DYNAMIC EXPRESSION OF THE ONECUT TRANSCRIPTION FACTORS HNF-6, OC-2 AND OC-3 DURING SPINAL MOTOR NEURON DEVELOPMENT

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Abstract—The Onecut (OC) transcription factors, namely Hepatocyte nuclear factor 6 (HNF-6), OC-2 and OC-3, are transcriptional activators expressed in liver, pancreas and nervous system during development. Although their expression and roles in endoderm-derived tissues and in the trigeminal ganglia have been investigated, their expression in the CNS has not been described yet. In this study, we report a qualitative and quantitative expression profile of the OC factors in the developing spinal motor neurons (MN). We provide evidence that OC expression is initiated in newly-born MN. At later stages, they are differentially and dynamically expressed in subsets of differentiating motor neuron within the four motor columns. We also show that the expression profile of HNF-6 in spinal MN is conserved in chick embryos. Together, our data unveil a complex and dynamic expression profile of the OC proteins in spinal MN, which suggests that these factors may participate in regulatory networks that control different steps of motor neuron development. © 2010 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: Onecut, transcription factors, spinal motor neurons, development.

In the embryonic spinal cord, motor neurons (MN) are the first cells to differentiate. Their differentiation program is dependent on a transcriptional code established by a gradient of Sonic Hedgehog signaling (Roelink et al., 1995; Ericson et al., 1996). This transcriptional code mainly involves cross-regulations between transcriptional repressors (Briscoe and Ericson, 1999; Briscoe et al., 2000). In the ventral spinal cord, it controls the generation of five cardinal progenitor domains called p0–p3 and pMN, which give rise to V0–V3 interneurons and MN, respectively (Ericson et al., 1997; Briscoe et al., 2000; Briscoe and Ericson, 2001). The pMN domain is defined by combined expression of Nkx6.1, Pax6, Olig2 and Neurogenin-2 (Neurog2). In this domain, neural progenitors generate MN which are initially characterized by Lhx3, Hb9 and Islet

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Abbreviations: CT, column of terni; HH, Hamburger–Hamilton stage; HMC, hypaxial motor column; HNF-6, Hepatocyte nuclear factor 6; Isl, Islet; LMC, lateral motor column; LMCl, lateral LMC; LMCm, medial LMC; MMC, median motor column; MN, motor neurons; nNOS, neuronal nitric oxide synthase; OC, Onecut; PBS, phosphate buffered-saline; PFA, paraformaldehyde; PGC, preganglionic column; S, somite stages; SEM, standard error of the mean.

(Isl)-1/2 expression (Tanabe et al., 1998; Arber et al., 1999; Takebayashi et al., 2002; Allan and Thor, 2003).

Then, upon differential activation of additional regulators, spinal MN undergo diversification into four different subpopulations that migrate to specific locations and gather in four discrete columns along the anteroposterior axis of the spinal cord (Sharma et al., 1998; Thor et al., 1999; Kania et al., 2000; Liu et al., 2001; Novitch et al., 2001; Allan and Thor, 2003; William et al., 2003; Dasen et al., 2005; Dalla Torre di Sanguinetto et al., 2008). These motor columns are characterized by combinations of transcription factors such as LIM–HD, Hox and Forkhead-box proteins (Dasen et al., 2003, 2005, 2008; Dalla Torre di Sanguinetto et al., 2008; Rousso et al., 2008). Each motor column controls the contraction of distinct groups of muscles or of visceral organs. Indeed, neurons of the median motor column (MMC) are characterized by expression of Isl-1, Hb9 and Lhx3 (Tsuchida et al., 1994; Sharma et al., 1998; Thor et al., 1999; William et al., 2003; Agalliu et al., 2009) and innervate axial muscles. MN of the hypaxial motor column (HMC), defined by Isl-1 and Hb9 expression (Briscoe and Ericson, 2001; Dasen et al., 2008; Rousso et al., 2008; Agalliu et al., 2009), innervate body wall muscles. Preganglionic motor column (PGC) neurons, which express Foxp1 and neuronal nitric oxide synthase (nNOS), innervate sympathetic ganglia which control visceral organ movements (Tsuchida et al., 1994; Shirasaki and Pfaff, 2002; Dasen et al., 2003, 2008; Rousso et al., 2008). Finally, lateral motor column (LMC) neurons, which innervate limb muscles, are divided into two subsets. Lateral LMC (LMCl) cells, which coexpress Foxp1, Lhx1/5 and Isl-2, innervate dorsal limb muscles while medial LMC (LMCm) cells, identified by Foxp1 and Isl-1 expression, innervate ventral limb muscles (Lin et al., 1998; Kania et al., 2000; Dasen et al., 2003, 2005, 2008; William et al., 2003; Rousso et al., 2008).

Onecut (OC) factors are expressed in the developing CNS of several species including sea urchin (Poustka et al., 2004), ascidian (Sasakura and Makabe, 2001), drosophila (Nguyen et al., 2000), zebrafish (Hong et al., 2002), frog (Haworth and Latinkic, 2009) and mouse (Landry et al., 1997; Vanhorenbeeck et al., 2002; Jacquemin et al., 2003b; Hodge et al., 2007). In mammals, the OC family is constituted by three genes called *Hnf6* (also called *Oc1*), *Oc2* and *Oc3*. These encode transcription factors containing a bipartite DNA-binding domain composed of a single cut domain and a divergent homeodomain (HD) (Lannoy et al., 1998; Jacquemin et al., 1999, 2003b; Vanhorenbeeck et al., 2002). These proteins act as transcriptional activators and control cell differentiation in

