Early Intervertebral Disc Differentiation Is Robust To Stopping Notochord Cell Proliferation, and Brachyury Shows Regional Activity

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INTRODUCTION:
The intervertebral disc is a beautiful, highly structured organ, composed of an outer fibrous tissue, the annulus fibrosus, encircling a proteoglycan rich core, the nucleus pulposus. It is derived from two embryonic tissues, the somitic mesenchyme and the notochord, which is the source of cells in the nucleus pulposus. The role of the mesenchyme and notochord on each other to pattern and differentiate this exquisite structure remains a mystery. Myself and others have recently published studies on the role of proliferative notochord cells on the formation and maintenance of the nucleus pulposus [Long et al., Dev Dyn, 2025 & Tan et al., Cell Reports, 2024]. This study studies the necessity of these cells in early development by preventing notochord cell proliferation. The second part of the study presents data on expression of a T-box transcription factor that may be active in growth, brachyury.

METHODS:
All animal studies were approved by the IACUC.
The first study measured dimensions of the annulus fibrosus and actin expression of E16 embryos (n = 2) from mice with alleles: 1) the Cre enzyme under the control of Shh; and 2) floxed cyclin dependent kinase 1 [Chaffee et al., Development, 2014]. Timed pregnant dams were injected with Tamoxifen (Sigma, 100mg/kg) at E13, effectively stopping cell division in the notochord.
The second study measured the presence of brachyury (R & D Systems, 1:100) at E13 & E14 (n = 4).
Segmentation progression was calculated by the ratio of the notochord diameter at the vertebra divided by the notochord diameter at the intervertebral space with 1 indicating no segmentation and 0 indicating complete segmentation. Statistics were the non-parametric Mann-Whitney test. Data are presented as median ± standard deviation.

RESULTS:
Cre recombination reporter (Ai9, red) suggests 50% of notochord cells are targeted (Figure 1). The area of annulus fibrosus with filamentous actin, indicated with phalloidin, was not different between the control wildtype littermate embryos and embryos with the homozygous floxed Cdk1 alleles, ShhCreERT2$^{+/-}$;Cdk1$^{fl/fl}$, (p = 0.08). The width of the ventral and dorsal (not shown) annulus fibrosus did not differ between genotypes (p>0.99) (Figure 1).
Brachyury is present in the notochord above the basioccipital bone primordium at E13 and E14 (Figure 2).
Brachyury is off in the distal tail at E13, when the notochord diameter is uniform, and the ratio is 1 (Figure 2).
Brachyury is on uniformly in the lumbar spine at E13, and off in the distal tail. One day later, brachyury is still off in the distal tail (posterior to 5 levels from sacrum), but is on in the intervertebral bulge and off in the fragment at all levels superior: thoracic, lumbar and proximal tail (Table 1).

DISCUSSION:
The first study did not detect a difference in annulus fibrosus differentiation, as defined as annulus fibrosus width and actin presence, between embryos with and without the floxed Cdk1 allele. Although this study is not powered to detect subtle changes (<20%), large changes were not detected. Follow up longer than 3 days was not possible due to embryonic death.
The brachyury pattern suggests regional brachyury expression as: 1) tail: off, then on in the bulge, 2) body: off, on then off in the fragment and 3) basioccipital: on. Recently identified region specific brachyury enhancers may explain irregular brachyury expression [Kemmler et al., Nat Comm 2023, Schifferl et al., Development 2023].

SIGNIFICANCE:
Understanding the role of proliferating cells on differentiation of annulus fibrosus can lend insight on necessary factors for tissue engineering. Understanding the embryonic activation of brachyury, a transcription factor, gives critical insight into understanding the formation of the spine.

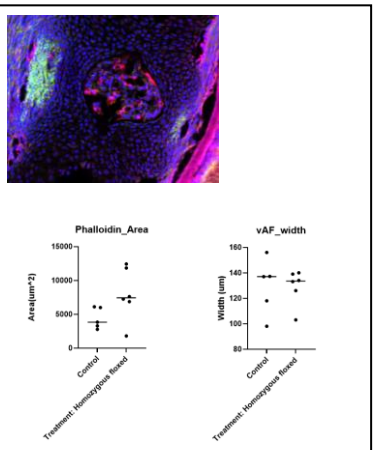
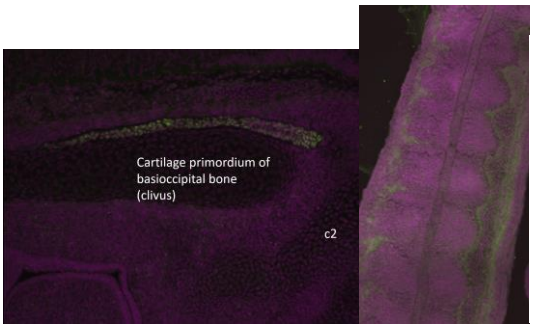


Figure 1. (top) Filamentous actin is present in the ventral and dorsal annulus fibrosus (green), and 50% of notochord cells are subject to Cre recombination (red), approx. 450um wide. (lower) the phalloidin area of wildtype controls was 3828 ± 1554 um^2 and 7429 ± 3875 um^2 for homozygous floxed embryos. The ventral annulus fibrosus width for wildtype controls was 137 ± 22 um and for was 134 ± 13.8 um for homozygous floxed embryos.



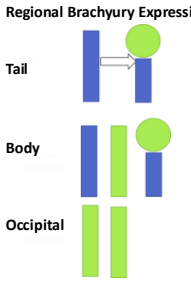


Figure 2. Brachyury (T) expression is constant above the basioccipital bone, but not in the body or tail at E13 and E14. At E13, T is present in the notochord above the basioccipital bone, approx. 300um in length (left), but not in the distal tail, approx. 700um in length (center). Cartoon: In the tail, T turns on in the intervertebral bulge; in the body, it turns on in the whole notochord then just the bulge; and above the basioccipital bone, it remains on (right).

Ratio vertebral fragment/notochord Expansion	0.1 (More mature)	0.14	0.24	0.4	0.8	1	1 (Less mature)
Stage, Location	E14 thoracic	E14 tail	E14 lumbar	E14 Tail	E14 distal Tail	E13 Lumbar	E13 distal Tail
Brachyury	ON in Bulge OFF in Vertebral fragment	ON in Bulge OFF in Vertebral fragment	ON in Bulge OFF in Vertebral fragment	ON in Bulge CC3-CC4 OFF in Vertebral fragment (CC4) OFF posterior to CC5	OFF in all notochord	ON in all notochord	OFF in all notochord

Table 1. At E13, T is off in the distal tail, but on in the lumbar spine. At E14, T is off in the fragment in the vertebra in the tail and body, except remains off in the distal tail.