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Molecular signaling in intervertebral disk development

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Abstract

The purpose of this investigation is to identify and study the expression pattern of pertinent molecular factors involved in the differentiation of the intervertebral disk (IVD). It is likely that hedgehog genes and the BMP inhibitors are key factors involved in spinal joint formation. Radioactive in situ hybridization with mRNA probes for pax-1, SHH, IHH and Noggin gene was performed on mouse embryo and adult tissue. Immunohistochemistry was performed to localize hedgehog receptor, "patched" (ptc). From 14.5 dpc until birth pax-1 mRNA was expressed in the developing anulus fibrosus (AF). During the same developmental period Noggin mRNA is highly expressed throughout the spine, in the developing AF, while ptc protein and SHH mRNA were expressed in the developing nucleus pulposus (NP). IHH mRNA was expressed by condensing chondrocytes of the vertebral bodies and later becomes confined to the vertebral endplate. We show for the first time that pax-1 is expressed in the adult intervertebral disk. Ptc expression in the NP is an indicator of hedgehog protein signaling in the developing IVD. The expression pattern of the BMP inhibitor Noggin appears to be important for the normal formation of the IVD and may prove to play a role in its segmental pattern formation.

Keywords: Spine; Intervertebral disk; BMP inhibitor; Noggin; Pax-1; Hedgehog; Embryology

Introduction

The intervertebral disk (IVD) has limited capacity for spontaneous repair. Due to the long-term mechanical and clinical short falls of current treatments for acutely disrupted or chronically degenerated discs [2,17,18] efforts are underway to develop alternative therapies that involve biological tissue regeneration and repair of nucleus pulposus (NP) and anulus fibrosus (AF). Studying developmental biology of the musculoskeletal system offers insight into the generation and maintenance of signals that coordinate the organization and remodeling of embryonic as well as mature tissue. It has been shown that these signaling events are often recapitulated in the healing process of mature tissue such as fractured bone [34].

Vertebral column development requires coordination of a series of cellular and molecular events [28]. Axial skeletal development is a multi-step process, starting with the formation of somites from the unsegmented paraxial mesoderm on both sides of the neural tube [6,28]. Upon somite differentiation the ventral parts de-epithelialize and form mesenchymal sclerotomes, which give rise to pre-cartilaginous structures as well as connective tissue. This process depends on signaling from the notochord [36,38]. Sclerotome cells then migrate ventrally and medially, surrounding the notochord, to form the perichordal tube. This structure eventually becomes segmented and gives rise to the vertebral bodies (VB's) and

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IVD's [33]. The IVD forms at discreet intervals as the notochord regresses, at the site of the future vertebral body and bulges at the site of developing NP. The AF is formed by cells of sclerotome origin and the NP is derived from cells that were once notochord tissue [33]. By 12.5–13.5 days post conception (dpc) in the mouse embryo, the beginning of IVD development is evident.

The pax genes form a family of developmental control genes that encode sequence specific transcription factors that contain the DNA binding paired domain [27]. Localized cell proliferation is often observed in organs expressing pax genes [28]. In the mouse, nine pax gene family members have been identified as major players in organogenesis [36]. Pax-1 is a sclerotomally derived gene that is required for normal development of ventral vertebral structures [36,37]. By 12.5 dpc in the mouse embryo, IVD anlagen is pax-1 positive, while VB and NP anlagen is pax-1 negative. Pax-1 may play a role in differentiating fibrous tissue and in the maintenance of the boundaries between skeletal elements.

A number of pax-1 mouse mutants exist, and have been phenotypically compared [36]. These mice are notable for vertebral column/tail abnormalities. Fusion of axial elements is typically seen in these mutants and it has been shown that they have significantly decreased mesenchymal proliferations in the vertebral column, which leads to disrupted vertebral body and disk tissue formation [28]. It has been speculated that pax-1 may be one of the key mediators of inductive signaling between the notochord and the sclerotome. Sustained notochord signaling is required for normal maintenance of pax-1 expression which may control downstream VB and IVD formation [36]. Currently it is not known how long into development that pax-1 expression persists.