liver and pancreas (Jacquemin et al., 2000, 2003a; Clotman et al., 2002, 2005; Pierreux et al., 2006; Margagliotti et al., 2007; Vanhorenbeeck et al., 2007), as well as patterning of the trigeminal sensory neurons (Hodge et al., 2007). Although previous studies have shown that OC transcription factors are expressed in the murine CNS during embryonic development, their expression pattern has not been fully investigated. In particular, cell types
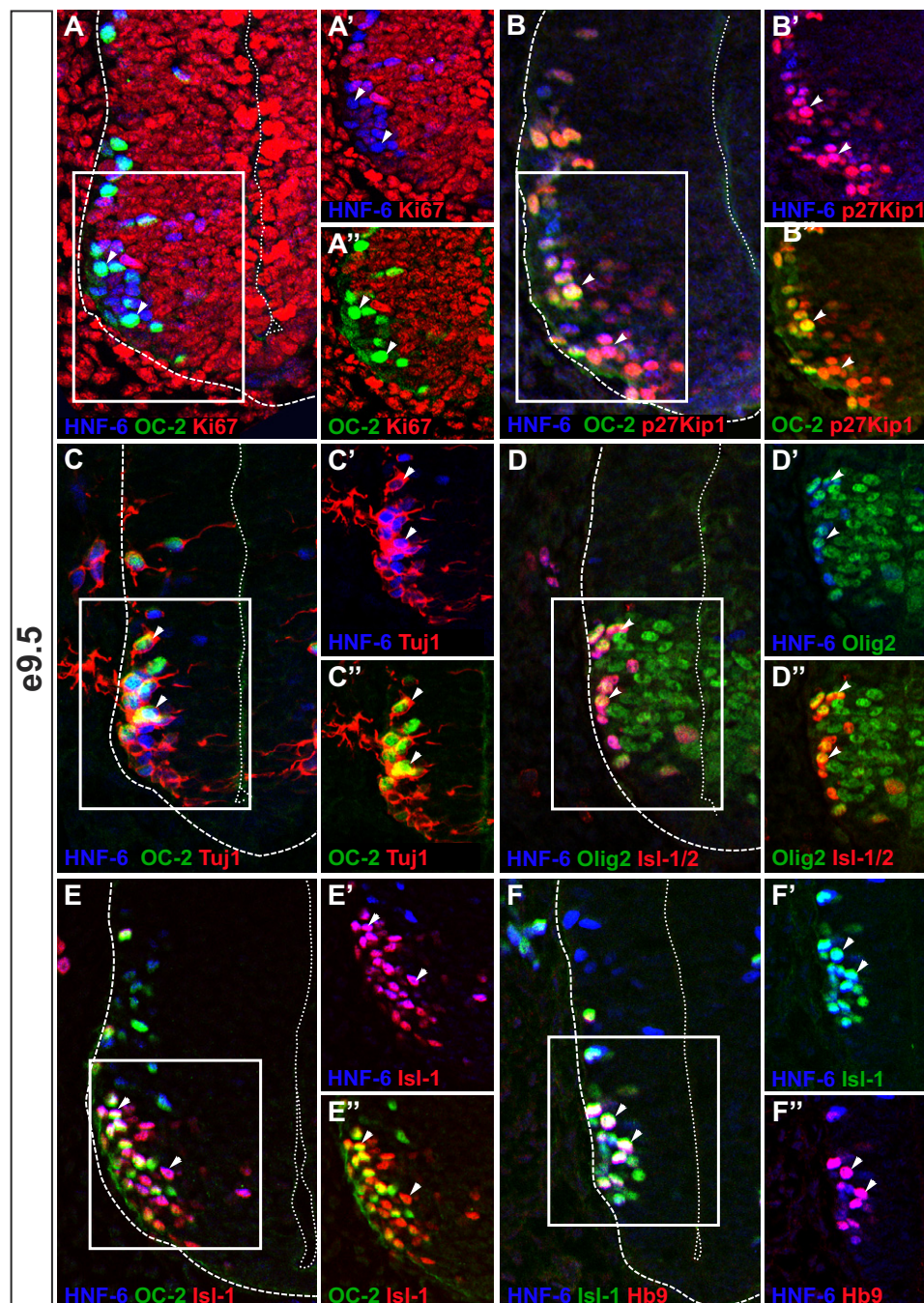
Fig. 1. Onecut (OC) factors are present in newly-born spinal motor neurons (MN). (A–A") Immunolabelings of Hepatocyte nuclear factor 6 (HNF-6,blue) and OC-2 (green) on transverse sections of ventral spinal cord at e9.5 showed that HNF-6 and OC-2 were not present in proliferating neural progenitors which expressed Ki67 (red) (examples shown by arrowheads). (B–B") In contrast, HNF-6 (blue) and OC-2 (green) were detected in post-mitotic cells that expressed $p27^{Kip1}$ (red) (examples shown by arrowheads). (C–C") OC^+ cells (blue/green) expressed the early neuronal differentiation marker β_{III}-tubulin (Tuj1 antibody, red) (examples shown by arrowheads). (D–D") HNF-6 (blue) was initially expressed in Isl-1/2$^+$ newly-born MN (red) wherein Olig2 (green) was still detected. (E–E") A majority of HNF-6$^+$ (blue) and OC-2$^+$ neurons (green) expressed Isl-1 (red), a marker of differentiating MN, as highlighted by arrowheads. (F–F'') HNF-6 (blue) was present in Hb9$^+$ (red) and in Isl-1$^+$ (green) newly-born MN. HNF6$^+$ and Isl-1$^+$ neurons are more numerous than Hb9$^+$ neurons. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.
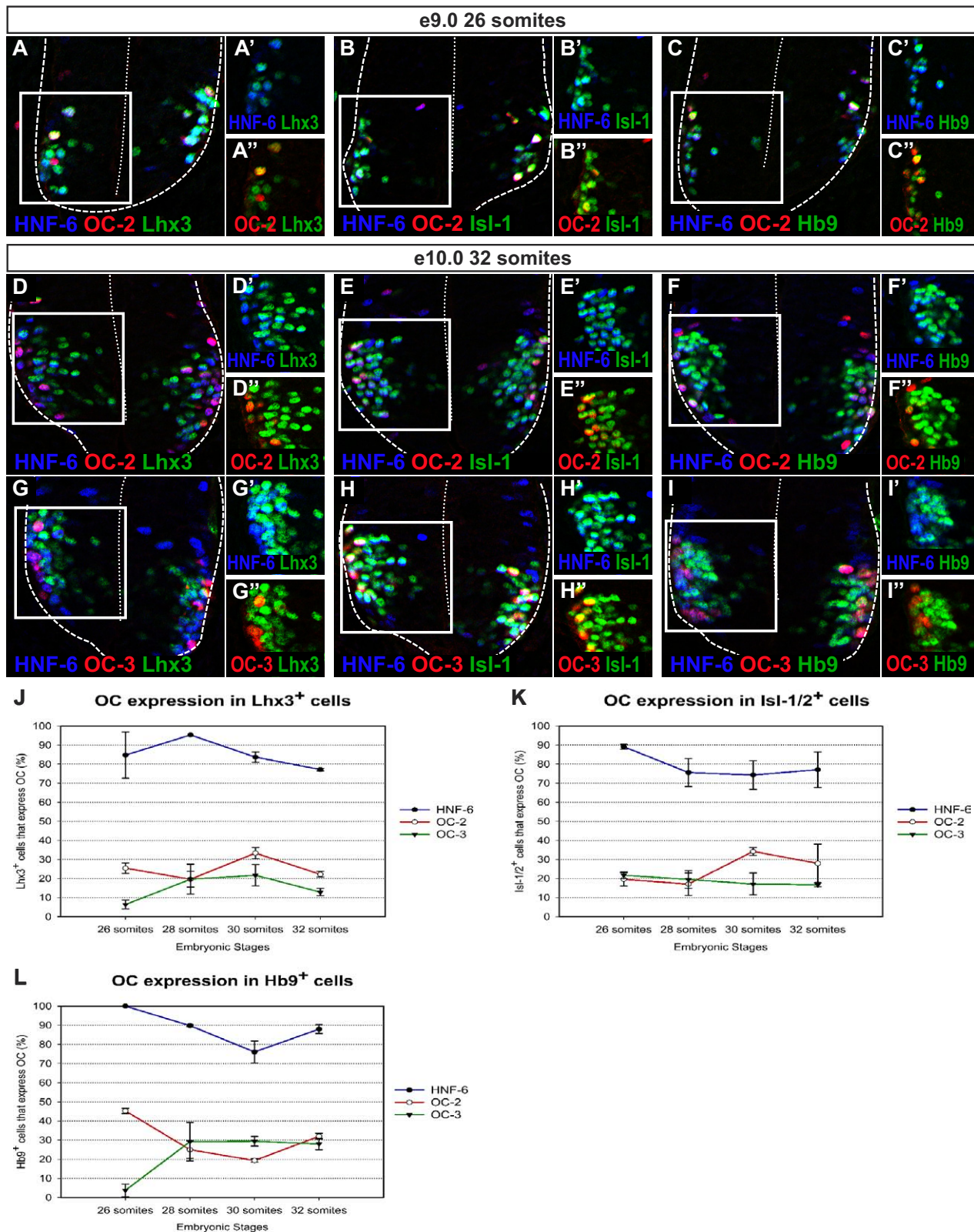
Fig. 2. Expression of the Onecut factors is sequentially activated in newly-born MN. (A–I′) Immunolabelings of HNF-6 (blue), OC-2 or OC-3 (red) and Lhx3, Isl1, or Hb9 (green) on transverse sections of ventral spinal cord from 26 to 32 somite stages (S). (A″, B″ and C″) At 26S, HNF-6 (blue) was present in most of the newly-born MN which expressed Lhx3, Isl1 and Hb9, whereas OC-2 (red) expression was initiated in a restricted subset of these cells. (D″, E″ and F″) OC-2 (red) expression expanded slightly at 32S in MN located close to basal lamina, while HNF-6 (blue) remained broadly

which express OC factors have not been characterized and data about the temporal evolution of OC expression are not available. In this study, we report that OC genes are differentially and dynamically expressed in the ventral spinal cord during multiple stages of MN differentiation.

