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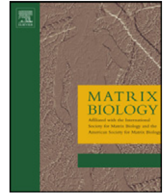

Lack of fibulin-3 alters regenerative tissue responses in the primary olfactory pathway

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ABSTRACT

The adult olfactory epithelium has maintained the ability to reconstitute its olfactory sensory neurons (OSNs) from a basal progenitor cell compartment. This allows for life-long turnover and replacement of receptor components as well as repair of the primary olfactory pathway in response to injury and environmental insults. The present study investigated whether fibulin-3, a glycoprotein in the extracellular matrix and binding partner of tissue inhibitor of metalloproteinases-3 (TIMP-3), plays a role in ongoing plasticity and regenerative events in the adult primary olfactory pathway. In wild-type control mice, fibulin-3 protein was detected on IB4^{+}CD31^{+} blood vessels, nerve fascicles and the basement membrane underneath the olfactory epithelium. After target ablation (olfactory bulbectomy), fibulin-3 was also abundantly present in the central nervous system (CNS) scar tissue that occupied the bulbar cavity. Using two different lesion models, i.e. intranasal Triton X-100 lesion and olfactory bulbectomy, we show that fibulin-3 deficient (*Efemp1*$^{-/-}$) mice have impaired recovery of the olfactory epithelium after injury. Ten days post-injury, *Efemp1*$^{-/-}$ mice showed altered basal stem/progenitor cell proliferation and increased overall numbers of mature (olfactory marker protein (OMP) -positive) versus immature OSNs. However, compromised regenerative capacity of the primary olfactory pathway in *Efemp1*$^{-/-}$ mice was evidenced by reduced numbers of mature OSNs at the later time point of 42 days post-injury. In addition to these neural differences there were consistent changes in blood vessel structure in the olfactory lamina propria of *Efemp1*$^{-/-}$ mice. Overall, these data suggest a role for fibulin-3 in tissue maintenance and regeneration in the adult olfactory pathway.
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1. Introduction

The primary olfactory pathway is a unique part of the mammalian nervous system where a small proportion of olfactory sensory neurons (OSNs) is continuously being turned over from basal stem/progenitor cells in order to maintain a receptive field for the sense of smell (e.g. Graziadei et al., 1979; Harding et al., 1977; Leung et al., 2007; for review, see Schwob, 2002). Axons of adult-born, immature OSNs leave the epithelium through gaps in the basement membrane, after which they enter the underlying lamina propria and track through the olfactory nerve towards the olfactory bulb. Here, in the glomerular layer of the olfactory bulb, they can successfully (re-) establish appropriate connections with the dendrites of second-order neurons, i.e. mitral and tufted cells. A similar process, though on a much larger scale, occurs following experimentally-induced *en mass* OSN turnover (e.g. Cummings et al., 2000).

Olfactory ensheathing cells (OECs) are intimately associated with bundles of OSN axons, from their peripheral origin to the target structures in the central nervous system (e.g. Doucette, 1984; Field et al., 2003). These specialised glial cells are thought to support the growth of new sensory axons, coming from the olfactory epithelium (OE), in a way similar to Schwann cells during peripheral nerve growth and regeneration. OECs upregulate expression of low-affinity nerve growth factor receptor p75 after injury (Gong et al., 1994), and it has been suggested that the cells maintain open channels that provide a passage for *de novo* growing axons from new OSNs (Li et al., 2005; Williams et al., 2004a,b). The extracellular matrix (ECM) constituents associated with OECs are likely to play an important role in olfactory neurite growth (Gong and Shipley, 1996; Tisay and Key, 1999; Treloar et al., 1996). In this context, we have recently reported that the glycoprotein fibulin-3 (also known as EFEMP-1, S1-5 or T16) is expressed along the primary olfactory pathway and produced by OECs *in vitro* (Vukovic et al., 2008).

Fibulin-3 belongs to a small family of glycoproteins that normally have widespread distribution in extracellular matrix structures such as basement membranes, microfibrils and elastic fibres (Argraves et al., 2003; Timpl et al., 2003; de Vega et al., 2009). A missense mutation in

Abbreviations: OE, olfactory epithelium; OMP, olfactory marker protein; OSN, olfactory sensory neuron; OEC, olfactory ensheathing cell.

