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► **To cite this version:**

Elisa Marti, Angeles Rabadan, Jordi Cayuso, Gwenvael Le Dreau, Catarina Cruz, et al.. Jagged2 Controls the generation of Motor Neuron and Oligodendrocyte progenitors in the ventral Spinal Cord. Cell Death and Differentiation, 2011, 10.1038/cdd.2011.84 . hal-00655646

HAL Id: hal-00655646

<https://hal.science/hal-00655646>

Submitted on 1 Jan 2012

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Tracking number CDD-11-0291R

**Jagged2 Controls the generation of Motor Neuron and
Oligodendrocyte progenitors in the ventral Spinal Cord**

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Key words: Sonic hedgehog, Notch, Jagged2, Olig2, Hes5, spinal cord, motor neurons, oligodendrocytes, neurogenesis, gliogenesis, chick, mouse.

Running title: MN to OLP switch by Jagged2

Character count: 00

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ABSTRACT

In the developing spinal cord, motor neurons and oligodendrocytes arise sequentially from a common pool of progenitors. However, the genetic network responsible for this neurogenesis to gliogenesis switch is largely unknown. A transcriptome analysis identified the Notch-ligand Jagged2 as a Shh-regulated factor transiently expressed in motor neuron progenitors (pMN). In vivo loss- and gain-of-function experiments show that Jagged2 schedules the differentiation of the pMN progenitors. At early developmental stages, Olig2 expressing pMN progenitors that enter the differentiation pathway exclusively generate motor neurons. At these times, the activation of the Notch pathway by Jagged2 maintains selected pMN progenitors in an undifferentiated state by two mechanisms; first inhibits MN generation by reducing Olig2 proteins levels, and second directly inhibits the premature generation of OLPs by maintaining high levels of Hes5. Later, extinction of Jagged2 from the pMN results in the loss of Hes5 expression, heralding the gliogenic phase of pMN progenitors. Strikingly, downregulation of Jagged2 in pMN progenitors is sufficient to promote the precocious generation of OLPs. Together these data provide evidence that Jagged2 is a key regulator of the the timely and ordered generation of two of the defining cell types in the spinal cord, motor neurons and OLPs.

INTRODUCTION

In many developing tissues, most notably the central nervous system, the identity of a cell is linked to the time at which it is generated. However the mechanisms that underpin the temporal specification of cell fate are poorly understood. A well defined example of this problem is the sequential generation of motor neurons (MNs) and oligodendrocytes (OLPs) in the developing spinal cord. These two cell types arise from a common domain of progenitors, termed the motor neuron progenitor (pMN) domain, which is located in the ventral neural tube. Initially, the pMN domain produces somatic MNs that send axonal trajectories out of the CNS to innervate peripheral targets. Subsequently progenitors in the same region produce oligodendrocytes, the myelinating cells of the CNS, which disperse throughout the grey matter of the spinal cord. The pMN domain is specified by the instructive activity of the secreted protein Sonic hedgehog (Shh), which, signalling through the canonical Gli pathway, provides positional identity to neural progenitors by directing a transcription network in responding cells (reviewed in 1). Amongst the members of this network is the basic-Helix-Loop-Helix (bHLH) transcription factor Olig2, which is expressed in the pMN domain and provides a molecular identifier for the pMN domain (2,3).

Early studies showed that Shh is both necessary and sufficient for the generation of spinal MNs (4,5,6). Subsequently Shh was shown to be required for OLPs to emerge at the appropriate developmental stages (7,8). This indicated a dual role for Shh in both MN and OLP generation. Consistent with this, Shh induces the expression of Olig2 and a related gene, Olig1, in the pMN domain. MNs and OLPs are depleted or eliminated in the spinal cord of mice and zebrafish lacking Olig2 (9,10,11,12). In addition Olig2 promotes the expression of the neurogenic bHLH factor Ngn2 that contributes to the terminal differentiation of MNs (13,14). These data led to a model in which MNs and OLPs derive from a common progenitor that expresses Olig2, which switches from MN to OLP generation at a defined developmental time point. However, the factors regulating the temporal switch from MN to OLP and maintaining the population of progenitors undifferentiated to ensure that the appropriate numbers of each cell type are generated, remain elusive.

The Notch pathway has a well recognized role in maintaining the balance between progenitor self renewal and differentiation (reviewed in 15). Ligands of the Notch pathway (Dll1, Jag1) are expressed in distinct patterns along the DV axis in the developing spinal cord (16,17,18,19). Notch signalling is a cell-cell communication system and activation of Notch signalling results from the interaction between the Notch receptor and its ligands, Delta and Jagged, expressed by adjacent cells. The ligand receptor interaction induces a proteolytic cleavage of the Notch protein and nuclear translocation of its intracellular domain (Notch ICD), which interacts with the DNA-binding protein CSL (RBP-Jk) to activate Notch downstream genes, such as the Hes transcription factors (15). These proteins have been demonstrated to mediate their functions, at least in part, by repressing the neurogenic bHLH factor Ngn2 that contributes to the terminal differentiation of MNs (13, 14), and by inhibiting the maturation of OLPs, at least in the optic nerve (20). Thus a plausible model might entail Notch activity preserving undifferentiated progenitors in the pMN during the period of MN generation so that sufficient are available when oligogenesis supervenes. Supporting this view, in the zebrafish embryo, Notch signalling is required during embryogenesis to maintain radial glia and permit oligodendrocyte development (21). However, whether Notch signalling plays a direct role in determining the timing, and the switch, from neurogenesis to gliogenesis is unknown.

Here we show that in the chick and the mouse embryo, the expression of the Notch ligand Jagged2 is restricted to the pMN domain during the period of motor neuron generation, and that this pattern of expression depends on Shh activity. In vivo experiments demonstrate that loss of Jagged2 resulted in the accelerated differentiation of MNs and the premature generation of OLPs. Conversely, gain of function experiments demonstrate that Jagged2 activity prevents the differentiation of pMNs, at least in part by lowering the levels of Olig2 protein, thus preserving Olig2+ progenitors for later oligodendrogenesis. In search for the Notch-dependent effector regulating this cell fate switch we found that Hes5, a Notch target and direct repressor of OLP specific genes, is regulated and mediates the activity of Jagged2. Together the data provide new insight into the genetic network responsible for the timely generation of spinal motor neurons and oligodendrocyte progenitors..

RESULTS

Shh signaling directs expression of Jagged2 to the pMN domain

An in vivo transcriptome analysis of neural progenitors responding to different levels of Shh activity in a cell autonomous manner (see methods) (22), focussed our attention on the Notch pathway, and the possibility that Notch signalling plays a role in patterning the neural tube. In response to activators of the Shh pathway expression of the Notch-ligand Serrate2/Jagged2 appeared up-regulated (Figure1A).

To examine the spatiotemporal distribution of Jagged2 in the developing spinal cord, we mapped its expression in relation to proteins defining different dorsoventral (DV) domains using immunohistochemistry and in situ hybridization at different developmental stages. The onset of expression occurred after neural tube closure in scattered cells (data not shown). From HH15 to HH18 Jagged2 was expressed within the motor neuron progenitor domain (pMN) (Figure 1B,C). At these stages Jagged2 was co-expressed with the MN progenitor protein Olig2 in a salt-and-pepper pattern (Figure 1F,G) and was excluded from the differentiated MNs. Additionally, Jagged2 was expressed in a subset of dorsal progenitors (Figure 1C,D). Expression of Jagged2 in the pMN was transient, however was maintained in the transition zone by HH23/24 (Figure 1D,H) and was extinguished by HH30 (Figure 1E,I). However, dorsal expression of Jagged2 is maintained at HH30, together with lower expression levels in some subpopulations of differentiated MNs. Interestingly, the down-regulation of Jagged2 expression in the pMN domain correlates with the time OLP generation supervenes (Figure 1E,J).

We tested whether the ventral expression of Jagged2 depends on Shh activity. Analysis of the *Shh*^{-/-} embryos showed the lack of Jagged2 expression in the ventral hindbrain and spinal cord (Supplementary Figure1). Additionally, inhibition of Shh signalling by a dominant negative version of Gli3 (Gli3^R, 23) resulted in the loss of Jagged2 expression from pMN (Supplementary Figure 1). Conversely, constitutive activation of the Shh pathway, using Gli3^{Act}, resulted in a dorsal expansion of Jagged2 (Supplementary Figure1). Together these results indicate that Shh signalling is required and sufficient for the ventral expression of Jagged2.

Experimental evidence supports a model in which the specific distribution of Notch ligands, along the DV axis of the neural tube, endows distinct progenitor domains with the ability to influence the pace of cell differentiation in a domain- and cell type-specific manner (19, 24, 25). This prompted us to ask whether Jagged2 activity influenced the generation of either MNs and/or OLPs from the pMN domain. To test the function of Jagged2 in the pMN domain, we first generated short-hairpin RNA (shRNA) to knock-down endogenous Jagged2. Analysis by qPCR of target RNA in transfected neural cells indicated that the shJagged2 (LOF-JAG2) reduces RNA levels to ~50% of normal without reducing the closely related ligand Jagged1 (Supplementary Figure 2). Additionally, knock-down of Jagged2 did not affect the relative distribution of proliferating progenitors and differentiated neurons in the spinal cord, neither changed the identity of progenitors along the DV axis (Supplementary Figure 2 and data not shown) similar to the results recently reported for Jagged1 (19). Thus this analysis indicated that shJagged2 did not affect the anatomical integrity of the neural tube, prompting us to analyse domain specific cell fate identities.

Jagged2 activity regulates the timely generation of MNs

In the spinal cord MNs are generated from the pMN domain in a narrow time-window (stage HH15 to stage HH23/24; 26). To test the role of Jagged2 in MN generation, brachial regions of embryos were electroporated at HH14, before the onset of MN generation, and assayed with the early MN marker MNR2/HB9 (14). Quantification of MNs and their spatial distribution was performed relative to the ventricular lumen (Figure 2A). MNR2⁺ cells positioned adjacent to the VZ correspond to newly generated MNs (MNR2⁺ cells in the Transition Zone, TZ), whereas those positioned towards the basal/pial surface correspond to terminally differentiated MNs (MNR2⁺ cells in the Marginal Zone, MZ). Knock-down of Jagged2 (LOF-JAG2) markedly changed the distribution of MNR2⁺ cells: more cells were positioned adjacent to the VZ and less terminally differentiated MNs were present. By contrast, forced expression of Jagged2 (GOF-JAG2) reduced the total number of MNR2⁺ cells at both locations (Figure 2B). This suggested that Jagged2 activity might regulate the tempo of MN generation and prompted us to analyse MN differentiation.