EXPERIMENTAL PROCEDURES

Animals

Mice were raised in our animal facility and were treated according to the principles of laboratory animal care of the local Animal Welfare Committee. All efforts were made to minimize the number of animals used and their suffering. CD1 strain mice were crossed, the day of vaginal plug was considered to be embryonic day (e) 0.5 and then embryos were collected at different stages. Chick embryos were obtained from fertilized eggs of *Gallus gallus domesticus* hens, stored at 14°C until experiment, then incubated at 38.5°C in a humidified incubator.

Mouse embryos were collected between e9.0 and e14.5 and fixed in phosphate buffered-saline (PBS)/4% paraformaldehyde (PFA) at 4°C for 10 to 35 min according to their developmental stage. Mouse embryos at e12.5 and e14.5 were decapitated before fixation. For postnatal day (P)1 stage, mice were perfused intracardially with cold PBS and then with cold PBS/4% PFA. Perfused mice were dissected and spinal cord at P1 were removed and washed in cold PBS. Chick embryos were collected between e3.0 (Hamburger-Hamilton (HH) 18 stage) and e6.5 (HH29 stage) of incubation, and fixed in PBS/4% PFA at 4°C for 30 to 45 min according to their developmental stage. Fixed embryos and spinal cord at P1 were washed thrice in cold PBS before incubation in PBS/30% sucrose overnight at 4°C. They were embedded in PBS/7.5% gelatin/15% sucrose before being frozen at −56°C. Embryos and spinal cord at P1 were cut at 10 μm in a cryostat and cryosections were stored at −20°C.

Immunofluorescent labelings

Cryosections were saturated with PBS/0.1% Triton/10% horse serum for 30 min and incubated with the primary antibodies diluted in the same solution overnight at 4°C. After three washes in PBS/0.1% Triton, the secondary antibodies, diluted in PBS/0.1% Triton/10% horse serum, were added for 30 min at room temperature. Slides were washed thrice in PBS/0.1% Triton before a final wash in PBS, and were mounted with Fluorescent mounting medium (DAKO, Via Real, Carpinteria, CA, USA).

The following primary antibodies and dilution were used: rabbit anti Hepatocyte nuclear factor 6 (HNF-6) at 1:50 (Santa Cruz, Santa Cruz, San Jose, CA, USA, #s.c.-13050), rat anti OC-2 at 1:400 (Clotman et al., 2005), guinea-pig anti OC-3 at 1:6000 (Pierreux et al., 2004), mouse anti Isl-1/2 at 1:6000 (DSHB, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA, #39.4D5), goat anti Isl-1 at 1:1000 (Neuromics, Edina, MN, USA, #GT15051), rabbit anti Hb9 at 1:5000 (Abcam, Cambridge, UK, #ab26128), mouse anti MNR2/Hb9 at 1:1000 (DSHB #81.5C10), mouse anti Lhx3 at 1:1000 (DSHB #67.4E12), mouse anti Lhx1/5 at 1:2000 (DSHB #4F2), mouse anti Nkx2.2 at 1:20 (DSHB #74.5A5), mouse anti Nkx6.1 at 1:2000 (DSHB #F55A10), mouse anti Evx1 at 1:2000 (DSHB #99.1-3A2), sheep anti Chx10 at 1:500 (Exalpha Biologicals, Watertown, MA, USA, #X1179P), mouse anti Gata3 at 1:400 (Santa Cruz #sc268), goat

anti Foxp1 at 1:1000 (R&D Systems, Minneapolis, MN, USA, #AF4534), rabbit anti nNOS at 1:2000 (Immunostar, Hudson, WI, USA, #24287), mouse anti Tuj1 at 1:6000 (Covance, Emeryville, CA, USA, #MMS-435P), mouse anti Ki67 at 1:500 (BD Biosciences Pharmingen, San Jose, CA, USA, #556003), mouse anti-p27^{Kip1} at 1:2000 (BD Biosciences Pharmingen #610242), rabbit anti-Pea3 at 1:1000 (kindly provided by Sylvia Arber), rabbit anti-SCIP/Oct-6 at 1:500 (kindly provided by Dies N. Meijer), guinea-pig anti-Olig2 at 1:8000 (kindly provided by Bennett Novitch) and guinea-pig anti Foxd3 at 1:2000 (kindly provided by Carmen Birchmeier and Hagen Wende).

Secondary antibodies from Invitrogen (Carlsbad, CA, USA) were used at 1:2000 and were donkey anti mouse/AlexaFluor 594 or 488 or 647, anti rabbit/AlexaFluor 594 or 488 or 647, anti rat/AlexaFluor 594, anti goat/AlexaFluor 488 or 594, anti sheep/AlexaFluor 594, or goat anti guinea-pig/AlexaFluor 594 or 488, anti mouse IgG$_1$/AlexaFluor 594 or anti mouse IgG$_{2a}$/AlexaFluor 488.

Quantitative analysis

Pictures were acquired on a Bio-Rad MRC1024 confocal microscope with the Lasersharp 2000 software and processed with ImageJ software before cell quantifications. Quantifications were performed on red or green or blue layer of acquired confocal images and double or triple labeling cells were processed by subtractive method. Labeled cells were quantified with count analysis tool of Adobe Photoshop CS3 software. Motor neurons and other neurons that express OC proteins were counted unilaterally on three sections at three different levels along the anteroposterior axis of embryonic ventral spinal cord from e9.0 to e10.5 and on three sections at three different levels of the brachial, thoracic and lumbar regions for stages later than e10.5. Raw data of quantifications were exported from Adobe Photoshop CS3 software to SigmaPlot v11 software and then processed in order to generate histogram and curve figures.

RESULTS

The OC factors are expressed in newly-born MN

We first analyzed the distribution of HNF-6, OC-2 and OC-3 in the mouse embryonic spinal cord at early developmental stages by immunofluorescence. The first OC factor to be detected was HNF-6, whose expression began at e9.5, around 22–24 somite stages (S) (Fig. 1 and Fig. S1). OC-2 expression was delayed for a few hours, starting between 24 and 26 S, while OC-3 expression began even later around 26–28S (Fig. 2). Therefore, the initiation of OC expression in the ventral neural tube occurred according to a HNF-6>OC-2>OC-3 temporal sequence, as observed in endoderm, liver and pancreas (Jacquemin et al., 2003a; Pierreux et al., 2004; Clotman et al., 2005; Margagliotti et al., 2007; Vanhorenbeeck et al., 2007).

At these early stages, OC were mainly detected in the marginal zone in the vicinity of the basal lamina, suggesting that they are expressed in newly-born neurons. To test this hypothesis, we performed immunolabelings for Ki67 (which labels the proliferating neural progenitors), p27^{Kip1} (a cell cycle inhibitor expressed in some post-mitotic cells, including MN) or β_{III}-tubulin (an early neuronal differentia-

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expressed in a majority of MN (green). (G", H" and I") At 32S, OC-3 (red) was detected in a few MN which were located close to basal lamina, as observed for OC-2. (J–L) Quantifications (mean percentage values±standard error of the mean (SEM)) of Lhx3$^+$ or Isl-1/2 or Hb9$^+$ MN that expressed HNF-6 or OC-2 or OC-3 from 26 to 32S (n=3). HNF-6 expression (blue) was maintained in ~80% of MN. OC-2 expression (red) amounted around 30% of Lhx3$^+$ or Isl-1/2 or Hb9$^+$ cells. OC-3 expression (green) was initiated at 26S and increased to 30% of Hb9$^+$ MN, but only to 20% and 10% of Isl-1/2 or Lhx3$^+$ cells, respectively. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

tion marker). Except for a few HNF6^{+} cells where a residual expression of Ki67 was still detected (Fig. 1A–A"), OC factors were present in cells that did not contain Ki67 (Fig. 1A–A"). Most of these cells contained p27[Kip1] (Fig. 1B–B"), suggesting that OC expression starts as early as neural progenitors exit from the cell cycle. Furthermore, all the OC^{+} cells contained β_{III}-tubulin (Fig. 1C–C"). Hence, OC proteins were present in post-mitotic cells that have initiated neuronal differentiation. As MN are the first spinal neurons to differentiate, we assessed whether the OC^{+} cells were MN by performing immunofluorescent labelings for Olig2 (marker of MN progenitors), Isl-1/2 and Hb9 (MN markers). Interestingly, we observed that HNF-6 expression was initiated in Isl-1/2[+] newly-born MN wherein Olig2 was still detected (Fig. 1D–D"). HNF-6 and OC-2 were expressed in a majority of Isl-1/2^{+} cells (Fig. 1E–E") and were present in all the Hb9^{+} neurons (Fig. 1F–F"). Thus, OC factors are present in newly-born MN.

