Targeting the annulus fibrosus of the intervertebral disc: *Col1a2-Cre(ER)T* mice show specific activity of Cre recombinase in the outer annulus fibrosus

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Abstract Degeneration of the intervertebral disc (IVD) is a major underlying contributor to back pain—the single leading cause of disability worldwide. However, we possess a limited understanding of the etiology underlying IVD degeneration. To date, there are a limited number of mouse models that have been used to target proteins in specific compartments of the IVD to explore their functions in disc development, homeostasis and disease. Furthermore, the majority of reports exploring the composition and function of the outer encapsulating annulus fibrosus (AF) of the IVD have considered it as one tissue, without considering the numerous structural and functional differences existing between the inner and outer AF. In addition, no mouse models have yet been reported that enable specific targeting of genes within the outer AF. In the current report, we discuss these issues and demonstrate the localized activity of Cre recombinase in the IVD of *Col1a2-Cre(ER)T;ROSA26mTmG* mice possessing a tamoxifen-dependent Cre recombinase driven by a *Col1a2* promoter and distal enhancer and the mTmG fluorescent reporter. Following tamoxifen injection of 3-week-old *Col1a2-Cre(ER)T;ROSA26mTmG* mice, we show Cre activity specifically in the outer AF of the IVD, as indicated by expression of the GFP reporter. Thus, *Col1a2-Cre(ER)T;ROSA26mTmG* mice may prove to be a valuable tool in delineating the function of proteins in this unique compartment of the IVD, and in

further exploring the compositional differences between the inner and outer AF in disc homeostasis, aging and disease.

Keywords Intervertebral disc · Outer annulus fibrosus · Inner annulus fibrosus · Tissue-specific gene deletion · *Col1a2*

Introduction

Back pain, the leading specific cause of disability in non-fatal health outcomes of disease and injury (Lim et al. 2012), has a lifetime incidence as high as 84 % (Walker 2000) and a point prevalence of 11.9 % (Hoy et al. 2012). Of the numerous spine-related pathologies associated with pain in the lumbar spine, intervertebral disc (IVD) herniation and degeneration are the most significant contributors and hence are targets for intervention (Kuslich et al. 1991; Deyo and Weinstein 2001). Yet, despite the large burden that IVD disease places on health care systems worldwide, the molecular mechanisms underlying IVD disease remain largely unknown; indeed, there are no clinical therapies targeting its pathophysiology. Thus, identifying the relative roles and function of proteins within the unique compartments of the IVD represents an important step towards understanding the pathogenesis of IVD disease and identifying therapeutic targets for this crippling condition.

The IVD, the connective tissue structure that lies between vertebrae of the spine, is essential for spine stabilization and movement (Adams and Roughley 2006). The disc consists of three distinct, highly specialized yet interdependent tissues: the nucleus pulposus (NP), the annulus fibrosus [AF; being further divided into the inner annulus fibrosus (IAF) and outer annulus fibrosus (OAF)] and the cartilage endplates. Precise regulation of matrix synthesis by distinct IVD cell types is essential for the IVD to fulfill its physiological function of absorbing compressive loading and conferring flexibility to

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the spine. Each tissue type of the IVD contains a unique protein composition that is hypothesized to play an important role in maintaining tissue health.

Many proteins that play crucial roles in functionally and compositionally related tissues (such as cartilage, ligament and tendon) have been identified as therapeutic targets in pathological conditions afflicting these tissues. However, we possess a limited understanding of the roles of many of these proteins in IVD health and dysfunction. Due in part to the limited number of genetic tools that specifically target distinct cell types within the disc, only a relatively small number of studies have used a targeted approach to assess the relative contribution of individual subpopulations of cell types to IVD function. Specifically, the inner and outer AF show distinct biochemical and structural properties that underlie differences in their respective biochemical and physiological functions (Antoniou et al. 1996; Hayes et al. 2001; Bruehlmann et al. 2002; Han et al. 2012; Li et al. 2014) (discussed below). Despite these observations, the vast majority of studies have examined AF structure and function in its entirety. An increasing body of evidence has shown that the OAF shows enriched expression of matricellular proteins including thrombospondin-2 (TSP-2) (Gruber et al. 2006), periostin (POSTN) (Gruber et al. 2011), tenascin (TN) (Gruber et al. 2002) and Secreted Protein, Acidic, and Rich in Cysteine (SPARC) (Gruber et al. 2004). Although they possess important roles in related tissues, the contribution of many such proteins to OAF function has not been rigorously assessed (Bedore et al. 2014).