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the fibulin-3 gene sequence has been linked to development of heritable macular degeneration in humans and mouse models (Stone et al., 1999; Fu et al., 2007; Marmorstein et al., 2007). Fibulin-3 deficiency, on the other hand, is hallmarked by reduced reproductivity, herniation, early ageing and reduced lifespan but not macular degeneration (McLaughlin et al., 2007). Additional *in vitro* work has shown that expression of fibulin-3 is significantly repressed in endothelial cells during capillary formation, i.e. vascular plasticity (Bell et al., 2001) while constitutive expression suppresses tumor angiogenesis (Albig et al., 2006). Based on its interaction with tissue inhibitor of metalloproteinases-3 (TIMP-3; Klenotic et al., 2004), a critical role for fibulin-3 in regulating matrix metalloproteinase (MMP) activity has been proposed (McLaughlin et al., 2007; Rahn et al., 2009). The latter molecules play an important role in matrix remodelling during periods of plasticity and growth.

To date, only three studies have mentioned fibulin-3 expression in context of the nervous system (Barkho et al., 2006; Thalmeier et al., 2008; Vukovic et al., 2008). Barkho et al. (2006) showed that fibulin-3 expression is downregulated in neurogenesis-promoting astrocytes. Our own earlier work has shown that manipulation of fibulin-3 expression in cultured OECs alters proliferation and migration (Vukovic et al., 2008). Specifically, knock-down of fibulin-3 resulted in reduced OEC proliferation whereas transgenic expression resulted in impaired migration of these glial cells. High levels of fibulin-3 also slowed down neurite growth. To further address a putative role for this glycoprotein in olfactory nerve growth and plasticity *in vivo*, the present study examined the role of fibulin-3 in olfactory nerve regeneration using comparative analysis between wild-type and fibulin-3 deficient (*Efemp1*$^{-/-}$) mice.

Two different lesion models were employed: 1) Triton X-100 irrigation of the nasal cavity (Verhaagen et al., 1990) and 2) unilateral ablation of the olfactory bulb (Graziadei et al., 1979; Carr and Farbman, 1992). In both scenarios, rapid death of OSNs occurs in the first few days after injury and degenerating OSNs are phagocytised by infiltrating macrophages (Suzuki et al., 1995). This is then followed by a more prolonged wave of basal cell proliferation and differentiation. Reversible Triton X-100 lesion allows these adult-born OSNs to mature and re-establish connections with target glomeruli in the olfactory bulb, with complete recovery after 6 to 7 weeks (Cummings et al., 2000). In contrast, olfactory bulbectomy does not lead to recovery of the OE to a pre-injury state, there being a persistent increase in epithelial proliferation and cell death (Carr and Farbman, 1992; Verhaagen et al., 1990). Regenerative responses following these lesions were assessed by quantitative counts of olfactory marker protein (OMP) -positive OSNs in the OE at different time points (i.e. 3, 10, 42 days post surgery); OMP selectively stains mature OSNs (Monti-Graziadei et al., 1977). In addition, (re-)innervation of glomeruli following lesioning with Triton X-100 was quantified using densitometric analysis. Changes in vascular structure in the lamina propria underneath the OE in response to injury and fibulin-3 deficiency were also investigated.

2. Materials and methods

2.1. Animals

Thirty-six *BALB/c* mice were used in this study (♂/♀, 8-week old), comprising 18 wild-type mice and 18 fibulin-3 deficient mice (*Efemp1*$^{-/-}$; McLaughlin et al., 2007). Animals were housed under standard conditions and maintained on a 12 h light/dark cycle with free access to water and food. Experimental procedures were approved by the animal ethics committees of The University of Western Australia and The University of Arizona, and conformed to NHMRC and NIH guidelines.