The OC factors are dynamically expressed during MN development

To gain further insight into OC expression during MN differentiation, we studied HNF-6, OC-2 and OC-3 distribution in spinal MN at different developmental stages from e9.0 to e14.5.

First, we quantified HNF-6, OC-2 and OC-3 expression in Lhx3^{+}, Isl-1/2[+] or Hb9^{+} MN from 26 to 32S (e9.5 to e10.0) along the rostrocaudal axis of the embryonic ventral spinal cord. At these early stages, Lhx3 is transiently expressed in all the newly-born MN while, at later stages, its expression is restricted to MMC neurons (see below). HNF-6 was present in a vast majority of Lhx3^{+} newly-born MN (Fig. 2A–A"and D–D"), although the amount of co-labeled cells slightly decreased between 28 and 32S (Fig. 2J). In contrast, OC-2 and OC-3 were stably expressed in a minority of Lhx3^{+} MN (Fig. 2A–A", D–D", G–G" and J). Similarly, the percentage of Isl-1^{+}HNF-6^{+} MN decreased from 89% to 77% out of total Isl1^{+} MN between 26 and 32S, while percentages of Isl-1^{+} cells that expressed OC-2 or OC-3 remained between 20% and 30 % (Fig. 2B–B", E–E", H–H" and K). Finally, HNF-6 expression in Hb9^{+} MN decreased from 100% to 88% of total Hb9^{+} MN, OC-2 expression decreased from 45% to 32% while the percentage of Hb9^{+} cells that express OC-3 increased from 3% to 28% (Fig. 2C–C", F–F", I–I" and L). At these stages, the proportion of HNF-6, OC-2 or OC-3 in MN did not vary along the rostrocaudal axis of the spinal cord (data not shown). Hence, at the beginning of MN differentiation, OC factors are present in a majority of newly-born MN.

Then, we quantified OC expression in Lhx3^{+}, Isl-1/2[+] or Hb9^{+} MN from e10.5 to e14.5. OC expression in Lhx3^{+} MN globally decreased, but OC factors remained expressed at low levels in some Lhx3^{+} MN located in a ventro-medial position (Fig. 3A–A", D–D", G–G" and J–J'), probably corresponding to MMC neurons (see below). From e10.5 to e14.5, HNF-6 expression in Isl-1/2[+] and in Hb9^{+} MN decreased from 74% to 15% and from 72% to 54%, respectively. Similarly, OC-2 expression decreased from 37% to 18% and from 30% to 6% (Fig. 3B–C", H–I"

and M–N"). In contrast, OC-3 expression was more stable, decreasing only from 39% to 28% and from 45% to 34% in Isl-1/2[+] or Hb9^{+} MN, respectively (Fig. 3E–F", K–L", M–N"). At all developmental stages, the expression pattern of the 3 OC genes was partially overlapping (Figs. 1–3, S2, S3). These observations indicated that OC expression is transient in most of the MN. In addition, combined expression of the OC factors may define subsets of MN populations, while MN that express HNF-6 (more than 40% at e12.5) may correspond to MN subpopulations that form some of the motor columns.
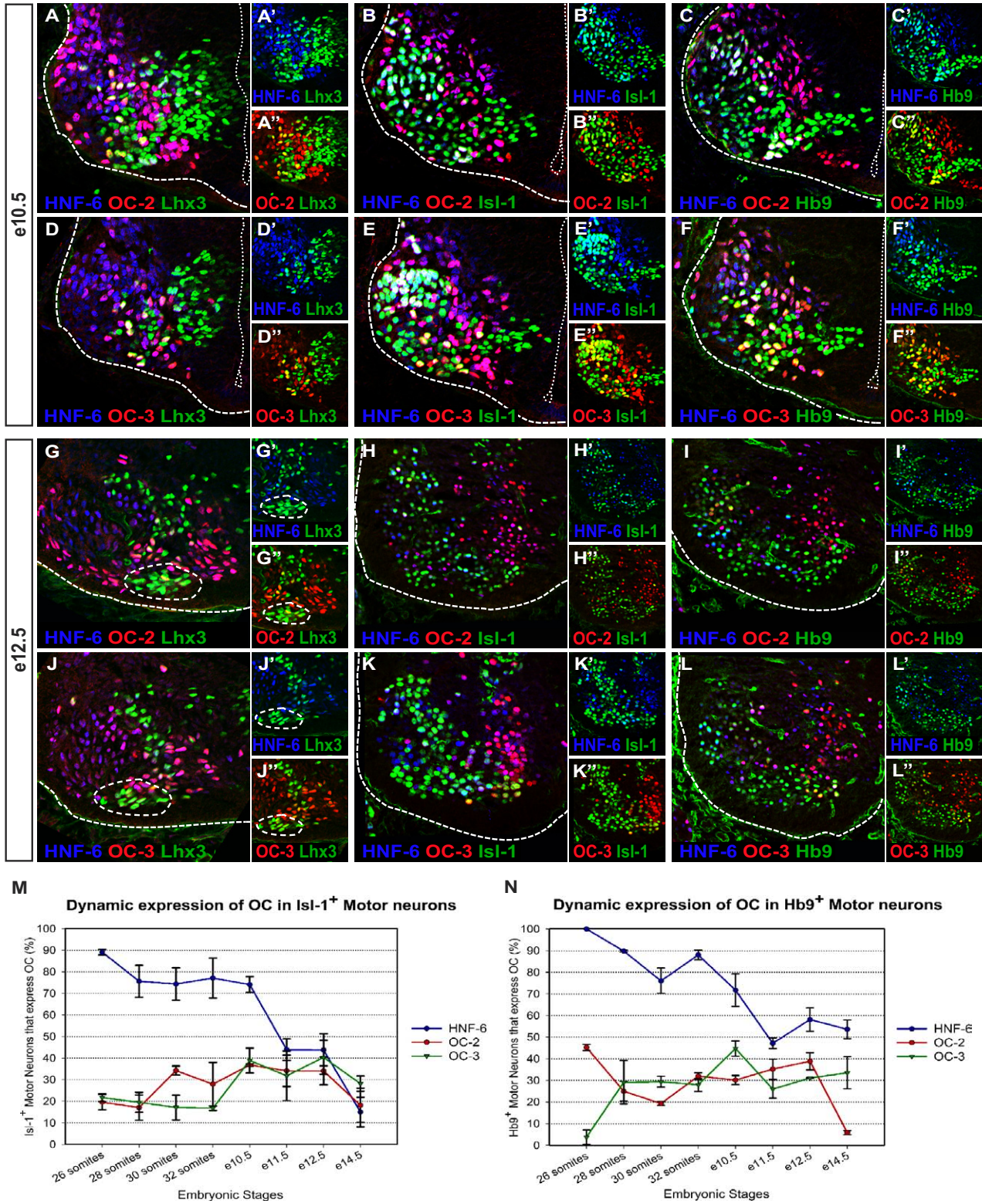
The OC factors are differentially expressed in subsets of MN column populations

