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Generation of an *OMgp* allelic series in mice

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Summary

The very limited ability to regenerate axons after injury in the mature mammalian central nervous system (CNS) has been partly attributed to the growth restrictive nature of CNS myelin. Oligodendrocyte myelin glycoprotein (OMgp) was identified as a major myelin-derived inhibitor of axon growth. However, its role in axon regeneration *in vivo* is poorly understood. Here we describe the generation and molecular characterization of an *OMgp* allelic series. With a single gene targeting event and Cre/FLP mediated recombination, we generated an *OMgp* null allele with a LacZ reporter, one without a reporter gene, and an *OMgp* conditional allele. This allelic series will aid in the study of OMgp in adult CNS axon regeneration using mouse models of spinal cord injury. The conditional allele will overcome developmental compensation when employed with an inducible Cre, and allows for the study of temporal and tissue/cell type-specific roles of OMgp in CNS injury-induced axonal plasticity.

Keywords

OMgp; conditional allele; axon regeneration; CNS repair; myelin inhibition

Following development, axons in the mammalian central nervous system (CNS, including the brain and the spinal cord) have largely lost the ability to regenerate in response to injuries (Ramón y Cajal, 1928). Spinal cord injury patients suffer permanent functional deficits due to this inability for axon regeneration in adult CNS, which is in contrast with the robust regenerative ability of axons in the developing CNS or in the adult peripheral nervous system (PNS). The inhibitory action of myelin on axon growth has been hypothesized as one primary reason for this loss of regenerative ability (Berry, 1982; Schwab and Bartholdi, 1996). Several myelin-derived inhibitory proteins have been identified (Filbin, 2003; Yiu and He, 2006). Oligodendrocyte Myelin glycoprotein (OMgp) was identified as a major myelin-derived inhibitor that possesses potent inhibitory activity on neurite outgrowth *in vitro* (Kottis *et al.*, 2002; Wang *et al.*, 2002). Together with two other myelin inhibitors, Nogo and myelin-associated glycoprotein (MAG), it signals through at least two receptors, NgR1 and PirB, to effect the inhibitory action of myelin (Atwal *et al.*, 2008; Yiu and He, 2006). However, the role of OMgp in axon regeneration *in vivo* is not well understood. In a published report, OMgp deficient mice were found to exhibit slightly enhanced axon regeneration in the ascending dorsal column sensory tract and the descending raphespinal serotonergic tract but not in the corticospinal tract (Ji *et al.*, 2008). It was unknown whether

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even this modest effect was caused directly by *OMgp* deletion, or indirectly by the effect of *OMgp* deletion on the expression of other genes since injury-induced upregulation of Nogo was abolished in this *OMgp* mutant (Ji *et al.*, 2008).

In contrast, compensatory gene expression changes have been reported in other germline mutants in the myelin inhibition pathway. Two distinct mutant lines of *NgR1* exhibit upregulated *Nogo* mRNA expression (Kim *et al.*, 2004; Zheng *et al.*, 2005). A *MAG* mutant exhibits upregulated NgR1 expression (Pernet *et al.*, 2008). Thus, mutations in either an inhibitory ligand or receptor in the myelin inhibition pathway can lead to changes in the expression of other genes in the pathway, which may mask the effect of gene deletion (in the case of the *NgR1* or *MAG* mutants) or complicates the interpretation of the results (in the case of the *OMgp* mutant). This is particularly a challenge for genetic studies of axon regeneration because the most relevant stage to examine the role of these molecules is the adult stage, when any developmental compensation or other gene expression changes have already occurred in germline mutants. Therefore, inducible gene deletion with the conditional knockout technology is highly desirable for studying these genes in CNS axon regeneration (Zheng *et al.*, 2006). Furthermore, OMgp is expressed by both neurons and glial cells (Habib *et al.*, 1998; Huang *et al.*, 2005; Wang *et al.*, 2002), and a conditional allele will allow for the study of its tissue/cell type-specific function.

In order to establish an inducible and tissue/cell-type specific *OMgp* gene deletion system, we generated a conditional allele of *OMgp*. From the same gene targeting event, we also generated two additional alleles: a null allele with a *LacZ* reporter gene and a clean null allele without any reporter gene. We had two primary considerations when generating this allelic series: 1) minimizing disruption to OMgp expression in the conditional mutants prior to Cre-mediated deletion (Kwan, 2002); and 2) minimizing disruption to the expression of *NF1*, which when mutated causes Neurofibromatosis type I in humans, since the *OMgp* gene is embedded in an intron of the *NF1* gene (Viskochil *et al.*, 1991; Vourc'h and Andres, 2004).

