos for 8 to 24 h. In our experiments, we used TSA concentrations of 1 or 2 mM. These solutions were calculated to deliver a total of 100 to 200 nmol of TSA in an injection volume of 0.1 μl, corresponding to regular doses of TSA used in cell culture experiments. When we analyzed the embryos after the reincubation period, we observed a significant increase in neural tube abnormalities in TSA treated embryos, compared to DMSO injected control embryos (Fig. 1A–J, summary in Fig. 1K and Table 1). After 8 h of HDAC inhibition, the neural folds were undulated and wrinkled or unusually far apart, depending on the TSA concentration and the developmental stage at the time of injection. In this respect, embryos injected with a 1 mM TSA solution displayed mainly undulated neural folds (Fig. 1A, higher magnification in Fig. 1A′), whereas embryos injected with a 2 mM TSA solution additionally manifested more widely separated neural folds than control embryos (Fig. 1B,