To assess whether *Hnf6* expression indeed corresponds to specific MN columns, we analyzed its distribution, as well as that of OC-2 and of OC-3 in MMC, HMC, PGC and LMC from e11.5 to e14.5. In the MMC, HNF-6 expression decreased from 63% to 39% out of total MMC neurons between e11.5 and e14.5. OC-2 expression decreased from 48% to 43% between e11.5 and e12.5, then increased to 71% at e14.5, while OC-3 expression decreased from 55% to 29% between e11.5 and e14.5 (Fig. 4G). Thus, OC-2 appeared to become the predominant OC in MMC neurons whereas HNF-6 and OC-3 expression was downregulated (Fig. 4A–F"). The OC expression profile was qualitatively similar in HMC. Indeed, HNF-6 expression slightly decreased from 51% to 44% between e11.5 and e14.5, while OC-2 expression increased from 16% to 83%. OC-3 expression increased from 48% to 80% between e11.5 and e12.5, then decreased to 15% between e12.5 and e14.5 (Fig. 4H).

Next, we characterized OC distribution in PGC neurons from e11.5 to e14.5 (Fig. 5A–F). At e11.5, HNF-6 was expressed in 80% of the PGC neurons (Fig. 5A–A" and F) while OC-2 and OC-3 were not present (Fig. 5B–C" and F). HNF-6 expression decreased dramatically from 80% to 2% between e11.5 and e14.5 (Fig. 5D–D" and F), while the expression of OC-2 and OC-3 was only transiently detected at e12.5 in 14% and 11% of PGC neurons, respectively (Fig. 5E–E" and F).