The overall strategy for gene targeting and Cre/FLP mediated recombination is illustrated in Fig. 1a. The murine *OMgp* gene has two exons, with the second exon containing all coding sequence (Mikol *et al.*, 1993). We constructed a targeting vector with two *Frt* sites flanking a reporter/selection cassette, two *loxP* sites flanking (i.e. floxing) the second exon, and a *TK* negative selection marker (Fig. 1a). The two *Frt* sites flank a cassette containing a splice acceptor (*SA*), a *LacZ* reporter gene and a Neomycin resistance gene (*Neo*), such that the cassette can be deleted with the FLP recombinase. The *SA-LacZ* reporter element would be used to track *OMgp* expression. The *loxP* sites were placed in regions of the genomic DNA that are not highly conserved between the mouse and human genomes, and thus less likely to disrupt regulatory sequences (but see below). Cre-mediated deletion of exon 2 is expected to give rise to a null mutation.

Correct gene targeting was detected by Southern blot analysis with a 3' external probe (Fig. 1b) and verified with a 5' internal probe (Fig. 1c). The wild type allele and the targeted allele ($OMgp^{LacZNeoFlox}$) could be distinguished by PCR using a primer pair flanking the 3' *loxP* site (Fig. 1d). The targeted allele was transmitted through the germline and bred to a FLP deleter mouse (*ACTB:FLPe*) (Rodriguez *et al.*, 2000) to obtain the conditional allele, or to a Cre deleter mouse (*EIIA-Cre*) (Lakso *et al.*, 1996;Xu *et al.*, 2001) to obtain the null allele with the *LacZ* reporter gene ($OMgp^{LacZNeoNull}$) (Fig. 1a, e, f). Both the targeted mutant and the conditional mutant were genotyped using the primer pair flanking the 3' *loxP* site as in Fig. 1d, but they could be distinguished using primer pairs flanking the 5' *loxP* site (*f5* and *r4*, or *a2* and *r4* in Fig. 1a, data not shown). The conditional allele was then bred further to the *EIIA-Cre* line to obtain the clean null allele without the *LacZ* reporter gene

(*OMgp*^{CleanNull}) (Fig. 1a, g). Because the presence of a selectable marker, especially one with the bi-directional PGK promoter, may interfere with nearby gene expression (Manley *et al.*, 2001;Olson *et al.*, 1996;Scacheri *et al.*, 2001), deleting the selection cassette (*PGK-Neo-bpA*) would be expected to minimize any disruption to *NF1* gene expression in the *OMgp*^{CleanNull} mutants.

To determine whether the *LacZ* knockin reporter is functional, we stained adult brain and spinal cord sections with X-Gal for β-galactosidase activity in the *OMgp*^{LacZNeoNull} mutants (Fig. 2). β-galactosidase activity was detected in the cerebral cortex and corpus callosum at high levels, and, among other brain regions, in the cerebellum and superior colliculus at lower levels (Fig. 2a). In contrast to the proposed role of OMgp as a white matter inhibitor of axon regeneration, robust β-galactosidase activity was detected in the gray matter but not in the white matter of the adult spinal cord in either heterozygous or homozygous mutants (Fig. 2b-f). This suggests that the expression level of *OMgp* in oligodendroglia is much lower than that in spinal neurons. This observation is consistent with previous immunohistochemical data indicating that OMgp expression in the adult mouse spinal cord is predominantly in the gray matter (Habib *et al.*, 1998). mRNA *in situ* hybridization data from the Allen Brain Atlas also shows strong *OMgp* expression in the gray matter of adult mouse spinal cord, whereas a lower level of *OMgp* transcript is detected in the white matter (http://www.brain-map.org, with *OMgp* referred to as *OMG*). The detection of *OMgp* transcript (Allen Brain Atlas) but not β-galactosidase reporter activity (this study) in the spinal cord white matter likely reflects a higher sensitivity of mRNA *in situ* hybridization compared with X-Gal staining of the *LacZ* reporter gene product. Because the proposed role for OMgp in axon regeneration is primarily based on its expression in oligodendrocytes or oligodendrocyte-like cells (Huang *et al.*, 2005;Wang *et al.*, 2002), these observations indicate that it will be important to distinguish the role of OMgp in neurons from that in oligodendroglia in future.