The Hedgehog gene family encodes a secreted signaling peptide with a consensus signal sequence at its N-terminus [14] that acts by way of the "patched" (ptc) receptor complex [7]. Members of this gene family direct an enormous variety of developmental events in vertebrates [15]. Sonic and Indian Hedgehog genes have been implicated in pattern formation and chondrogenesis in developing axial structures and limbs [7,11,12]. Sonic hedgehog has also been shown to be an inducer of sclerotome and pax-1 gene expression by signaling from the notochord and floor plate to adjacent tissues [7].

Patched (ptc) is a multi-pass transmembrane protein with structural similarity to channels and transporters [4]. In Drosophila ptc is expressed in all cells that are responsive to hedgehog (HH) signal and the level of ptc appears to rise in response to HH signaling. This feature is conserved among vertebrate signaling pathways [4]. Ptc can bind and sequester HH which then leads to changes in cellular behavior such as gene transcription and negative feedback [4].

Members of the bone morphogenetic protein (BMP) family are important in the development of both the early somite and late vertebral body chondrogenesis and ossification [24–26]. BMP interacts with a number of molecules such as hedgehog proteins and BMP inhibitor Noggin to create a complex array of signals capable of differentially patterning tissues [3,10]. Noggin protein directly binds to BMP's thus preventing them from binding to their receptors [39]. It is capable of blocking various members of the BMP family including BMP-2, -4, and -7 [39] and it has been shown to act downstream of SHH to inhibit BMP-4 in somite patterning [10]. Through their direct inhibition of BMP's, inhibitors such as Noggin have been shown to be essential mediators in joint formation [3].

Currently little is known about the signals that regulate the development of the IVD. Much of the study of pertinent genes such as pax-1 and SHH stops short at the notochord and sclerotome level. It is likely that hedgehog genes, along with the BMP inhibitors are key factors involved in spinal joint formation and continue to be involved in the maturing tissue. The purpose of this investigation is to study the expression pattern of pertinent molecular signals in the growth and development of the IVD and to identify factors that differentiate it from vertebral body in the spinal column.

Methods

All experiments were performed with the approval of the Sloan Kettering Institutional Animal Care and Use Committee and Institutional Review Board. Wild type and LacZ/Ptc heterozygous mice were studied. The heterozygous mice were created by "knocking in" the lacZ promoter into the 108B2 locus of the ptc 1 gene. They were generously provided by J. Eggenschwiler and created by Scott et al. [9]. Date of pregnancy was determined by the presence of a mucous plug in the vaginal region of the female mouse. Mice were checked daily and upon documentation of a plug, the age of embryos was considered to be 0.5 days post conception (dpc). LacZ/Ptc heterozygote males were bred with WT females to produce LacZ/Ptc offspring in an expected ratio of 1:1 WT:LacZ/Ptc. X-gal staining of LacZ/Ptc tissues was performed according to the methods published by Tam et al. [31].

Mouse embryos were harvested at day 14.5–17.5 dpc. Neonate mice were sacrificed 2 days post delivery. Adult mice were at least 6 months old. Neonate and adult spines were dissected and embedded in segments that contained 2–5 IVD's.

Histology

Embryos and spinal segments were first fixed in freshly made 4% PF in PBS buffer at 4 °C overnight. Volume of fixative exceeded tissue volume by at least 20×. After fixing, the tissues were washed with running cold tap water for 1 h. Embryos age 14.5–16.5 dpc were not decalcified. Embryos and tissue from age 17.5 dpc-adult were next decalcified in 0.5 M EDTA pH 7.8. for 7 days at 4 °C. After decalcification, tissues were washed in cold PBS twice for 30 min in each.

Paraffin embedding

All tissues were dehydrated and embedded in paraffin. Embryos and tissues were then embedded in a sagittal orientation in paraffin blocks and allowed to cool overnight in a water bath. Sectioning was performed on a microtome at 8–10 μm thickness. All sections containing spinal anatomy were collected on microscope slides.