Finally, we examined the distribution of each OC factor in brachial and lumbar LMCm and LMCl from e11.5 to e14.5 (Fig. 6A–F"). The ratio of OC^{+} cells in LMCm or in LMCl was very similar at brachial and lumbar levels. During LMCm development, HNF-6 expression was maintained in more than 80% of the MN between e11.5 and e12.5, but decreased to 39% at e14.5. In contrast, OC-2 and OC-3 expression increased from 38% to 76% and from 30% to 70% between e11.5 and e12.5, then decreased at e14.5 to 9% and 39%, respectively (Fig. 6G). In the developing LMCl, expression of HNF-6 decreased from 84% to 20% between e11.5 and e14.5. In contrast, OC-2 expression increased from 24% to 44% between e11.5 and e12.5 then decreased to 2% at e14.5. As observed for HNF-6, OC-3 expression dramatically decreased from 15% to 0% (Fig. 6H). This distribution of OC factors in subsets of LMC neurons suggested that OC expression may correspond to motor pools which innervate specific sets of locomotory muscles.
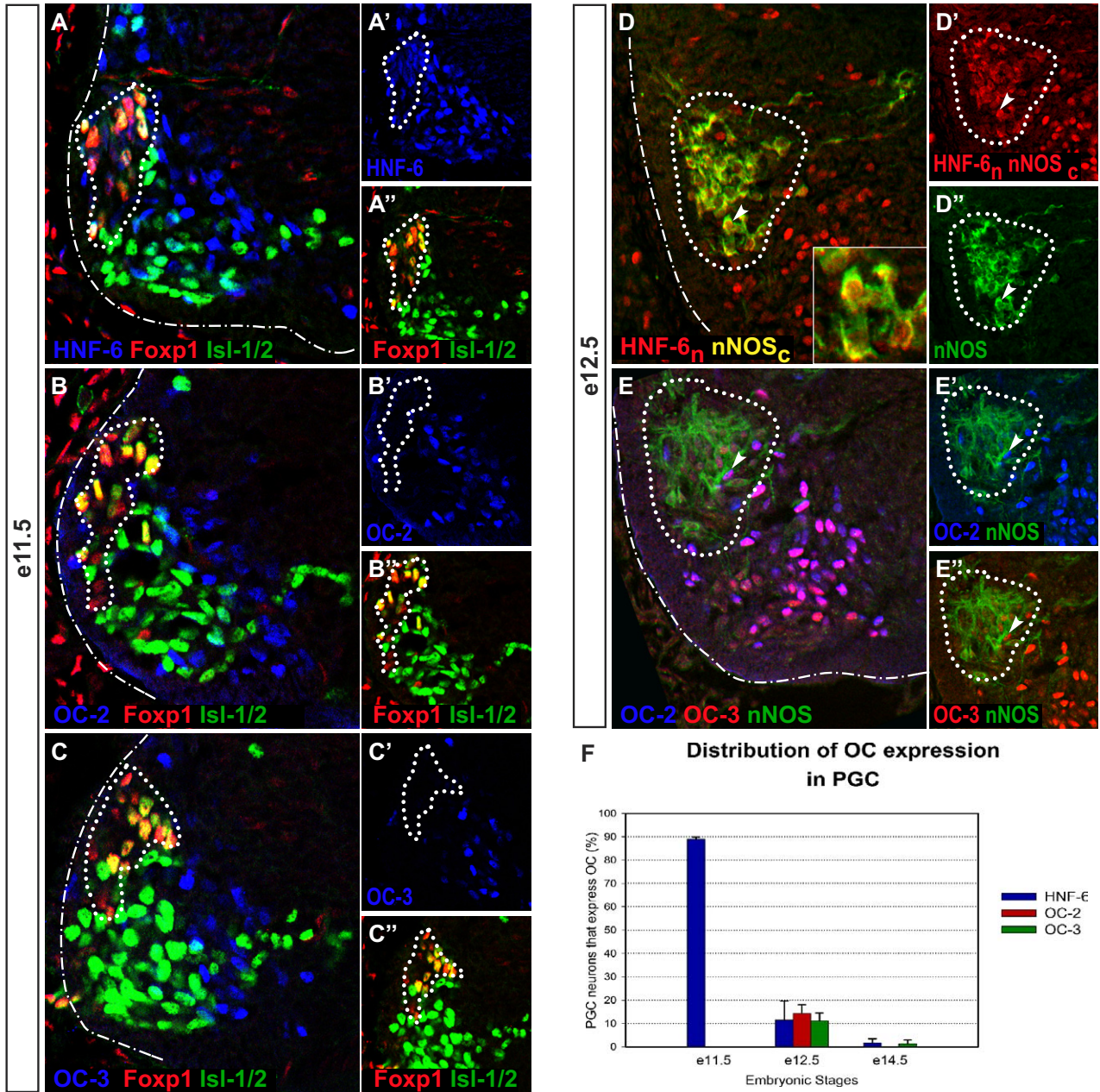
Fig. 3. The Onecut factors are dynamically expressed in differentiating MN. (A–L") Immunolabelings of HNF-6 (blue), OC-2 or OC-3 (red), in Lhx3+ or Isl-1+ or Hb9+ MN (green) on transverse sections of ventral spinal cord at brachial levels from e10.5 to e12.5. (A–F") At e10.5, HNF-6 was present in a majority of Lhx3+ or Isl-1+ or Hb9+ MN. In contrast, OC-2 and OC-3 exhibited a limited expression in Lhx3+ MN (A" and D") and were present in a minority of Isl-1+ or Hb9+ MN (B", C", E" and F"). Expression of HNF-6, OC-2 and OC-3 was partially overlapping. (G–L") At e12.5, HNF-6, OC-2 and OC-3 expression was strongly limited in Lhx3+ MN (G–G" and J") which correspond to the MMC (delineated by white dashes). HNF-6 was present in large subsets of Isl-1+ or Hb9+ MN, while OC-2 and OC-3 exhibited a more restricted expression pattern (H", I", K" and L"). (M and N) Quantifications (mean percentage values±SEM) of Isl-1+ or Hb9+ MN that expressed HNF-6 or OC-2 or OC-3 from 26S to e14.5 ($n=3$). HNF-6 expression (blue) decreased significantly in Isl-1/2+ and in Hb9+ MN. OC-2 expression (red) transiently increased from 20% to 37%, then decreased to 18% in Isl-1/2+ MN, while it decreased gradually from 45% to 6% in Hb9+ MN OC-3 expression (green) remained around 30% in Isl-1/2+ and in Hb9+ MN. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.
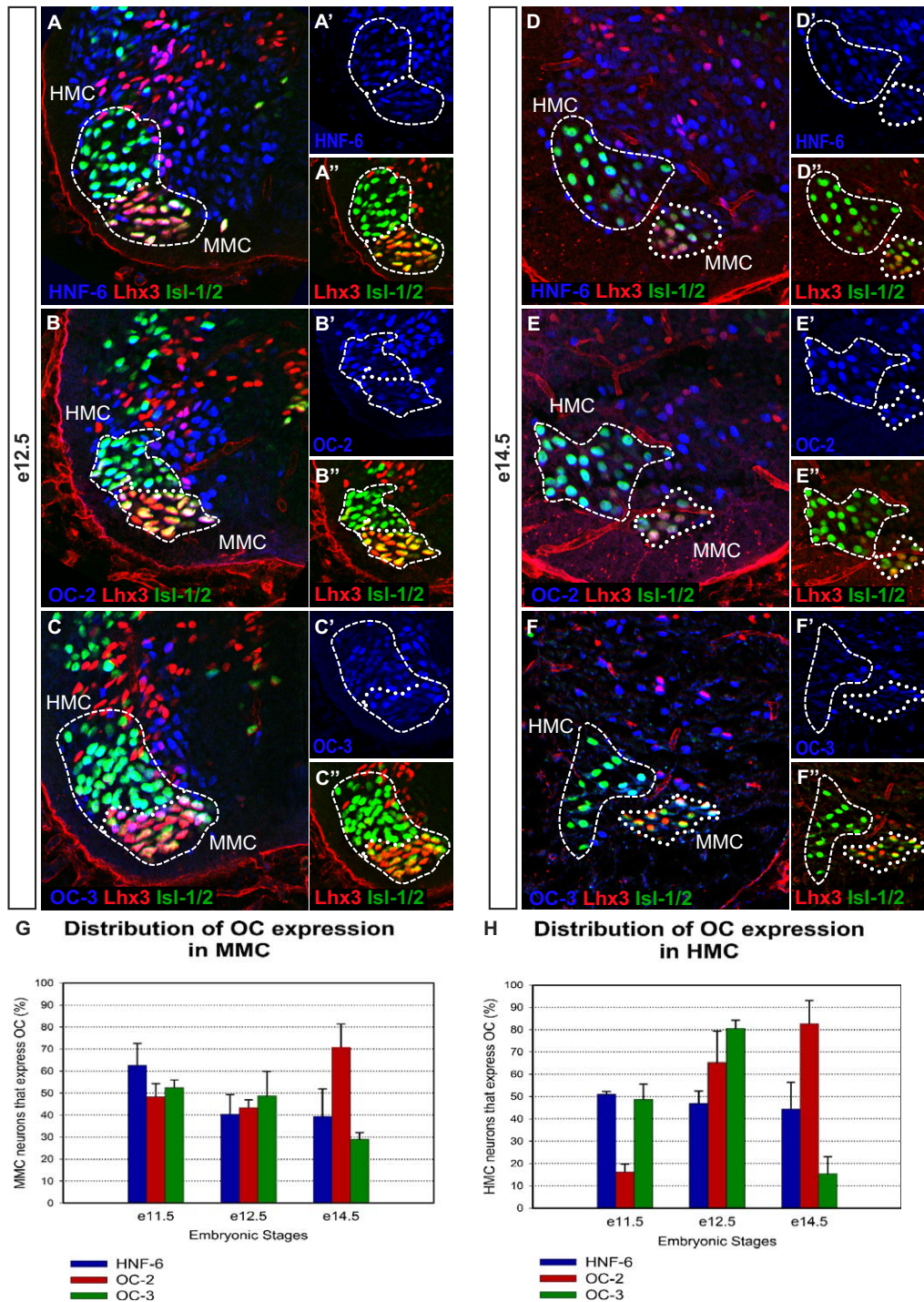
Fig. 4. The Onecut factors are differentially and dynamically expressed in MMC and hypaxial motor column (HMC) neurons. (A–F") Immunolabelings of HNF-6 or OC-2 or OC-3 (blue), Lhx3 (red) and Isl-1/2 (green) on transverse sections of ventral thoracic spinal cord at e12.5 and e14.5. MMC and HMC domains were delineated by white dashes. MMC neurons (yellow) were characterized by the coexpression of Lhx3 and Isl-1/2. HMC neurons (green) expressed Isl-1/2 but not Lhx3. (A–A") At e12.5, HNF-6 was expressed in a portion of MMC and HMC cells; (B–B") OC-2 was present in a small portion of MMC neurons, but in more numerous HMC cells; (C–C") OC-3 was more broadly expressed in MMC and HMC neurons than OC-2. (D–D") At e14.5, HNF-6 expression was observed in MMC and HMC; (E–E") OC-2 was broadly expressed in MMC and HMC; (F–F") OC-3 expression was restricted to a few MN in MMC and HMC. (G and H) Quantifications (mean percentage values±SEM) of MMC or HMC neurons that expressed HNF-6 or OC-2 or OC-3 from e11.5 to e14.5 ($n=3$). (G) In MMC neurons, HNF-6 expression decreased gradually from 63% to 39%, while OC-2 expression increased from 48% to 71%. OC-3 decreased gradually from 55% to 29%. (H) In HMC neurons, HNF-6 expression decreased slightly from 51% to 44%, while OC-2 increased significantly from 16% to 83%. OC-3 expression increased from 48% to 80% between e11.5 and e12.5, but it expression decreased dramatically to 15% at e14.5. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.
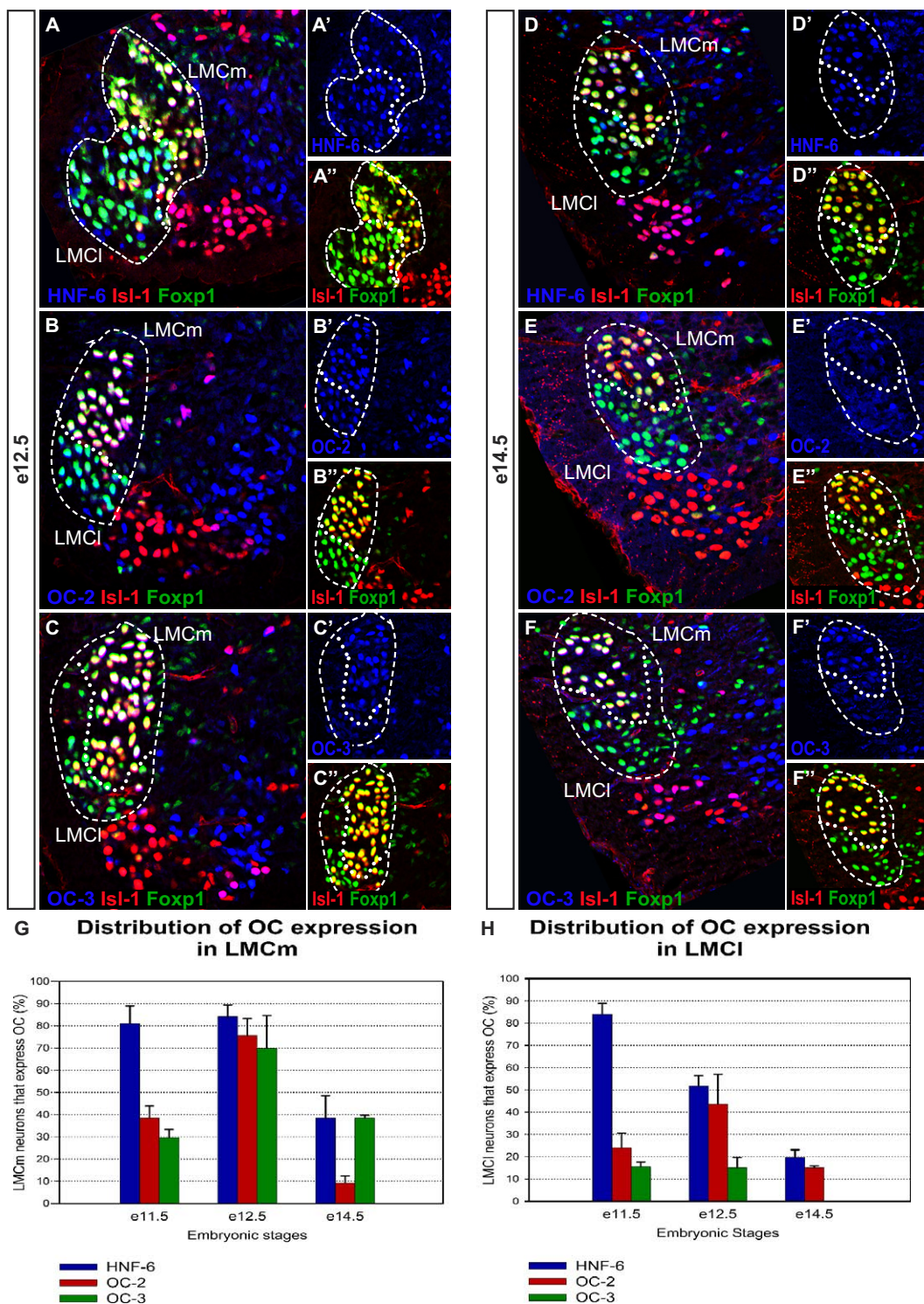
Fig. 5. The Onecut factors are differentially and dynamically expressed in preganglionic column (PGC) MN. (A–E") Immunolabelings of Onecut factors on transverse sections of ventral thoracic spinal cord at e11.5 and e12.5. The PGC, delineated by a white dotted line, was characterized by the expression of Foxp1 (red) and Isl-1/2 (green) at e11.5 (A–C'), and by the expression of nNOS (green) at e12.5 (D–E"). Rabbit HNF-6 and nNOS antibodies were sequentially detected with different anti-rabbit secondary antibodies, resulting in HNF-6 nuclear labeling in red and in nNOS cytoplasmic labeling in red and green (D–D"). (A–A") At e11.5, HNF-6 (blue) was broadly expressed in PGC neurons (yellow); (B–C") At e11.5, OC-2 (blue) or OC-3 (blue) was not detected within PGC neurons (yellow). (D–D') At e12.5, HNF-6 (nuclear labeling, red) expression was restricted to a few PGC neurons (nNOSc, cytoplasmic labeling, yellow in A, red in A' and green in A"), as indicated by arrowhead. (E–E") OC-2 (blue) and OC-3 (red) were present in very few PGC cells (green), as indicated by arrowheads. (F) Quantification (mean percentage values±SEM) of PGC neurons that expressed HNF-6 or OC-2 or OC-3 from e11.5 to e14.5 ($n=3$). HNF-6 was expressed in 80% of PGC MN at e11.5, then its expression strongly decreased to 12% at e12.5 and to 2% at e14.5. OC-2 and OC-3 were only detected at e12.5 in 14% and 11% of PGC cells, respectively. At e14.5, OC-2 was not detected while OC-3 was present in less than 1% of PGC MN. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