To unequivocally identify the birth date of MNs, we administered a single dose of BrdU at HH20 (24h-PE, the time at which ~50% should be post-mitotic; 26) and harvested the embryos at HH23/24 (48h-PE). Double immunohistochemistry for BrdU and the

definitive MN marker *Islet1* allowed us to identify MNs (*Islet1*⁺/*BrdU*⁻) that had exited the cell cycle and initiated differentiation prior to HH20 (Figure 2C,D). Embryos that were electroporated at HH14 (before the onset of MN generation) with the control vector contained 43±0,015% of *Islet1*⁺/*BrdU*⁻ cells, confirming that ~50% of MNs were generated prior to HH20 (Figure 2E,F). By contrast, in embryos in which *Jagged2* activity was depleted from HH14, the percentage of *Islet1*⁺/*BrdU*⁻ MNs increased significantly to 61±0,017% (~2 fold increase) Furthermore, the total number of *Islet1*⁺ cells appeared reduced, 48h-PE of LOF-JAG2 (Figure 2E,F). This suggests that loss of *Jagged2* activity promotes the premature differentiation of MNs.

Conversely, gain-of-function experiments with *Jagged2* (GOF-JAG2) resulted in the significant decrease in MN differentiation and the percentage of *Islet1*⁺/*BrdU*⁻ decreased to 33±0,02% (~1.5 fold decrease) (Figure 2E,F). Importantly, these experiments indicated that the ability of *Jagged2* to activate Notch is limited to pMN progenitors, since differentiation of other neuronal identities was largely unaffected (Supplementary Figure 3). This is in concordance to the recently reported roles for *Dll1* and *Jagged1* (19,25).

Jagged2 activity inhibits the differentiation of *Olig2*⁺ progenitors.

Intriguingly both LOF- and GOF- in vivo experiments resulted in a reduction of the final numbers of differentiated MNs. To better understand this result, we took advantage of the data generated by the determination of the percentages of born motor neurons (Figure 2F), to model the behaviour of the pMN domain for three cell divisions (~48 hours). We took the simplifying assumption that normally 50% of divisions were asymmetric. We next examined the effect of the changes in the rate of neurogenesis measured in the LOF- and the GOF- experiments. Plotting the ratios of predicted differentiated MNs in each experimental condition showed the initial increase in MN in the absence of *Jagged2*, while at the end of the 48h period, the final MN number decreased in both conditions. These profiles were similar to those obtained empirically (Figure 3A). Moreover, the model predicted specific changes in progenitor numbers, prompting us to experimentally test this prediction.

Olig2 is a basic-Helix-Loop-Helix (bHLH) transcription factor expressed in the pMN domain that provides a molecular identifier for the pMN domain and is a key component of the genetic network operating for the generation of MNs (2,3,14).

Consistent with the prediction of the model, the forced expression of Jagged2 (GOF-JAG2) resulted in an increase in Olig2⁺ progenitor cells within the VZ (Figure 3B). Conversely, reduced Jagged2 activity (LOF-JAG2) caused a gradual loss of Olig2⁺ cells from the VZ (Figure 3B). Noticeably, these experimental data match the changes in progenitor numbers predicted by our model. All together these data suggests that Jagged2 controls MN differentiation through the regulation of Olig2⁺ cells.

We observed that forced expression of Jagged2 laterally inhibit Olig2⁺ cells that are retained as progenitors within the VZ (Figure 3C). Furthermore, it also came to our attention the fact that Olig2⁺ cells, retained in the VZ by the activity of Jagged2, appeared to express lower levels of Olig2 protein, when compared to the control contralateral Olig2⁺ progenitors (Figure 3C). To further assess this observation, we next quantified total protein levels by WB in neural tube extracts that were electroporated with either Olig2 alone or together with Jagged2. Co-electroporation of Jagged2 caused a ~50% reduction on both Olig2 protein bands (Figure 4A). This observation suggested a mechanism by which Jagged2 reduced Olig2 protein to a level below the threshold require to generate MNs, thus resulting in the lateral inhibition of MN differentiation. Indeed, over-expression of Olig2 alone was sufficient to promote MN generation as shown by the ectopic MNR2⁺ cells (Figure 4C) (14). However, exposure to Jagged2 inhibits the MN differentiation activity of Olig2⁺ (Figure 4C). Altogether, our data suggest a model in which Jagged2 function in the pMN domain to inhibit laterally the Olig2-expressing progenitor from becoming MNs. Olig2-expressing progenitors that do not contact with Jagged2, were primed to enter the differentiation pathway to generate MNs. Lateral contact to Jagged2, prevents MN differentiation, at least in part, by reducing the Olig2 protein levels.

Jagged2 activity is necessary to prevent premature OLP generation

Once neurogenesis of MNs is terminated, progenitors in the pMN domain switch to the generation of oligodendrocyte progenitors (OLPs). We therefore analysed the effect of Jagged2 depletion on the oligodendrogenesis. Strikingly, after knock-down of Jagged2 function at HH14, Olig2 expressing cells were observed migrating away from the pMN (mean 19,14± 1,99 cells/section, n=20) (Figure 5A,B). This contrasted with control embryos in which Olig2 was robustly expressed in the pMN domain and no migratory Olig2⁺ cells were detectable. Furthermore Olig2⁺ migratory cells were significantly reduced when the human-Jagged2 (GOF-JAG2) was co-electroporated, supporting the

specificity of the phenotype (rescue experiments: mean $9 \pm 2,27$ cells/section, $n=15$) (Figure 5C,H). In both mouse and chick spinal cord, Olig2 expressing cells that migrate away from the VZ have been characterized as OLPs (2,3,9,10), but these are not normally generated until HH30 (7), after the period of MN generation. In order to characterize the molecular nature of these migratory Olig2⁺ cells we co-immunostained with the OLP specific marker O4 (7). This revealed that most of the migratory Olig2⁺ cells co-expressed O4 (Figure 5G), consistent with these cells being OLPs.

Furthermore, the SoxE proteins have a prominent role in OLP generation. Sox9 is required for OLP specification and is expressed transiently in early migrating OLP. Sox8 and Sox10 are also expressed in early migrating OLPs and Sox10 activates myelin genes as part of the terminal differentiation program of oligodendrocytes (27, 28). Consistent with migratory cells being OLPs, knock down of Jagged2 resulted in the premature migration of Sox9⁺ cells (mean $28,57 \pm 5,73$ cells/section, $n=16$) and the concomitant reduction of Sox9⁺ cells from the pMN. Again in a Jagged2-LOF background, the number of Sox9⁺ migratory cells was significantly reduced when NT was co-electroporated with the human Jagged2 cDNA (GOF_JAG2) that was resistant to the short hairpin (rescue experiments; mean $7,2 \pm 2,1$ migratory cells/section) further supporting the specificity of this phenotype (Figure 5D-F,H). Furthermore, premature migratory OLPs expressed expressed *Sox8* (not shown), *Sox10* and the myelin specific gene *PLPDM20* (Figure 5I-N). Knock-down of Jagged2 additionally increased levels of platelet derived growth factor receptor alpha (*PDGFRa*) and *PLP* expression, as assessed by real-time PCR (Figure 5O), as well as the activity of the Myelin Basic Reporter as assessed by Luciferase activity (Figure 5P). Altogether, these data indicate that knockdown of Jagged2 activity results in the precocious generation of OLPs from the pMN domain.

Converse gain-of-function experiments allowed the maintenance of high levels of Jagged2 expression at the time of OLP generation, when Jagged2 expression is normally extinguished from pMN. This resulted in the loss of migratory OLP identified by double immunohistochemistry for Olig2⁺/Nkx2.2⁺ and Olig2⁺/O4⁺, at the stage at which migratory OLPs were abundant in the control contralateral side (Figure 6A-E) (7, 29, 30). This result is consistent with the finding that JAG2 has a negative role on Olig2 expression. Together these data supports the idea that MNs and OLPs are sequentially

generated from a single progenitor and that Jagged2 activity ensures progenitors do not switch from neurogenesis to gliogenesis prematurely.

Jagged2 controls OLP generation through the regulation of Hes5 expression

How might Jagged2 prevent precocious OLP generation? Members of the Hes family of bHLH transcription factors are the best characterized Notch targets that function as effectors of the cell fate decisions mediated by Notch signalling. In the chick spinal cord Hes5 is the only member expressed in the pMN domain (Figure 7A,B) (31) and its expression is reduced from these progenitors as they switch from MN to OLP generation (Figure 7C). Moreover, mice lacking Hes5 generate excess oligodendrocytes (32). These data raised the possibility that Hes5 is the transcriptional effector that regulates the timing of OLP generation.

We first tested whether Jagged2 regulates Hes5 expression in the pMN. Reduction of Jagged2 function caused the loss of Hes5 expression (Figure 7D,F). Moreover, even though Hes5 is broadly expressed, the loss of Hes5 expression was restricted to the pMN. Conversely, gain-of-function experiments showed that forced expression of Jagged2 resulted in the maintenance of high levels of Hes5 in the pMN domain (Figure 7F).

We next asked whether Hes5 activity could rescue the premature generation of OLP observed in the absence of Jagged2. Indeed, in embryos in which Jagged2 was depleted by shRNA, the forced expression of Hes5 significantly reduced the premature migration of Olig2⁺/Sox9⁺ cells, caused by depletion of Jagged2 activity (Olig2⁺ from 19,14±1,99 to 3,62±0,53 cells/section; Sox9⁺ from 28,57±5,73 to 7,09±1,28 cells/section, Figure 7G-K). Consequently, Olig2 and Sox9 expressing cells were maintained in the VZ of the pMN domain (Figure 7G,H). This supports the conclusion that Hes5 acts downstream of Jagged2 to control the premature generation of OLPs.