Both the homozygous *OMgp*^{Flox} and homozygous *OMgp*^{CleanNull} mutants were viable, fertile and morphologically indistinguishable from their wild type littermates. We next examined the expression of OMgp and NF1 in homozygous *OMgp*^{Flox} and *OMgp*^{CleanNull} mutants by Western blot analysis on total spinal cord extracts (Fig. 3). As expected, homozygous *OMgp*^{CleanNull} mutants did not express OMgp protein (Fig. 3a). However, the level of OMgp protein in homozygous *OMgp*^{Flox} mice is reduced, to ~35% of the wild type levels (Fig. 3a, b). This indicates that even though we followed all possible steps to minimize the possibility of altered gene expression in the *OMgp* conditional mutants prior to gene deletion (Kwan, 2002), the expression of OMgp was affected by the limited sequence changes at the *loxP* insertion sites flanking exon 2. This may reflect the fact that the sequence of the single intron in *OMgp* is highly conserved between mice and humans (Mikol *et al.*, 1993).

Importantly, the expression of NF1 protein remained at the same level in homozygous *OMgp*^{CleanNull} mutants as in wild type controls (Fig. 3c, d). There is a trend for a slight increase of NF1 expression in homozygous *OMgp*^{Flox} mutants that did not reach statistical significance (Fig. 3c, d). In this regard, we noted that a previously published *OMgp* null allele carries a *GFP-Neo* fusion gene, and it is not known whether NF1 expression is altered in this mutant (Ji *et al.*, 2008). Thus, our *OMgp*^{CleanNull} allele may offer a unique advantage when assessing the baseline phenotype of an *OMgp* null mutant by avoiding any confounding changes in NF1 expression.

To determine whether inducible knockout can be achieved, we bred the *OMgp* conditional allele (*OMgp*^{Flox}) to an inducible Cre line with a ubiquitous promoter, *CAGGCre-ER*™ (Hayashi and McMahon, 2002). The Cre activity from this transgene is dependent on the

presence of tamoxifen (TM), which binds to the mutated form of estrogen receptor (ER), leading to the nuclear translocation and activation of the CreER fusion protein (Feil *et al.*, 1996). Mice homozygous for the conditional allele and carrying the *CAGGCre-ER*™ transgene were analyzed for OMgp protein levels by Western blot analysis following tamoxifen treatment (Fig. 4). Two weeks following the completion of tamoxifen treatment, there was already a significant reduction of OMgp in homozygous $OMgp^{Flox}$ mice with the *CAGGCre-ER*™ transgene when compared with homozygous $OMgp^{Flox}$ littermate controls without the *CAGGCre-ER*™ transgene. Four weeks following tamoxifen treatment, OMgp levels in the inducible knockout mice reduced to ~8% of that in littermate controls (Fig. 4), or ~3% of wild type levels. Thus, even though mice homozygous for the $OMgp^{Flox}$ allele exhibited a lower level (~35%) of baseline OMgp protein expression than wild type mice, inducible gene deletion is still effective with this conditional allele. Provided that ~35% of the OMgp protein is sufficient for its normal function and does not cause significant compensatory expression of other genes, the lower baseline level of OMgp in homozygous $OMgp^{Flox}$ mice may even be advantageous for conditional gene deletion: since there is less OMgp protein to start with, even an incomplete conditional gene deletion may be sufficient to disrupt the normal function of OMgp.

In summary, we generated an allelic series for the gene encoding OMgp, a major myelin-derived inhibitor of axon growth. The clean null allele, $OMgp^{CleanNull}$, will be useful in analyzing the baseline phenotype of an *OMgp* null mouse without significantly affecting the expression of NF1. The $OMgp^{LacZNeoNull}$ allele will be a useful reporter line to track *OMgp* gene expression with the *LacZ* knockin reporter. The conditional allele, $OMgp^{Flox}$, will be useful in analyzing OMgp function in axon plasticity in adult CNS with minimized compensatory gene expression, in establishing the temporal roles of OMgp in different phases post-injury, and in investigating the tissue/cell-type specific function of OMgp.