The above reports suggest that tools to specifically genetically target proteins in the OAF would be extremely useful. One such model might be the *Col1a2-Cre(ER)T* transgenic mouse which drives the expression of a tamoxifen-inducible Cre recombinase under the control of a specific distal enhancer element localized ~20 kb upstream of the endogenous *Col1a2* promoter (Bou-Gharios et al. 1996; Ponticos et al. 2004). In these mice, Cre is expressed in all *Col1a2*-expressing cells within many tissues, with the exception of osteoblasts derived from endochondral ossification. Although annulus fibrosus cells of intervertebral disc are known to express high levels of type I collagen (Eyre and Muir 1976; Antoniou et al. 1996; Clouet et al. 2009; Li et al. 2014), whether Cre is active in the IVD of *Col1a2-Cre(ER)T* mice has not been previously examined. In this report, we examined the activity of this *Col1a2* promoter and distal enhancer element following Cre induction in 3-week old mice.

Methods

Mice hemizygous for a tamoxifen-dependent Cre recombinase under the control of the *Col1a2* promoter and far upstream enhancer elements (Ponticos et al. 2004) were bred with mice harboring a double-fluorescent reporter transgene (*mTmG*)

integrated into the *Gt(ROSA)26Sor* locus (Muzumdar et al. 2007) to generate *Col1a2-Cre(ER)T;ROSA26mTmG* mice, as previously described (Parapuram et al. 2015). The *mTmG* transgene confers expression of membrane-targeted tandem timer (td)Tomato prior to Cre-mediated excision and membrane-targeted green fluorescent protein (GFP) after excision. In this model, cells expressing Cre at the time of tamoxifen induction are permanently labeled by expression of GFP, while all other cells are labeled by expression of (td)Tomato (Muzumdar et al. 2007).

To induce Cre recombinase, 3-week-old littermate *Col1a2-Cre(ER)T;ROSA26mTmG* mice were given 0.1 ml intraperitoneal injections of a 10 mg/ml tamoxifen solution in corn oil, or corn oil alone as a vehicle control, for five consecutive days. One hundred days later, mice were sacrificed and individual IVDs were dissected out of the lumbar spine. IVDs were fixed in formalin overnight, incubated at 4 °C in 30 % (v/v) sucrose, embedded in OCT mounting medium (VWR, Mississauga, ON) and sectioned at a thickness of 8 µm. Mounting was performed with VECTASHIELD Mounting Medium with DAPI (Burlingame, CA), and images were acquired using a Leica Microsystems DM16000B fluorescent microscope and DFC360FX camera. Images of whole IVDs were produced from 100x magnification images using AutoStitch image reconstruction software (Brown and Lowe 2006). All aspects of this study were conducted in accordance with the policies and guidelines set forth by the Canadian Council on Animal Care and were approved by the Animal Use Subcommittee of the University of Western Ontario, London, ON.

Results

To localize the activity of the *Col1a2* promoter/enhancer in the IVD, 3-week-old *Col1a2-Cre(ER)T;ROSA26mTmG* mice were injected with tamoxifen, and 100 days later localization of reporter expression was detected by visualization of tdTomato and GFP fluorescence from cryosectioned lumbar discs. Examination of lumbar intervertebral discs from tamoxifen-treated *Col1a2-Cre(ER)T;ROSA26mTmG* mice (Fig. 1) showed GFP expression, indicating Cre-mediated recombination, specifically in cells of the outer annulus fibrosus (OAF), but not inner annulus fibrosus (IAF) or nucleus pulposus (NP).

Discussion

Whole-body knockout mice provide useful tools to characterize the functions of specific proteins in cellular and physiological processes in vivo. However, the use of such mice to study pathologies is particularly challenging as candidate regulators of IVD function may also be involved broadly in

Fig. 1 Cre recombinase activity in the intervertebral disc of *Col1a2-Cre(ER)T;ROSA26mTmG* reporter mice. Reporter mice expressing a tamoxifen-inducible cre recombinase under the control of a *Col1a2* promoter/enhancer (Ponticos et al. 2004) show expression of membrane-targeted tdTomato (tdTom) prior to Cre-mediated recombination and membrane-targeted GFP following Cre-mediated recombination. Three-week-old mice were injected with tamoxifen, and 100 days later localization of reporter expression was detected by visualization of tdTom and GFP fluorescence in cryosectioned lumbar discs. Tissue was counterstained with DAPI to allow visualization of

nuclei. **a** Using image reconstruction software, visualization of an entire lumbar intervertebral disc of tamoxifen-treated *Col1a2-Cre(ER)T;ROSA26mTmG* mice shows GFP expression indicating Cre-mediated recombination specifically in the outer annulus fibrosus (OAF), but not in the inner annulus fibrosus (IAF) or nucleus pulposus (NP). TdTom expression is detected in untargeted cells (i.e., cells not expressing Cre under the control of the *Col1a2* promoter/enhancer at the time of tamoxifen injection). **b** 200x magnification of NP/IAF/OAF boundary. Scale bars indicate 250 and 50 μm , respectively