To determine whether this is the case, we assessed OC expression at e12.5 in Pea3$^+$, SCIP$^+$ or Nkx6.1$^+$ MN pools at brachial and lumbar levels. We were not able to study HNF-6 expression in pools of MN which are defined by Pea3 or SCIP, since the anti-HNF-6 antibody was made in rabbit as for the anti-Pea3 or anti-SCIP antibodies. OC factors were expressed in subsets of cells within pool of MN that expressed Nkx6.1 (Fig. 7A–C"). OC-2 as OC-3

Fig. 6. The Onecut factors are differentially and dynamically expressed in medial and lateral LMC neurons. (A–F") Immunolabelings of HNF-6 or OC-2 or OC-3 (blue), Isl-1 (red) and Foxp1 (green), on transverse sections of ventral spinal cord at brachial levels. LMC was delineated by white dashes and medial LMC (LMCm) and LMCl were separated by a white dotted line. LMCm neurons (yellow) were characterized by the expression of Foxp1 and Isl-1, while LMCl neurons (green) expressed Foxp1 but not Isl-1. (A–C") At e12.5, HNF-6, OC-2 and OC-3 were broadly expressed in LMCm and LMCl neurons. (D–D") At e14.5, HNF-6 expression was limited to a few LMCm or LMCl neurons; (E–E") OC-2 expression was strongly reduced in both LMCm and LMCl; (F–F") OC-3 expression was still observed in a few LMCm neurons but was absent from LMCl cells. (G and H) Quantifications (mean percentage values±SEM) of LMCm or LMCl neurons that expressed HNF-6 or OC-2 or OC-3 from e11.5 to e14.5 ($n=3$). (G) In LMCm, HNF-6 expression was maintained in ~80% of the neurons, then decreased to 39% between e12.5 and e14.5; OC-2 expression increased from 38% to 76% between e11.5 and e12.5, then decreased to 9% at e14.5; OC-3 expression increased from 30% to 70% between e11.5 and e12.5, then decreased to 39% at e14.5. (H) HNF-6 expression decreased gradually from 84% to 20% in LMCl; OC-2 expression increased from 24% to 44% between e11.5 and e12.5, then decreased to 2% at e14.5. From e11.5 to e14.5, OC-3 expression gradually decreased from 15% to 0%. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.
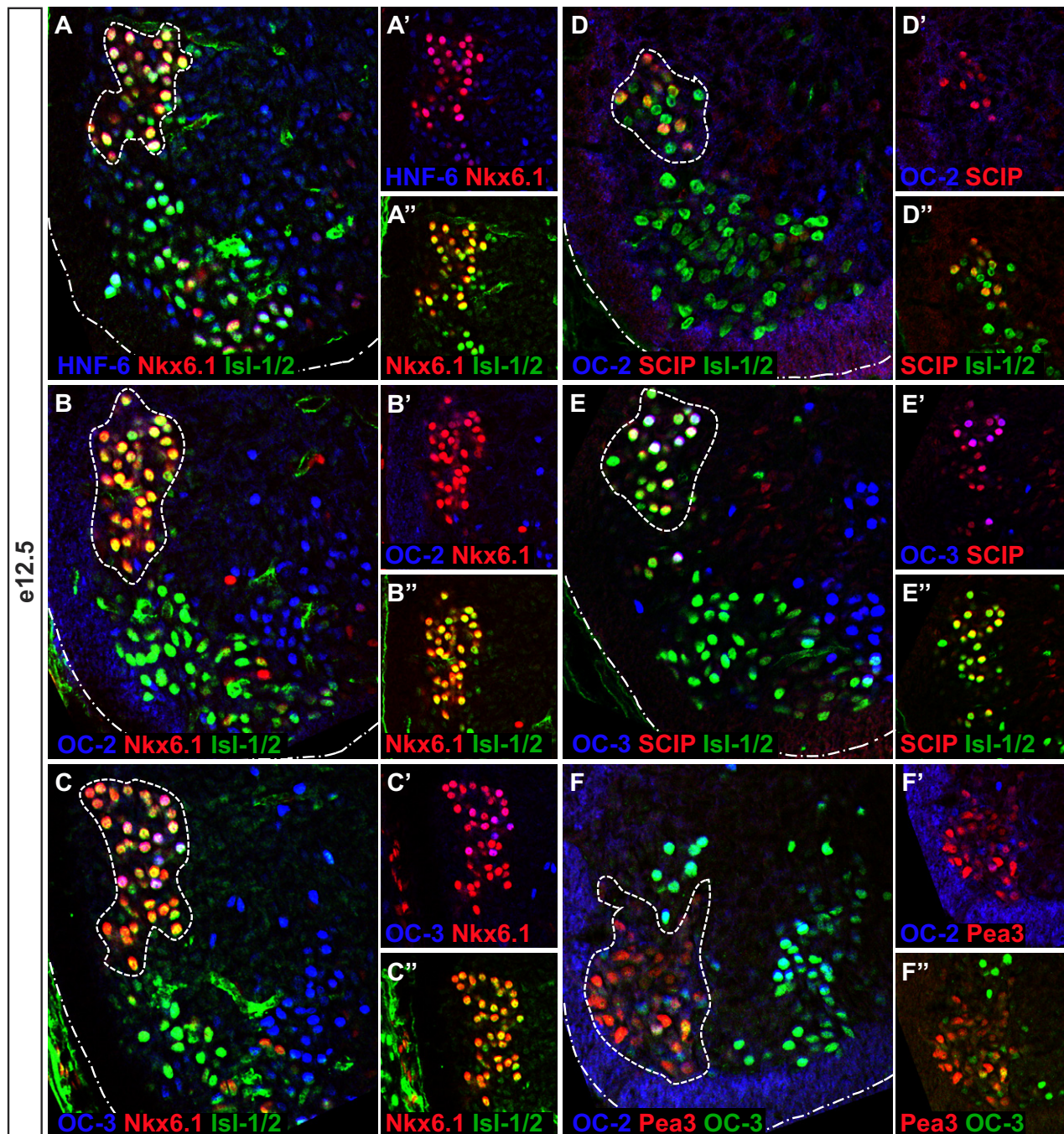
Fig. 7. The Onecut factors are expressed in subsets of MN pools. (A–F") Immunolabelings of HNF-6 or OC-2 or OC-3 (blue), Isl-1 (green) and Nkx6.1 or SCIP or Pea3 (red), on transverse sections of ventral spinal cord (brachial levels) at e12.5. MN pools that expressed Nkx6.1 or SCIP or Pea3 were delineated by white dashes. (A–C") HNF-6, OC-2 and OC-3 were present in subsets of neurons within a MN pool characterized by the expression of Nkx6.1. (D–D") OC-2 was expressed in a few SCIP$^+$ MN; (E–E") OC-3 was expressed in a subset of neurons within the MN pool defined by SCIP expression. (F–F") OC-2 (blue) and OC-3 (green) were present in subsets of neurons within the MN pool that expressed Pea3 (red). For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