MATERIALS AND METHODS

Gene targeting and molecular characterization

A replacement gene targeting vector was constructed with a 3.9 kb left arm homology, a *Frt*-flanked *SA-LacZ-Neo* reporter gene and positive selection cassette, a *loxP* site, a 2.7 kb middle arm homology, a second *loxP* site (in the same orientation as the first one), a 4.1 kb right arm homology, and a herpes simplex virus thymidine kinase gene (*TK*) as the negative selection marker. The selection cassette and the first (*5′*) *loxP* site replaced a 97 bp sequence in the intron that is not conserved between the mouse and human genomes. The second (3') *loxP* site is marked with a *Bgl*II site so that the conditional allele (resulting from a crossover at the left arm and a crossover at the right arm) can be distinguished from the non-conditional allele (resulting from a crossover at the left arm and a cross over at the middle arm) as well as the wild type allele on a Southern blot with *Bgl*II digest hybridized with the 3' external probe: wild type, 10.2 kb; conditional allele, 5.9 kb; non-conditional allele, 13.7 kb (data not shown). The homology arms were amplified from isogenic 129S5 genomic DNA with a high fidelity PCR amplification kit (Roche) and sequence verified. The *SA-LacZ* (or *SA*-β-*Galactosidase*) cassette was a gift from Philippe Soriano (Fred Hutchinson Cancer Center) (Friedrich and Soriano, 1991). The floxed *PGK-Neo-bpA* cassette was a gift from Richard Behringer (M.D. Anderson Cancer Center). Sequences of primers used for constructing the homology arms or genotyping are provided as supplementary information (Supplementary Table 1).

The linearized targeting vector was electroporated into AB2.2 embryonic stem cells followed by positive selection with Geneticin (for *Neo*) and negative selection with FIAU (against *TK*). The sequence of all primers used in constructing the targeting vector, genotyping and detecting the Cre/FLP recombination products is provided as supplementary

information. Targeted ES cells harboring the *OMgp*^{LacZNeoFlox} allele were injected into blastocyst embryos (by the UCSD Transgenic Mouse Core). Chimeric mice were bred to C57BL/6 females for germline transmission.

Histology

3-5 month old mice were euthanized with anesthetic overdose and perfused transcardially with 4% paraformaldehyde. Brain and spinal cord were extracted and post-fixed in 4% paraformaldehyde for two hours followed by cryoprotection in 30% sucrose overnight. Specific regions of the spinal cord were embedded in OCT, frozen over dry ice and 20 μm cryosections thaw-mounted onto Superfrost Plus slides. To stain for LacZ, slides were incubated overnight at 37°C in X-gal staining solution (5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 40 mg/ml X-gal in 1× PBS), and then dehydrated through graded alcohol washes and cover-slipped.

Tamoxifen injection and Western blot

Tamoxifen (50 mg/ml) was diluted in 90% sunflower oil and 10% ethanol and mice were injected once daily (0.125 mg/g of body weight) intraperitoneally for five consecutive days. At 2 or 4 weeks after the last injection, mice were sacrificed for analysis. Spinal cord tissue was homogenized in protein extraction buffer, centrifuged and protein concentration in supernatant quantified using Lowry assay (Bio-Rad DC Protein Assay kit). 30 μg protein samples from different OMgp mutant mice were separated in a 5% resolving gel using SDS-PAGE and then transferred onto a nitrocellulose membrane (Amersham Biosciences). Membranes were washed, blocked (5% milk in TBS-T) and incubated in the appropriate antibodies overnight at 4°C. Next day, membranes were washed and incubated in the appropriate HRP-conjugated secondary antibodies (Pierce) and detected using Supersignal West Dura ECL solution (Pierce). Antibodies for Western blot analysis: rat anti-OMgp at 1:1000 (R&D); rabbit anti-Neurofibromin 1 (or NF1) at 1:200 (Santa Cruz); mouse anti-α-Tubulin at 1:1000 (Sigma). Scanned images were quantified using ImageJ software (normalized to α-Tubulin levels) and tested for statistical significance using GraphPad Prism software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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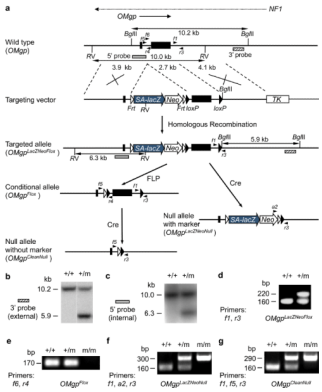

Fig. 1.