DISCUSSION

In this study we provide evidence that the generation of MN and OLP in the ventral spinal cord depends on the activity of the Notch-ligand Jagged2. The activity of

Jagged2 appears to prevent the neighbouring Olig2-expressing progenitor from entering the differentiation pathway. In pMN progenitors in which the Notch pathway is activated by Jagged2 MN differentiation program is prevented, at least in part, by reducing the Olig2 protein levels. This preserves a pool of progenitors and, at the same time, maintains high levels of Hes5 expression that in turn directly inhibit the oligodendrocyte generation during the neurogenic phase.

In the absence of Jagged2 however, pMN progenitors enter the differentiation program to generate first MNs and next, when Hes5 levels are sufficiently reduced, to generate oligodendrocytes. Together the data support a model in which MN and OLP arise sequentially from a common progenitor population residing in the pMN. Jagged2 controls the timing of the switch from MN to OLP production by suppressing OLP generation during the neurogenic phase.

During the process of writing these results, two independent studies show post-transcriptional modifications of Olig2 that changed the activity of Olig2 and thus the outcome of the Olig2+ progenitors in the pMN. On the one hand, Li and cols (33) find a phosphorylation site in the bHLH domain of Olig2 that favours the homodimerization capability of Olig2, and thus its transcriptional activity. Mice carrying a mutant version of Olig2 (Olig2S147A) show the loss of progenitors at the pMN domain and the loss of differentiated MNs, a phenotype that is highly comparable to the over-expression of Jagged2. This suggested that changes in the MN-differentiation capacity of Olig2 that we reported in the presence of Jagged2, might be the result of a similar post-transcriptional modification. On the other hand, Sun and cols (34) find a triple serine phosphorylation motif at the N-terminal part of the Olig2 protein, which are required for the Olig2 mediated proliferation of neural progenitors however are dispensable for the Olig2-mediated specification of OLPs. Altogether these data indicate a pivotal role played by post-transcriptional modifications of Olig2 in the outcome of the pMN progenitors, and our data indicate that Jagged2 might be among the upstream signals regulating these modifications.

The generation of both MNs and OLPs requires Shh signalling. Olig2 expression depends on Shh and is required for the generation of both MNs and OLPs suggesting an explanation for the requirement for Shh in pMN derivatives. Here we provide evidence that, in addition to Olig2, the regulation of Jagged2 expression by Shh signalling also

contributes to the generation of both MNs and OLPs. Shh activity is required and sufficient for the restricted expression of Jagged2 to the pMN domain. Moreover, the involvement of Jagged2 in regulating the timing of the transition from MN to OLP production links the process of spatial patterning, which positions the pMN domain in the ventral neural tube, with temporal patterning that determines when each cell type is generated. Whether Jagged2 expression is directly or indirectly regulated by Shh signalling, remains to be determined. Furthermore, the timely loss of Jagged2 expression from the pMN domain appears as an important pre-requisite for OLP formation. Molecular mechanism operating directly to pause Jagged2 expression might include transcriptional repression and/or epigenetic silencing, among many others, and understanding these mechanisms remains as an important unresolved question.

Whether the Notch pathway is required simply to maintain pools of undifferentiated progenitor cells, or whether it plays instructive roles in promoting specific cell fates has been the subject of debate. Our data indicate that Notch signalling, activated by Jagged2, maintains pMN progenitors in an undifferentiated state but also provides an instructive signal that blocks the fate switch from MN to OLP. This suggests a dual role for Notch signalling and is consistent with other studies of glial development. The Notch effector Hes1 promotes Muller and radial glia identities (35), while Hes5 directly regulates expression of myelin genes (32). Moreover, Notch signalling has been implicated in the diversification of neuronal identities in the neural tube (24, 36). The observation that Notch-ligands are expressed in discrete domains along the DV axis of the CNS is in line with the idea that Notch-ligands play specific roles in the cell fate decisions of progenitors. Thus our data support the idea that Notch ligands can serve both to prevent the untimely differentiation of neural progenitors and to generate diversity within a population of progenitors (21, 24, 36, 37).

Our results suggest a model in which, early in the developing spinal cord, a common neuro-glioblast progenitor is present in the pMN domain and this has the capacity to generate MN and OLP (33, 34). During MN generation, Hes5 induced by Jagged2 prevents premature OLP differentiation and, at the same time, maintains a proportion of these progenitors in an undifferentiated state. The switch to OLP generation depends on the downregulation of Jagged2 expression which facilitates the differentiation of the remaining progenitors and disinhibits OLP generation. When Jagged2 activity is

artificially depleted in vivo, the precocious generation of OLP is accompanied by a reduction in MN generation. This argues against a model in which separate OLP and MN precursors are present in the pMN and suggests that in the absence of Notch activity the progenitors of MN adopt a gliogenic identity. Nevertheless, our results cannot formally rule out the presence of separate lineage-restricted neuroblasts and glioblasts within the pMN that are derived from a single common precursor (38). In this case, Jagged2 activity would act on these populations separately to inhibit differentiation and OLP generation. High resolution analysis of the behaviour of individual pMN progenitors will be necessary to conclusively test this possibility. Notwithstanding this question, the data presented here demonstrate a novel role for Jagged2 and Notch signaling in the temporal control of cell type identity in the vertebrate CNS.

MATERIALS AND METHODS

Fluorescence Associated Cell Sorting (FACS) and Microarray analysis

Plasmid DNA encoding either GFP alone, a deleted form of mouse Patched lacking the second large extra cellular loop (mPtc1 Δ^{loop2}) or an activated version of Gli3 (Gli3^{Act} aa1402-4743) were inserted into pCAGGS-ires-EGFP vector as reported (23), for in ovo electroporation into HH11-12 stage embryos. Neural tubes were dissected out 14 or 36 hours later, and single cell suspension was obtained by 10-15 minutes incubation on Trypsin-EDTA (SIGMA). GFP fluorescence was determined by flow cytometry using a MoFlo flow cytometer (DakoCytomation, Fort Collins, Colorado, USA). The resulting cell population, consisting of ~90% of GFP+ cells, was used for total RNA extraction. GeneChip Chicken Genome Arrays (Affymetrix) containing 32,773 chicken transcripts were used for the hybridization of the samples. Unpaired analysis of the data, based on the comparison between the average values of the logRed and logGreen intensities, was performed using SOLAR System v2.0 (Alma Bioinformatics S.L.). Results were filtered using unpaired t-tests using thresholds of ≥ 1.5 fold change and p-value ≤ 0.05 (Cruz et al., 2010)

Mouse and Chick embryos

Mice homozygous for the *Shh* null allele were as published (6). Eggs from White-Leghorn chickens were staged according to Hamburger and Hamilton (HH) (39). For chick embryos electroporation, plasmid DNA was injected into the lumen of neural tubes and electroporation carried out using an Intracel Dual Pulse (TSS10) electroporator.

For BromodeoxyUridine (BrdU) labelling, 5µg/µl BrdU was injected into the neural tubes at the indicated times prior fixation. Transfected embryos were allowed to develop to the indicated stages, then dissected, and processed for immunohistochemistry, in situ hybridisation, or FACS sorting and qPCR analysis.

Immunohistochemistry and in situ hybridization

For immunohistochemistry, embryos were fixed 2-4 hours at 4°C in 4% PF in PB. For BrdU detection, sections were incubated in 2N HCl for 30 minutes followed by 0.1M Na₂B₄O₇ pH8.5 rinses further PBT rinses and anti-BrdU incubation. Immunostainings were performed following standard procedures on either vibratome or cryostat sections. Antibodies against the following proteins were used; green fluorescence protein (GFP) (Molecular Probes), caspase3 (BD), a unique beta-Tubulin (Tuj-1, MEDPASS S.a.r.l.), phospho-Histone 3 (p-H3, Upstate Biochemicals), Olig2 (Zhou et al., 2001), Pax2 (Zymed), Sox9 (M. Wegner), O4 (C. Soula). Monoclonal antibodies to BrdU, MNR2, Nkx2.2, Islet1 and Islet2 were obtained from the Developmental Studies Hybridoma Bank (DSHB). Alexa488- and Alexa555-conjugated anti-mouse or anti-rabbit antibodies (Molecular Probes) were used. After single or double staining, sections were mounted, photographed using a Leica SP5 Confocal microscope, and processed with Adobe Photoshop CS3.

For in situ hybridisation, embryos were fixed overnight at 4°C in 4% PF in PB, rinsed and processed for whole mount RNA in situ hybridization following standard procedures using probes for chick *Jagged2*, *Hes5*, *Sox8*, *Sox9*, *Sox10* and *PLPDM20* (from the chicken EST project, UK-HGMP RC). Mouse *Jagged2* probe was used (JL de la Pompa). Hybridized embryos were developed either with NBT/BCIP, or with FAST-RED Chromogen (Roche) for Fluorescent in situ, rinsed in PBT and vibratome sectioned and photographed on a Leica DMR microscope, or a Leica SP5 confocal.

DNA Constructs

A deleted form of mouse Patched (mPtc1 Δ^{loop2}), deleted forms of human Gli3 (Gli3^{Rep} aa1-768 and Gli3^{Act} aa1402-4743), human full-coding sequences for *Jagged2* and *Hes5* were cloned into pCAGGS-ires-GFP for in vivo gain-of-function (GOF) experiments. The full-length mouse Olig2 cDNA into pCDNA3.1 was also used (14).

For in vivo loss-of-function (LOF) experiments, short RNA hairpin-based expression vectors for RNA interference were generated using the pSUPER RNAi systemTM (OligoEngine Inc.) A pair of custom oligonucleotides that contained a unique 19-nt sequence derived from the chicken *Jagged2* mRNAs were designed using the RNAi Design Tool (www.oligoengine.com). Forward and reverse 64-nt oligos (Sigma) were used for annealing and cloning into the pSUPER vector (Supplementary Figure 1A), according to the manufacturer's guidelines. Control short hairpin RNA was generated for a human HDAC sequence, cloned into the pSUPER vector and electroporated at same developmental stages for each experiment.