2.2. Surgical procedures

2.2.1. Olfactory epithelium lesions

Transient deafferentation of the olfactory bulb in both wild-type and *Efemp1*$^{-/-}$ mice was achieved via intranasal irrigation with 0.7% Triton X-100 (Promega, WI, USA) in sterile phosphate-buffered saline (PBS; 10 mM, pH 7.4). Mice were hand-held and a blunt 25-gauge needle was inserted into the right nostril to expel 100 µl of Triton X-100 solution into the nasal cavity (Verhaagen et al., 1990). Experimental mice ($n = 3$ for each genotype and time point) were allowed to survive for 3, 10 or 42 days after injury.

2.2.2. Olfactory bulbectomy

Regenerative responses in the OE of wild-type and *Efemp1*$^{-/-}$ mice were also investigated following target ablation, i.e. removal of the olfactory bulb (e.g. Carr and Farbman, 1992; Graziadei et al., 1979). These mice were anaesthetized via intraperitoneal injection with 3% Avertin (2,2,2-tribromoethanol; Sigma) solution at a dose of 300 mg/kg. Next, heads were shaved and an incision made in a rostrocaudal direction to expose the skull covering the olfactory bulbs. A hole was then drilled through the bone to create a window onto the right olfactory bulb. The exposed olfactory bulb was carefully separated from the forebrain with a sharpened 27 Gauge needle and subsequently removed via vacuum aspiration. The scalp was sutured and animals were left to recover on a warm plate (37 °C). All animals received post-operative injections of saline and buprenorphine (0.1 mg/kg; Reckitt and Colman, UK) and were allowed to survive up to similar time points as specified for Triton X-100 lesioned mice.

2.3. Specimen preparation and staining procedures

2.3.1. Tissue processing

Mice were euthanized 3, 10 or 42 days post-injury. Animals were killed with an overdose of sodium pentobarbitone (150 mg/kg; Virbac), followed by transcardial perfusion with 10 ml of saline (0.9% NaCl) and 30 ml of phosphate-buffered 4% paraformaldehyde (PFA) solution pH7.4, respectively. The olfactory bulb and epithelium were carefully dissected out and the specimens post-fixed overnight in 4% PFA at 4 °C. In preparation for cryosectioning, dissected tissue was incubated overnight at 4 °C with 250 mM EDTA in PBS to decalcify bone structures. Next, the tissue was cryoprotected via overnight incubations in 10% and 30% sucrose solutions and then snap-frozen in 2-methyl butane that was cooled on dry-ice. Horizontal serial sections (20 µm) were cut on a cryostat and collected on consecutive Superfrost Plus slides. Slides were air-dried for 2 h at room temperature and stored at −80 °C.

2.3.2. Histological stains

One series of sections through the dorso-ventral axis of the olfactory epithelium and bulb from each animal in the lesion experiments was used for haematoxylin and eosin (H&E) staining in combination with Alcian blue, which stains for glycoconjugates in cartilaginous structures within the tissue. These sections were used to verify the success of lesioning procedures and to compare gross morphology of the OE, lamina propria, olfactory nerve and bulb in wild-type and *Efemp1*$^{-/-}$ mice.

2.3.3. Immunofluorescent and histochemical staining procedures

Staining for fibulin-3 required treatment with Proteinase K (10 µg/ml; Sigma) for 5 min at 37 °C. Following three rounds of washing in PBS, the sections were blocked for 1 h at RT in PBS containing 1% BSA (Sigma) and 0.2% Triton X-100. Next, polyclonal rabbit anti-mouse fibulin-3 antibody (1:100; Kobayashi et al., 2007) was applied to the sections and the slides left overnight at 4 °C in a humidified chamber. The following day, sections were thoroughly washed and incubated with FITC-conjugated donkey anti-rabbit secondary antibody (1:200; Jackson ImmunoResearch, PA, USA) for 1 h at RT. Biotinylated isolectin B4 (IB4, 1:50; Vector Laboratories, CA, USA) and rat anti-mouse CD31 (1:200; BD Pharmingen, NJ, USA) were used to study the relationship between vascular structures and fibulin-3 in the lamina propria underneath the olfactory epithelium. Binding of IB4 was visualised via streptavidin-Alexa546 (Invitrogen) while CD31 immunoreactivity was revealed by subsequent incubation with biotinylated donkey anti-rat (1:400; Jackson ImmunoResearch) and streptavidin-Alexa546. Stained sections were washed several times in PBS to

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