Gene targeting to generate an allelic series of *OMgp*. (**a**) The general scheme for gene targeting and Cre/FLP mediated recombination. The two black bars represent the two *OMgp* exons. *R*V, *Eco*RV; *SA*, splice acceptor; *Neo*, neomycin resistance gene; *TK*, herpes simplex virus thymidine kinase gene. Black triangle, *loxP* site; open triangle, *Frt* site. Small arrowheads indicate primers used to confirm gene targeting or Cre/FLP-mediated recombination and for genotyping. (**b**) Southern blot to detect targeted clones with a 3' external probe. (**c**) Southern blot to confirm targeted clones with a 5' internal probe. (**d-g**) PCR analyses to genotype the $OMgp^{LacZNeoFlox}$ (**d**), $OMgp^{Flox}$ (**e**), $OMgp^{LacZNeoNull}$ (**f**) and $OMgp^{CleanNull}$ (**g**) alleles. +, wild type allele; m, mutant allele.

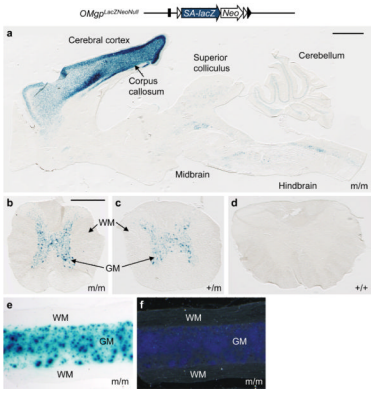

Fig. 2.

Reporter gene expression with the *OMgp*^{LacZNeoNull} allele. Sagittal sections of the adult brain (**a**), transverse (**b**, **c**, **d**) and sagittal (**e**, **f**) sections of adult spinal cord from the *OMgp*^{LacZNeoNull} mutants and controls were stained for *LacZ* reporter expression with X-Gal. +, wild type allele; m, mutant allele. (**f**) is a dark field view of the same section as in (**e**) to better delineate the boundary between the gray matter (GM) and the white matter (WM). Scale bar = 2 mm (**a**), 500μm (**b-f**).

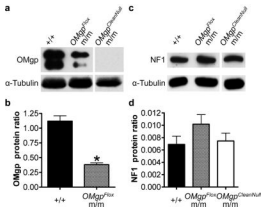

Fig. 3.

Western blot analysis on OMgp and NF1 in *OMgp*^{Flox} and *OMgp*^{CleanNull} mutants. (**a**, **b**) Total protein extracts from the adult spinal cord were blotted for OMgp (**a**) or NF1 (**b**), and α-Tubulin as the loading control. Representative data from one of three independent sets of biological samples are shown. +, wild type allele; m, mutant allele. (**c**, **d**) Quantification of OMgp and NF1 normalized to α-Tubulin. *$p < 0.05$, Student's *t*-test.

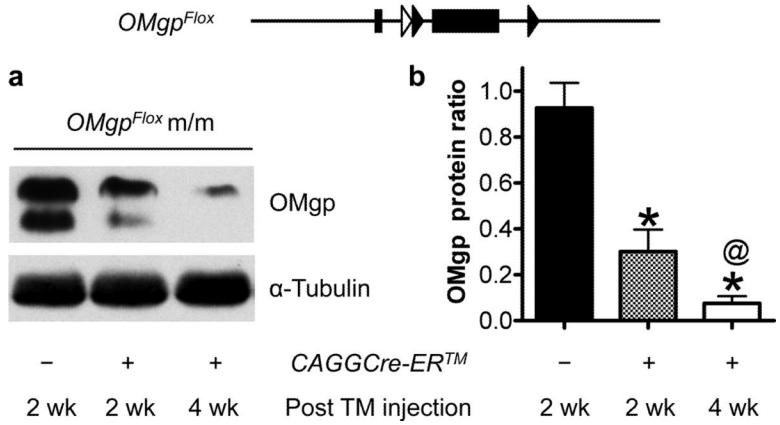

Fig. 4.

Inducible deletion of OMgp in *OMgp*^{Flox} mutants. Adult homozygous *OMgp*^{Flox} mutants with or without the *CAGGCre-ER*™ transgene were injected with tamoxifen (TM) and spinal cord tissue extracted at 2 and 4 weeks after the completion of the injections. (**a**) Total protein was immunoblotted for OMgp protein and α-Tubulin control. Representative data from one of three independent sets of biological samples are shown. (**b**) Quantification of OMgp normalized to α-Tubulin. *$p < 0.05$ compared to mice without the *CAGGCre-ER*™ transgene; @$p < 0.05$ compared to mice with the *CAGGCre-ER*™ transgene at the 2-week time point, Student's *t*-test.