RT-Quantitative Real-Time-PCR

EGFP-containing plasmid DNAs were electroporated and neural tubes dissected out 24-48 hours later. Single cell suspension was obtained by 10-15 minutes incubation on Trypsin-EDTA (SIGMA). GFP expressing cells were FACS sorted and the resulting cell population was used for total RNA extraction. PCR reactions were carried out by triplicate using fixed amounts of template DNA.

Oligonucleotides were obtained from QIAGEN, (QuantiTect Primer Assays) for use in real-time RT-PCR with SYBR green detection. References for QuantiTect Primer Assay were as follows: GgJagged1 QT00598003, GgJagged2 QT01512749, GgPDGFRalpha QT00598969, GgPLP QT00598955. A standard curve was made for each amplicon by plotting the number of cycles at which the fluorescence crossed the threshold (crossing values) against increasing amounts of DNA template. All experimental values were normalized to those obtained for GAPDH.

Western Blot

HH Stage 14 embryos were electroporated with a Myc-Tagged-Jagged2, a V5-Tagged-Olig2, and pCAGGS-ires-RFP for transfection control. Neural tubes dissected 12h-PE were collected in PBS and directly lysed in 1X SDS loading buffer (10% glycerol, 2%SDS, 100mM DTT and 60 mM Tris-HCl pH 6.8) and the DNA disrupted by sonication. Selected samples were treated with Calf Intestin Alkaline Phosphatase

(CIAP) for 30 minutes, normalized by total protein content, separated by SDS-PAGE gel electrophoresis and transferred to nitrocellulose membranes; blocked with 8% non fat dry milk in TTBS (137mM NaCl; 0.05 Tween 20 and 20 mM Tris-HCl pH 7.4) and probed with in house produced monoclonal anti-Myc antibody (9E10) and polyclonal mouse anti- RFP or rabbit polyclonal anti-Olig2 (Zhou et al., 2001). The blots were developed using anti-rabbit or anti-mouse coupled to IRDye800CW or IRDye680LT and scanned with the Odyssey Infrared Imaging System (LI-COR Biosciences).

Luciferase-reporter Assay

A transcriptional activity assay of Jagged2 activity on the Myelin Basic Protein (MBP) reporter was performed *in vivo*. Chick embryos were electroporated with indicated DNAs or empty pCAGGS-GFP vector as control; together with a firefly-luciferase reporter construct containing the MBP-reporter (M. Wegner) and a renilla-luciferase reporter construct (Promega) for normalization. Embryos were harvested after 24 hours incubation *in ovo* and GFP-positive neural tubes were dissected and homogenized with a douncer in Passive Lysis Buffer on ice. Firefly- and renilla-luciferase activities were measured by the Dual Luciferase Reporter Assay System (Promega).

Quantifications and statistical analysis

Cell countings were made on 10-20 different sections of at least 4 different embryos after each experimental condition, from confocal images. Quantitative data were expressed as mean±s.e.m.

AKNOWLEDGEMENTS

The authors thank Susana Usieto and Mónica Pons for invaluable research assistance. For DNAs and antibodies we thank Drs. D. Rowitch, M.Wegner, C. Soula, JL de la Pompa. Monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. Work in the EM lab is supported by Grant BFU2010-18959. Work in the SP lab is supported by grant BFU-2008-02424BFI. Work in the JB lab is supported by MRC

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TITLES AND LEGENDS TO FIGURES

FIGURE 1.- *The Notch-ligand Jagged2 is transiently expressed in the ventral spinal cord.*

(A) A transcriptional profiling strategy for Shh identified patterning determinants and components of the Notch pathway. Chick embryos were electroporated with activator or inhibitors of the Shh pathway (see methods), transfected GFP-expressing cells were FACS sorted, and extracted RNA was used to hybridize full genome genechips. In response to activators of the Shh pathway (Gli3Act), genes expressed in the ventral NT were upregulated, while dorsal genes appeared down-regulated. Additionally, expression of the Notch-ligand Serrate2/Jagged2 appeared upregulated.

(B-E) Expression of *Jagged2* revealed by in situ hybridization in sections of chick embryos at the indicated stages. **(C)** Shows double labelling with the intermediate neuronal subtypes (Pax2). **(F-H)** Expression of *Jagged2* revealed by fluorescent in situ (red), double labelled with the pMN marker Olig2 (F) or the MN marker islet1. **(I)** Double immunohistochemistry for Olig2/Nkx2.2 show the generation of OLP. **(J)** Summary of the expression of *Jagged2* in relation to the generation of MNs and OLPs from the pMN domain.

FIGURE 2.- *Jagged2 activity controls the timely differentiation of MNs.*

(A) Embryos electroporated (EP) at HH14, with the indicated DNAs, were analysed 72 hour post-EP with the early MN marker MNR2 (red). EP side is shown to the right, GFP (green) show transfected cells. Quantification of motor neurons and their spatial distribution was performed analyzing confocal images. **(B)** Histograms represent the number of MNR2 positive cells depending on the distance to the lumen: TZ: transition zone; MZ: mantle zone. **(C)** Schematic representation of the MN birthdating experiments following *Jagged2* manipulations. Embryos EP at HH14 were labelled with a single BrdU pulse 24h-PE and analysed at 48h-PE for BrdU/MN markers. **(D)** Schematic representation of motor neurons birthdates in the brachial spinal cord of the chick embryo, data adapted from Hollyday and Hamburger 1977).

(E) Example sections showing double staining for the MN marker Islet1 (green) and BrdU (red). EP side is shown to the right, GFP (Blue) show transfected cells. **(F)**

Quantitative data showing the proportion of born MNs, plots corresponds to the ratio of EP vs non-EP side in each experimental condition (control: black dots, GOF-Jag2: red dots, LOF-Jag2: green dots). Time is represented as hours of incubation and HH stages. First dot corresponds to the EP time, second to the BrdU-/Islet1+ cells (MNs born at the time of the BrdU pulse), third dot represents the total Islet1+ cells (MNs terminally differentiated 48h-PE). Bars correspond to the standard error (SEM).

FIGURE 3.- *Over-expression of Jagged2 retain Olig2+ cells in a progenitor stage* **(A)**

Model representing the fate of pMN progenitors along three cell cycles, in control situation in which 50% divisions are asymmetric, giving rise to one MN (green) and one progenitor cell (red). Percentages of asymmetric divisions are adapted from data provided by BrdU incorporation experiments in the presence or the absence of Jagged2. Drawing shows the resulting proportion of neurons (N) and progenitors (P). Plots represent the ratio of theoretical MNs generated in each condition, dots correspond to each cell cycle. Plots represents the ratio of theoretical progenitors that remain in each condition, dots correspond to each cell cycle.

(B) Quantitative data showing the ratio of Olig2+ cells, plots corresponds to the ratio of EP vs non-EP side in each experimental condition (control: black dots, GOF-Jag2: red dots, LOF-Jag2: green dots). Time is represented as hours of incubation and HH stages. Dots correspond to 12h-, 24h-, 48h- and 72h-PE. Bars correspond to the standard error (SEM). **(C)** Sample sections, EP with indicated DNAs, and immunostained with Olig2 (red). EP sides are shown to the right, GFP (green) show transfected cells.

FIGURE 4.- *Jagged2 reduced Olig2 protein levels and inhibits the Olig2 capacity to differentiate MNs*

(A) Western blots from NT total lysates were immunoblotted with anti-Olig2, anti-Myc and anti-RFP antibodies. Embryos co-EP at HH14 with Jagged2-Myc, Olig2 and pCAGGS-ires-RFP, were dissected 12h-PE. Note that Olig2 protein is distributed within two separate bands that were both shifted but maintained in the presence of CIAP, showing that both forms are phosphoproteins. These two bands might reflect alternate translation initiation sites (Lu et al., 2000). Anti-Myc reveals the presence or absence of Jagged2- Anti-RFP, as EP control. **(B)** Quantitative analysis of upper and lower Olig2 bands, in the presence or the absence of Jagged2, normalized to RFP levels. Olig2 protein levels are significantly reduced in NT co-transfected with Jagged2.

(C) The Olig2 capacity to differentiate MNs is prevented by Jagged2 activity. Embryos EP at HH14 with the indicated DNAs were analysed 12h-PE for the MN progenitor marker Olig2 (red) and the MN definitive marker MNR2 (green). GFP is shown in blue. EP of Olig2 is sufficient for the generation of ectopic MNR2+ MNs. Quantification of ratio of MNR2+ cells in EP vs control non-EP side revealed a ratio of $0,85\pm 0,07$ in control EP embryos, while in Olig2-EP increased to $2,02\pm 0,03$. EP of Jag2 prevents the generation of MNR2+ cells to $0,58\pm 0,02$, while co-EP of Olig2 and Jag2 also reduced the appearance of MNR2+ cells to $0,51\pm 0,05$ in the normal ventral domain, but also prevented the generation of ectopic dorsal MNs (D) Summary model of Jagged2 activity in the pMN domain, Jagged2 activity splits Olig2+ve progenitors into two different identities.

FIGURE 5.- *Depletion of Jagged2 activity at the time of MN generation results in premature OLP generation.*

(A-H) Embryos electroporated at HH14 with the indicated DNAs were analysed 48h-PE with OLP markers. EP side is shown to the right, GFP (green) show transfected cells (A,B) Olig2 is expressed at pMN in control NT, while GOF-JAG2 EP shows migratory Olig2+ cells. (C) Co-EP with human Jagged2 (GOF-JAG2) restores Olig2+ cells to pMN. (D,E) Sox9 is expressed throughout the VZ in control NT, while LOF-JAG2 electroporated NT shows depletion of Sox9+ cells at pMN and migratory Sox9+ cells. (F) Co-EP with GOF-JAG2 restores Sox9 expression in pMN. (G) Olig2+ migratory cells (red) co-expressed the OLP marker O4 (green). (H) Quantitative analysis of Olig2+ and Sox9+ migratory cells, 48h-PE after electroporation of the indicated DNAs. Numbers are shown as migratory marker expressing cell in each experimental condition. Bars correspond to the standard error (SEM). (I-N) 72h-PE of LOF-JAG2, Sox9 is extinguished from the VZ, and premature expression of the OLP markers *Sox10* and *PLPDM 20* are detected by in situ hybridization. In control electroporated spinal cords *Sox10* and *PLPDM20* are only expressed in the ventral roots. (O) Premature OLP generation as revealed by the Real-Time PCR detection of a significant increase in the expression of PDGF Receptor alfa and in the myelin specific gene PLP, 48h-PE of LOF-JAG2 (P) Premature OLP generation as revealed by the increased activity of the MBP-Luc reporter 24h-PE of LOF-JAG2.