embryogenesis and/or tissue homeostasis. As a consequence, deletion of these genes is often embryonically lethal. To circumvent such problems, tissue-specific and inducible Cre systems have been developed to drive or delete a gene of interest in the desired tissue in a time- and or tissue-specific manner in the IVD (Table 1). To target genes of interest specifically in the NP, *Shh* (Choi et al. 2012; Maier et al. 2013), *Foxa2* (Frank et al. 2007; Merceron et al. 2014) and *Noto* (McCann et al. 2012; Bedore et al. 2013) Cre transgenic mice have been used, whereas *Agc* Cre transgenic mice have been used to

target genes of the IAF and NP (Henry et al. 2009). In addition, the *Col2a1* Cre (Hsieh et al. 2009; Chen et al. 2014) shows specific activity and has been used to target genes in the IAF. Lastly, the *Gdf5* Cre shows activity in both the IAF and OAF (Mundy et al. 2011). Importantly, no transgenic mice had been identified that enable the analysis of protein function specifically in the OAF.

Salient differences have been reported between the IAF and OAF (Fig. 2) with respect to cellular morphology and matrix composition (Bruchlmann et al. 2002), biochemical, structural

Table 1 Identified tissue-specific transgenic mouse models with Cre recombinase activity in the IVD

Cre	NP	IAF	OAF	Reference
<i>Gdf5</i>	-	+	+	(Mundy et al. 2011)
<i>Acn</i>	+	+	-	(Henry et al. 2009)
<i>Noto</i>	+	-	-	(McCann et al. 2012)
<i>Shh</i>	+	-	-	(Choi et al. 2012)
<i>Col2al</i>	-	+	-	(Chen et al. 2014)
<i>Foxa2</i>	+	-	-	(Frank et al. 2007)

+ Cre activity detected; -Cre activity not detected

and biomechanical properties (Hayes et al. 2001; Han et al. 2012; Li et al. 2014), as well as relative protein composition changes during degeneration (Antoniou et al. 1996). Type I collagen is more abundant and collagen fibrils are larger in diameter and more tightly packed in the OAF than IAF, which collectively confers a higher stiffness to the tissue (Han et al. 2012). Conversely, the IAF shows a higher aggrecan and type II collagen content (Li et al. 2014), narrower fibril diameter and larger extrafibrillar spacing (Han et al. 2012), which

together allows the IAF to more readily conform its shape in response to changes in osmotic pressure within the NP. Thus, differences in structure and protein composition of the inner and outer annulus are complementary in conferring mechanical properties to the entire AF that allow it to fulfill both of its major functions: to effectively absorb osmotic pressure from the NP, and prevent structural failure leading to pain (Aprill and Bogduk 1992). Another notable difference between IAF and OAF relevant to pathophysiological studies is that in a healthy IVD the OAF is innervated, whereas nerve fibers do not reach into the IAF (Bogduk et al. 1981). In addition, within the OAF cells show fusiform (spindle-like) morphology, whereas IAF cells are more spherical in shape (Bruehlmann et al. 2002). This evidence suggests that delineation of the IAF and OAF is critical in the study of the pathogenesis of IVD degeneration, particularly when considering the major compositional and structural changes that occur in the AF during ageing and disc degeneration (Antoniou et al. 1996; Ye et al. 2015). Thus *Col1a2-Cre(ER)T* mice may serve as a valuable tool for identifying the function of proteins that reside in this unique tissue, and hence will allow us to gain a better understanding of the processes and pathways regulating OAF health

Fig. 2 Schematic representation illustrating key differences between inner and outer annulus fibrosus tissues of a healthy intervertebral disc. The inner annulus fibrosus (IAF) and outer annulus fibrosus (OAF) show marked yet complimentary distinctions in their biochemical and structural properties. OAF cells have a fusiform (spindle-like) morphology, whereas IAF cells are more spherical in shape. Type I collagen is more abundant in the OAF than the IAF, whereas the IAF shows higher aggrecan and type II collagen content. In addition, collagen fibrils are larger in diameter and more tightly packed in the OAF than the IAF. The OAF is also innervated, whereas nerve fibres do not reach into the IAF in a healthy IVD. The pattern of fluorescent protein expression in IVDs from the *Col1a2-Cre(ER)T;ROSA26mTmG* mouse is depicted in this schematic. NP nucleus pulposus, VB vertebral body

and function in tissue homeostasis and disease. Given that the expression of type I collagen is dynamic in development, homeostasis and aging, further assessment of the potential utility of *Colla2-Cre(ER)T;ROSA26mTmG* mice will require a more detailed temporal analysis of the activity of the *Colla2* promoter/enhancer activity in these mice.

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Compliance with ethical standards

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Competing interests The authors declare that they have no competing interests.

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