FIGURE 6: *Persistent over-expression of Jagged2 inhibits OLP generation.*

Embryos EP at HH18 with Jagged2 (GOF-JAG2), or control empty vector (Control), were analysed 4,5 days after EP for oligodendrocyte generation. **(A,B)** Oligodendrocytes are identified as migratory cells departing from the pMN expressing Olig2 (red) and Nkx2.2 (green). Electroporated side is shown to the right, GFP (blue) show transfected cells. Controls show numerous migratory OLPs. GOF-JAG inhibits OLP migration. **(C,D)** Oligodendrocytes are identified as migratory expressing Olig2 (red) and O4 (green). Electroporated side is shown to the right, GFP (blue) show transfected cells. Controls show numerous migratory OLPs. GOF-JAG inhibits OLP migration. **(E)** Quantitative analysis of Olig2⁺ migratory cells, 72h and 108h after electroporation of the indicated DNAs. Numbers are shown as migratory marker expressing cell in each experimental condition. Bars correspond to the standard error (SEM).

FIGURE 7: *Hes5* acts down-stream of *Jagged2* to prevent OLP generation **(A-C)** In situ hybridization of wild type embryos show that *Hes5* is transiently expressed in pMN at the time of MN generation and extinguished MN generation. **(E)** Over-expression of *Jagged2* (GOF-JAG2 48h-PE at HH14) maintains *Hes5* expression at pMN. **(F)** Depletion of *Jagged2* (LOF-JAG2 48h-PE of sh*Jagged2* at HH14) inhibits *Hes5* expression. **(G-J)** *Hes5* over-expression retains Olig2⁺ and Sox9⁺ cells at pMN, even in the absence of *Jagged2* function. Electroporated side is shown to the right, GFP (green) show transfected cells. **(K)** Quantitative analysis of migratory Olig2⁺ and Sox9⁺ cells. Numbers are shown as total migratory cells on the electroporated side, in each experimental condition. Bars correspond to the standard error (SEM). **(L)** Summary of the genetic network operating for OLP generation

SUPPLEMENTARY FIGURE 1.- *Patterned expression of Jagged2 depends on Shh activity.*

(A) *Jagged2* expression in a 10.5 dpc *Shh*^{+/+} embryo. **(B,C)** *Jagged2* expression is lost in *Shh*^{-/-} littermates. **(D)** Constitutive repression of *Shh* activity, by electroporation (EP) of *Gli3Rep*, resulted in the loss of *Jagged2*. EP side of the NT is shown to the right. **(E)** Constitutive activation of *Shh*, by EP of *Gli3Act* resulted in the dorsal expansion of *Jagged2*.

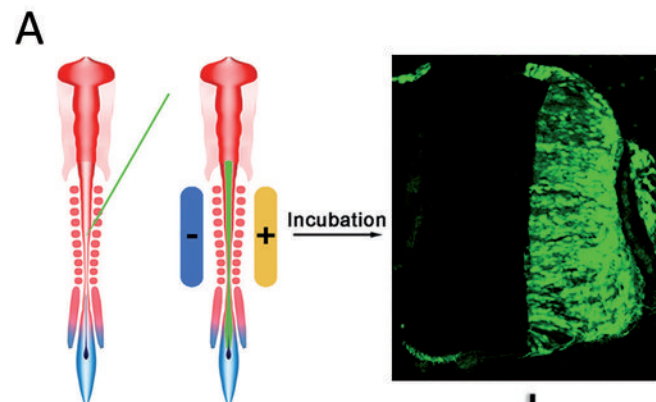
SUPPLEMENTARY FIGURE 2.- *Generation and in vivo characterization of short hairpin RNA for chick Jagged2.*

(A) Sequence of the three pairs of custom oligonucleotides that contained unique sequences derived from the chicken *Jagged2* mRNA designed and tested for in vivo *Jagged2* knock down. (B,C) Embryos were electroporated (EP) at HH14 with the empty pSUPER vector or with a human shHDAC, as controls, and with the three generated sh*Jagged2*.1, sh*Jagged2*.2 and sh*Jagged2*.3. Cells were GFP-sorted 48 hours after electroporation (48h-PE), RNA extracted and analysed by qRT-PCR for level of endogenous *Jagged2* expression. sh*Jagged2*.3 efficiently reduced ~50% *Jagged2* expression, and was used for the rest of the experiments. (D) Reduction of *Jagged2* expression, consistently increased to ~20% the expression of the closely related Notch ligand *Jagged1*. (E,F) In vivo loss-of-function of *Jagged2* activity, at the time of MN generation, preserved spinal cord morphology. (E) Embryos EP with sh*Jagged2* at HH14 were analysed 48h-PE for proliferation of neural progenitors by BrdU incorporation. GFP (green) shows EP cells, BrdU (red) shows proliferative cells restricted to the ventricular zone (VZ), in both the EP and non-EP sides of the spinal cord. (F) EP embryos were analysed for proliferation/differentiation using the mitotic marker pH3 (blue) and the pan-neural marker Tuj1 (red). GFP (green) shows EP cells. Mitosis (pH3+, blue) are all located at the apical side of neuroepithelial cells while differentiated neurons (Tuj1+, red) cells migrate to the marginal zone (MZ).

SUPPLEMENTARY FIGURE 3: *Jagged2 over-expression resulted in a specific MN loss.*

(A-F) Embryos electroporated (EP) at HH14 with GOF-JAG2 or control empty vector were analysed 72 hours later by immunohistochemistry for specific neuron subtype markers; dI1 by anti-Lhx2/9, dI3 as dorsal Islet1+ cells, and MNs as MNR2+ or ventral islet1+ cells. EP sides are shown to the right, GFP (green) show transfected cells, specific markers are shown in red. (G) Quantitative data from immunostaining shown in A. Numbers are shown as ratio of cells in EP vs control non-EP side in each experimental condition; 72h PE Lhx2/9+ cells (dI1) in control embryos were $0,98\pm 0,03$ and in *Jag2* were $0,92\pm 0,03$. Dorsal Islet1+ cells (dI3) were $1,02\pm 0,05$ and in *Jag2* were $0,85\pm 0,06$. MNR2+ cells were $0,99\pm 0,06$ and in *Jag2* were $0,52\pm 0,03$. Ventral Islet1+ cells (MNs) were $0,99\pm 0,04$ and in *Jag2* were $0,43\pm 0,03$. Bars correspond to the standard error (SEM).

FIGURE 1



FACS sorting
RNA extraction
Hybridization
Data mining

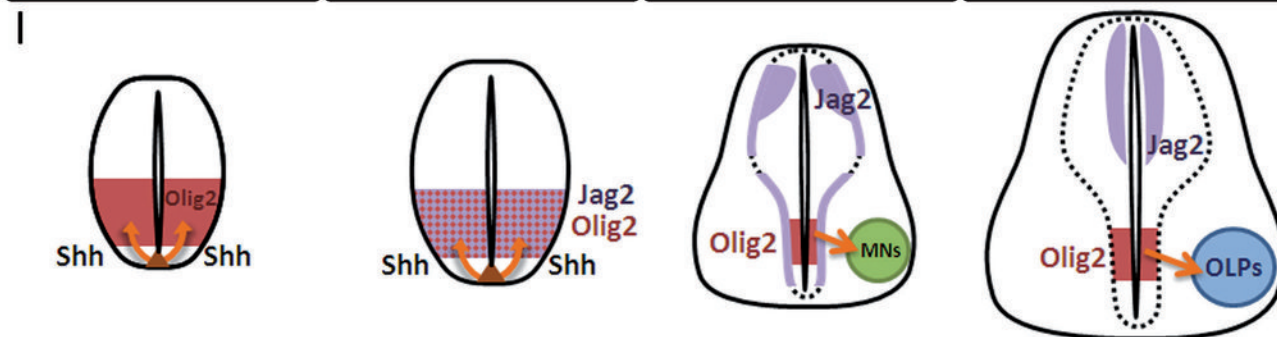
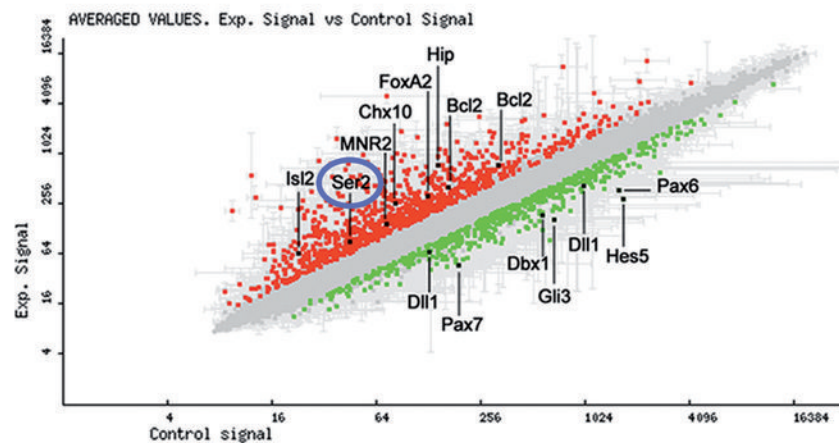
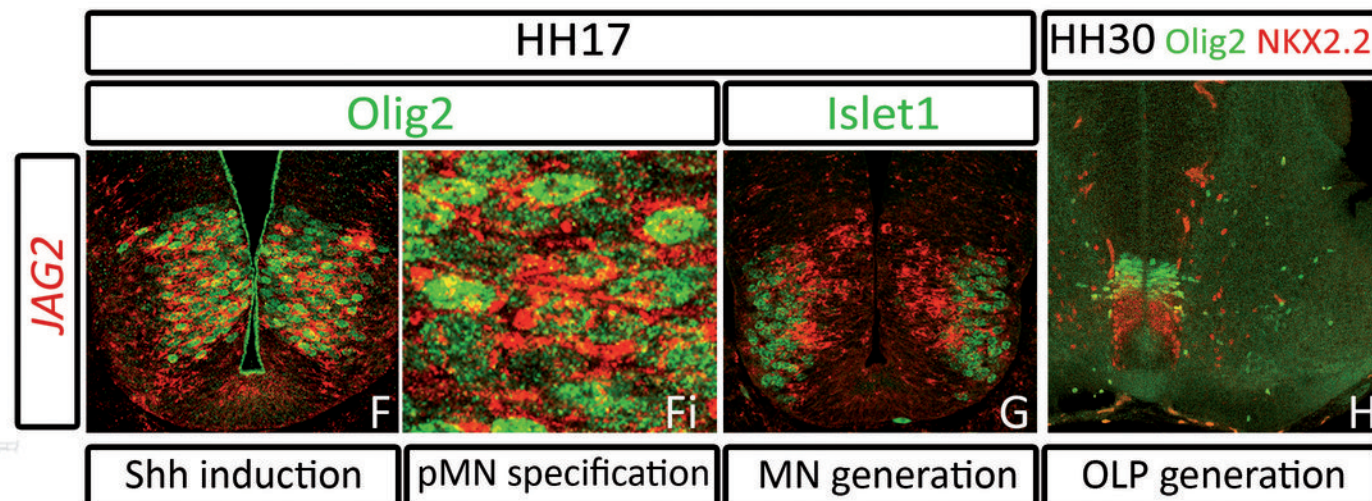
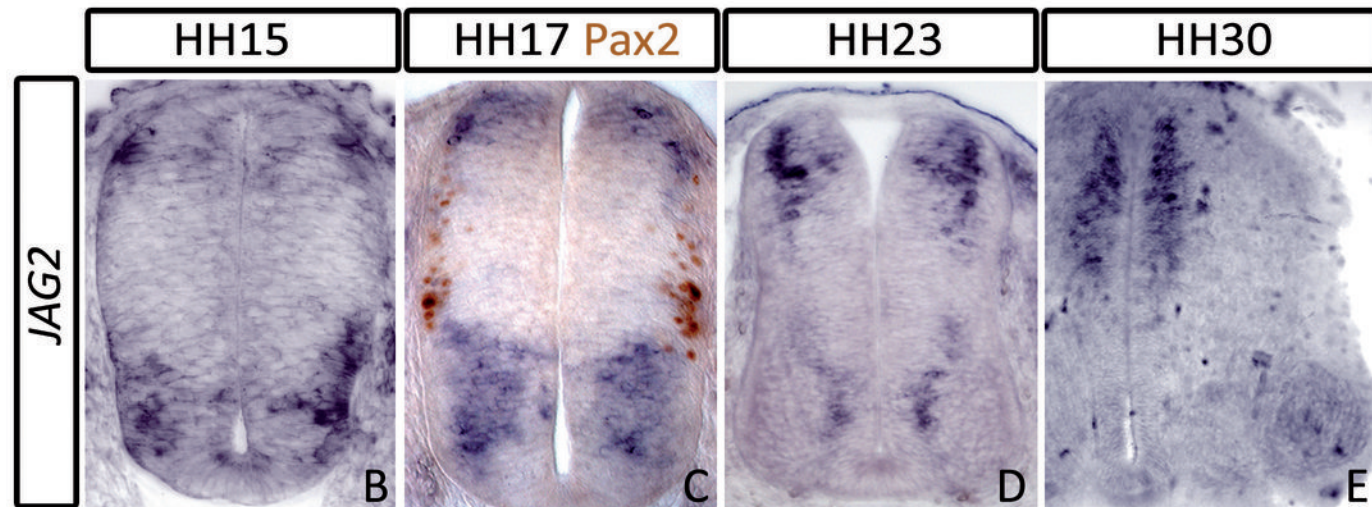


FIGURE 2

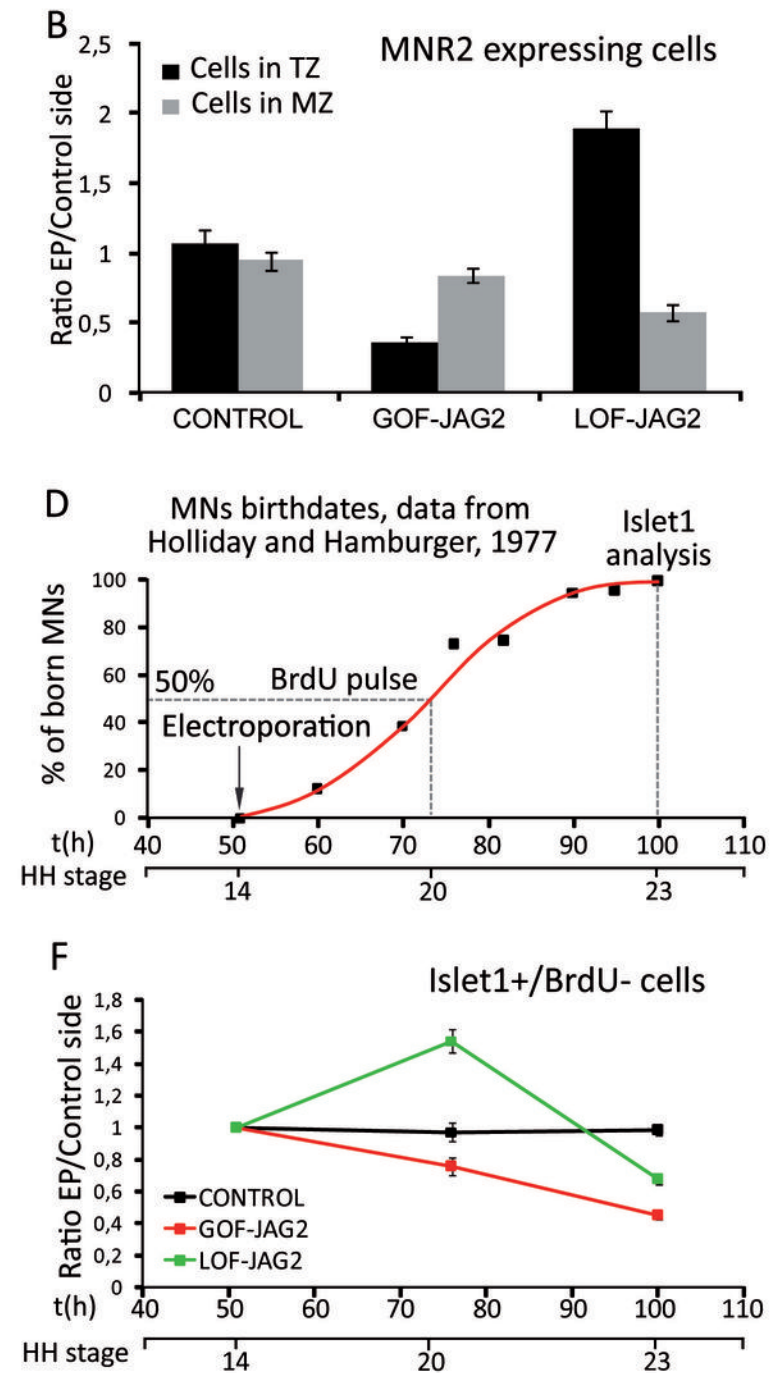
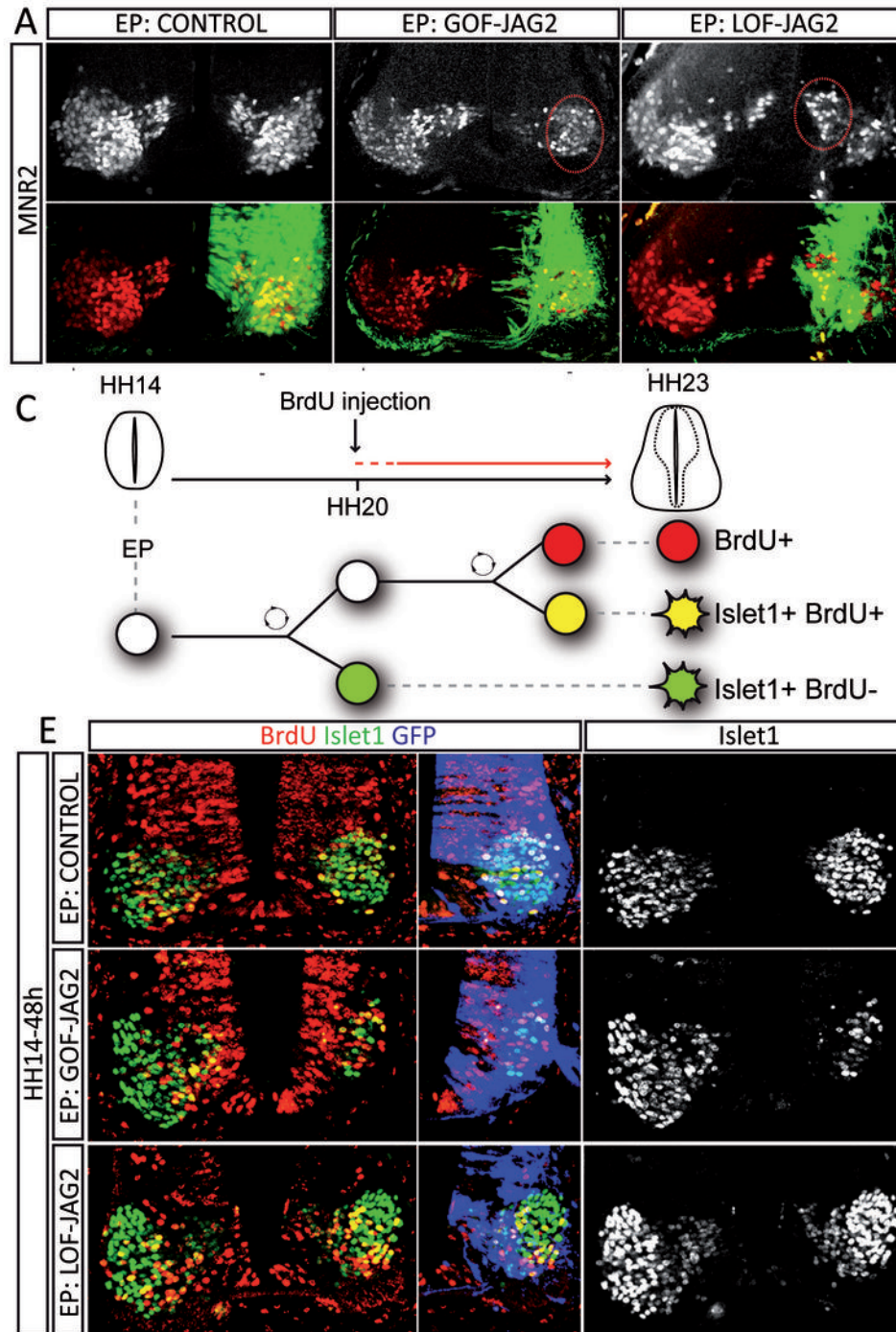


FIGURE 3

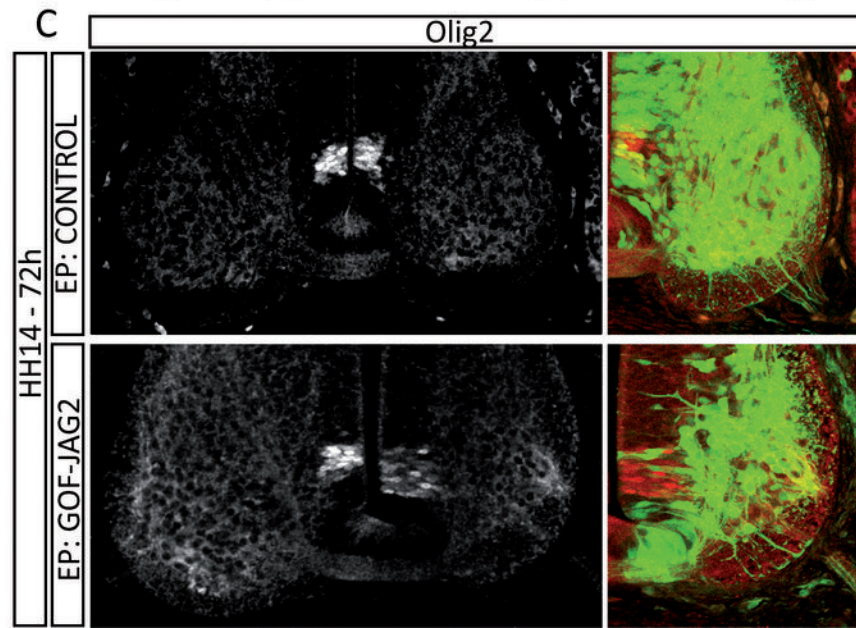
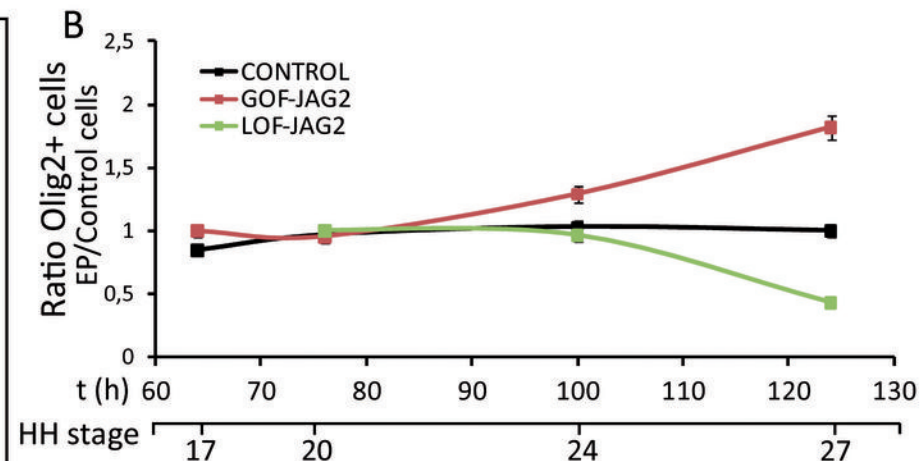
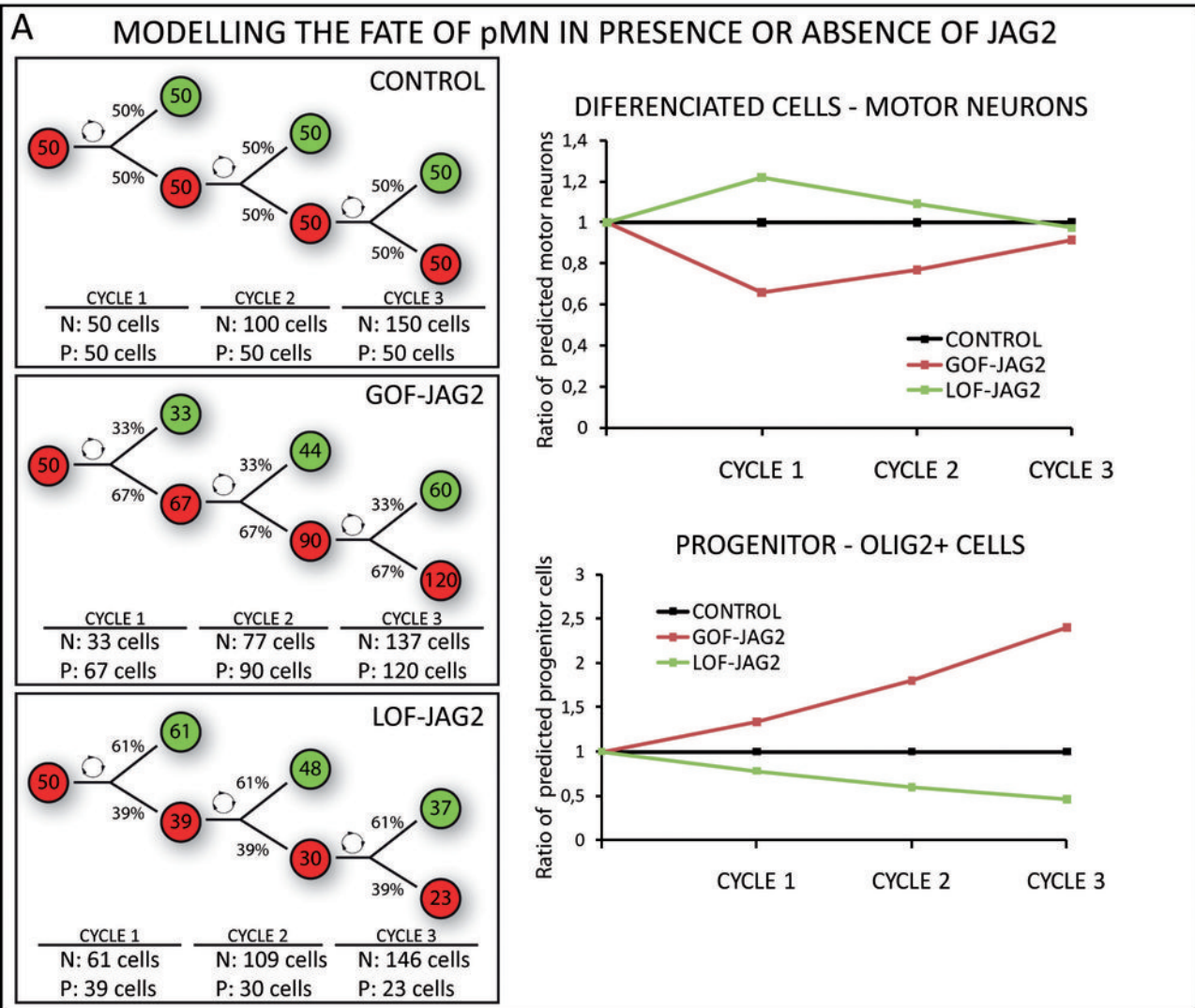


FIGURE 4

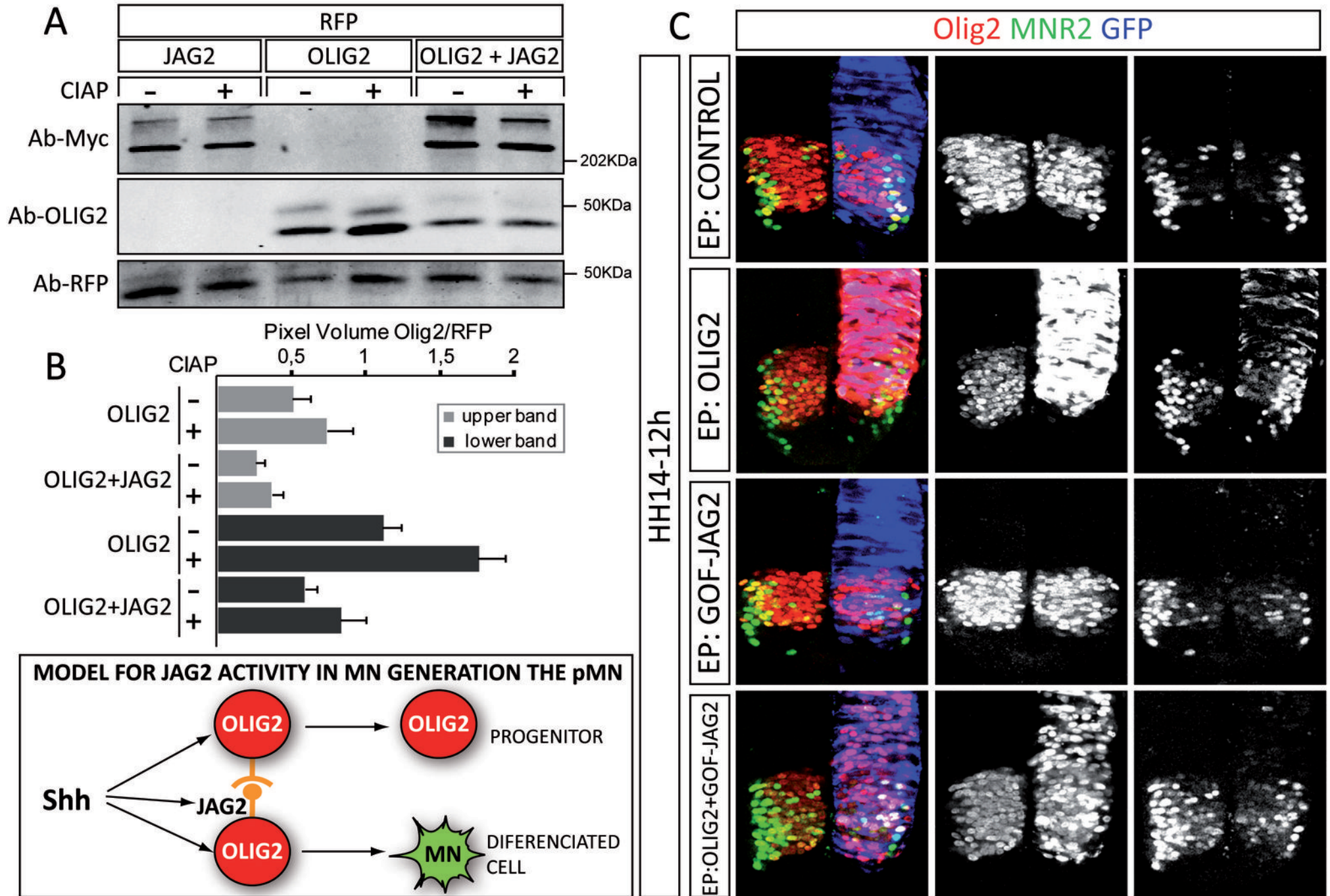


FIGURE 5

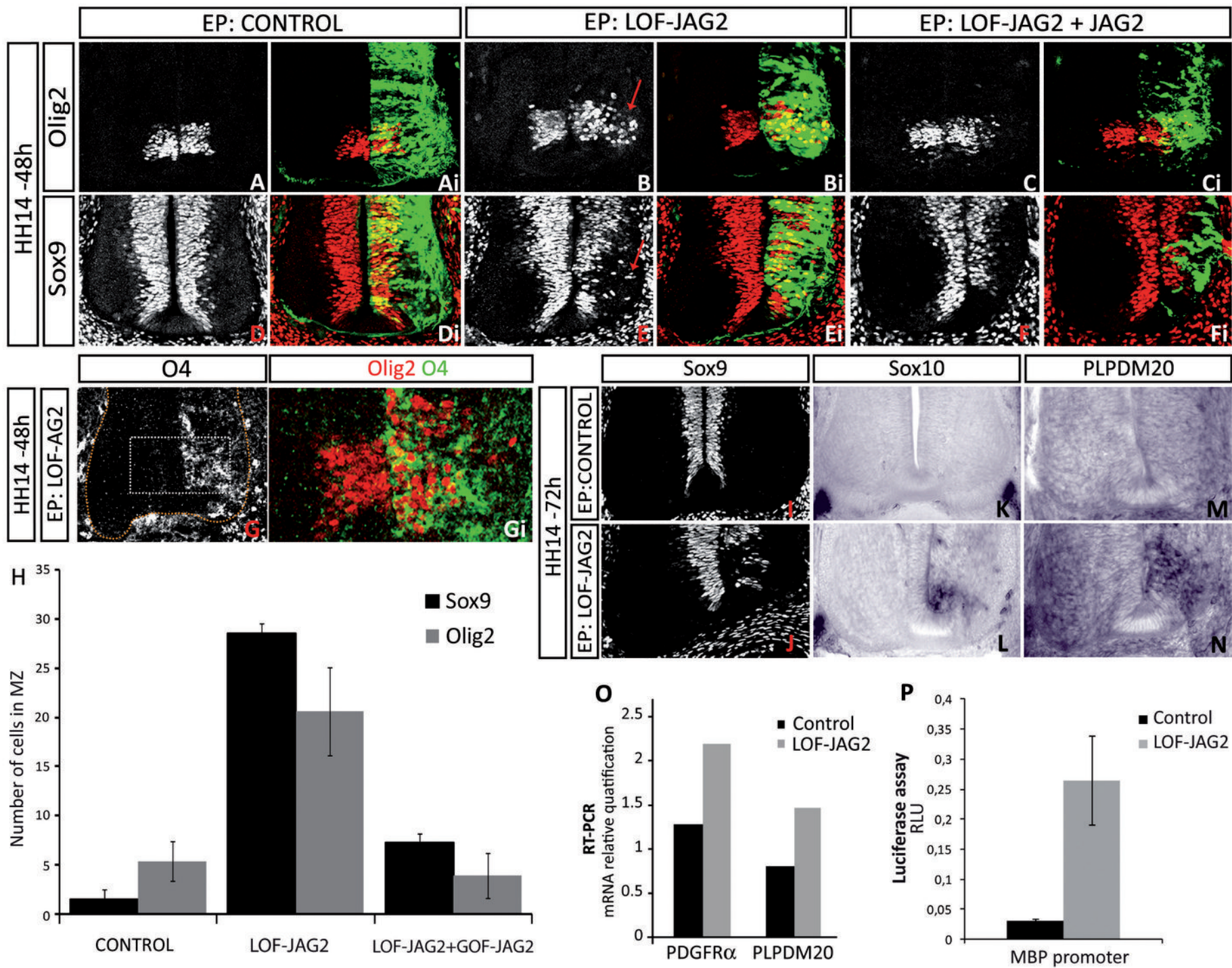


FIGURE 6

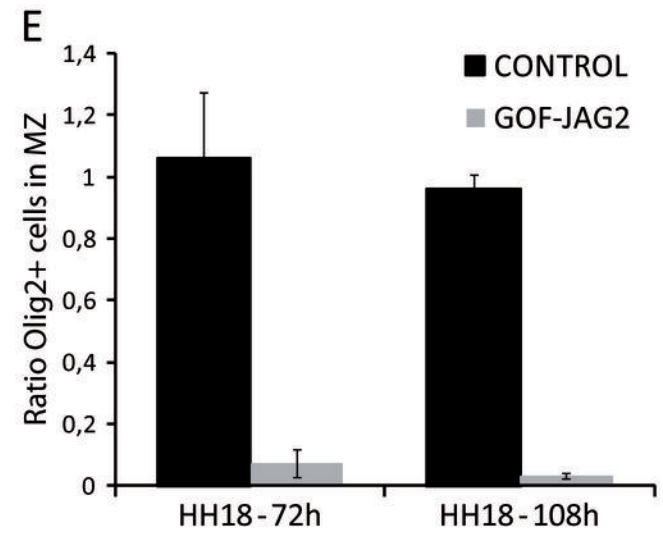
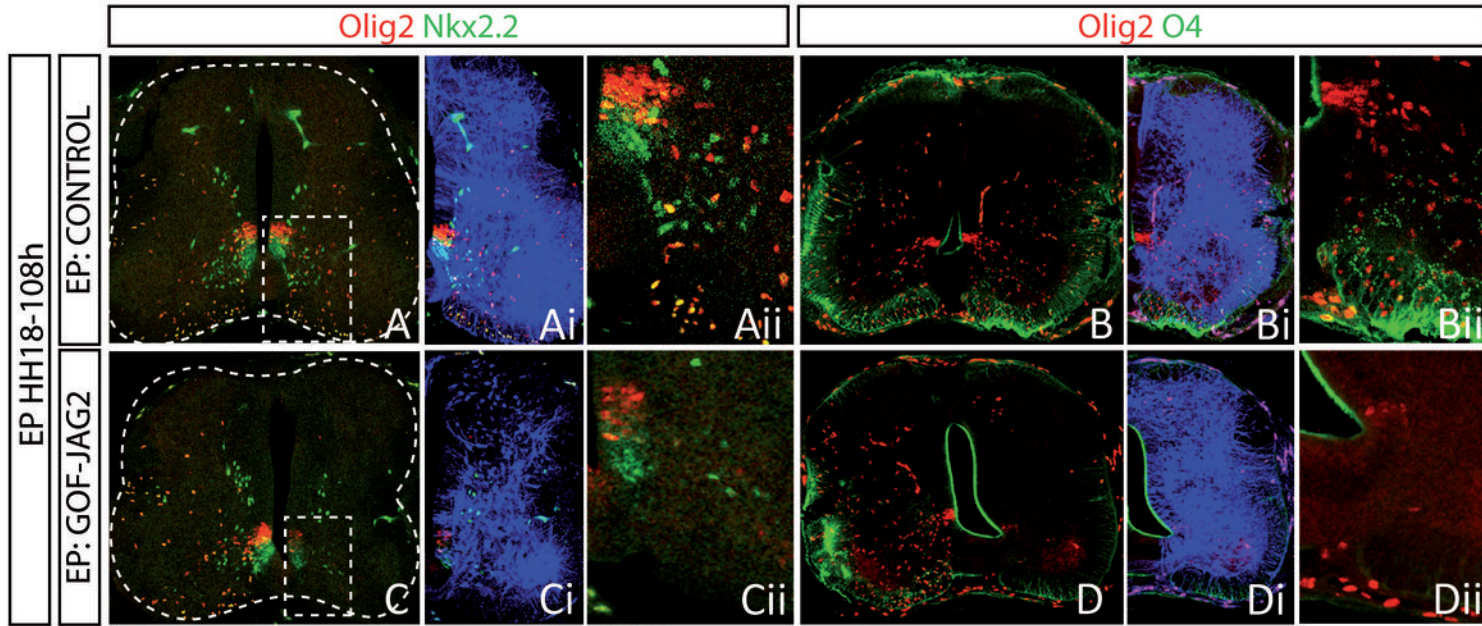


FIGURE 7