were present in subsets of MN within SCIP and Pea3 MN pools (Fig. 7D–F"). Hence, our results indicated that subsets of MN that express OC did not correspond to known MN pools.

Taken together, these results showed that OC genes are dynamically and differentially expressed in subsets of MN. They also confirmed that OC expression in MN is transient, as it is progressively restricted to a minority of these cells during the course of development. In addition to MN, OC factors were also detected in cells of the ventral spinal cord that do not express MN markers (Figs. 1–7). To determine the identity of these cells, the distribution of OC

proteins in each ventral interneuron population was assessed. Co-labelings for OC and Evx1, Foxd3, Chx10, Gata3 or Nkx2.2, which define V0, V1, V2a, V2b or V3 interneurons respectively, showed that OC factors were expressed in a subset of each interneuron population (Fig. S4), as observed for MN. Finally, to assess whether OC might be re-expressed in MN at later stages, we determined the distribution of HNF-6, OC-2 and OC-3 in the ventral spinal cord at postnatal day (P)1. We observed that OC factors were broadly expressed in the ventral horn, and that HNF-6 and OC-3 were re-expressed in a large proportion of MN (Fig. S5).

The expression profile of HNF-6 in spinal MN is conserved between chick and mouse

Finally, we addressed the question of the conservation of OC expression pattern during MN development in other vertebrate species. Our interest focused on chick because chick embryo is widely used in MN development studies. First, we assessed whether the OC antibodies we used on mouse tissues do bind proteins on chick spinal cord sections. Only the HNF-6 antibody gave a specific signal (Fig. 8), whereas antisera raised against OC-2 or OC-3 did not (data not shown). This suggested either that a single OC gene related to *Hnf6* exists in the chick, or that additional chick OC proteins exist but are not recognized by the OC-2 and OC-3 antisera. Analysis of the chick genome showed a single region annotated as OC gene (Onecut1 ENSGAL00000021321, on chromosome 10: 9.894.475-9.895.108). Although this sequence was incomplete, preventing any consistent comparison with the mammalian OC gene, this suggested that the HNF-6 antibody recognizes the chick protein cOC-1. Therefore, the HNF-6 antibody was used to assess the distribution of cOC-1 on spinal cord sections from HH18 to HH29 embryos (from e3.5 to e6.5).

We observed that cOC-1 expression was initiated around HH18 in cells located in the marginal zone in the vicinity of the basal lamina. cOC-1 was present in postmitotic neurons, since all the cOC-1$^+$ cells expressed the cell cycle inhibitor p27^{Kip1} and an early neuronal differentiation marker, β_{III}-tubulin (Fig. 8A–A"). To determine whether these cOC-1$^+$ neurons corresponded to newly-born MN, we performed immunolabelings of Olig2 (marker of MN progenitors) and Isl-1/2. This experiment provided evidence that cOC-1 was not present in Olig2$^+$ MN progenitors but was expressed in Isl-1/2$^+$ newly-born MN (Fig. 8B–B"). Hence, cOC-1 is present in MN in chick spinal cord, at the early steps of MN differentiation.

Next, we examined whether cOC-1 was also expressed during later steps of MN differentiation. cOC-1 was present in a large majority of MN at HH24 (data not shown). At HH29, it was detected in equivalent amounts of cells in MMC and HMC neurons (Fig. 8C–C"), it was broadly expressed in LMCm and more restricted in LMCl neurons (Fig. 8D-D"), and was present in the columns of Terni, which are equivalent to the PGC in mice (Fig. 8E–E"). This distribution was similar to that observed in mouse embryos, indicating that the HNF-6 expression pattern in

MN is conserved between bird and mice. These results strongly suggest a conservation of HNF-6 functions during MN development.

DISCUSSION

The differentiation program of the spinal MN is under the control of multiple transcription factors. Most of these are transcriptional repressors (Olig2, Hb9, Nkx6.1, and Nkx6.2) which prevent irrelevant differentiation cascades to be activated, resulting in the activation of the proper differentiation program in the right cells (Arber et al., 1999; Thaler et al., 1999; Sander et al., 2000; Novitch et al., 2001). Their expression patterns often match to well-defined MN subpopulations corresponding either to newly-born MN, to motor columns or to pools of MN that eventually innervate specific sets of muscles (Lin et al., 1998; Dasen et al., 2005, 2008; Dalla Torre di Sanguinetto et al., 2008; Rousso et al., 2008). Here, we provide evidence that the OC transcriptional activators are dynamically and differentially expressed in subsets of these MN subpopulations at different stages of MN development.

OC expression starts in newly-born MN. The initiation of HNF-6 expression seems to correlate with that of Isl-1 and to precede the onset of Hb9 expression, consistent with the fact that Isl1 lies upstream of Hb9 in MN (Lee et al., 2004; Nakano et al., 2005). OC genes exhibit partially overlapping expression patterns characterized until e11.5 by a spatial HNF-6>OC-2>OC-3 hierarchy which parallels their temporal induction sequence. Indeed, the *Oc3* expression domain is included in the *Oc2* domain, which is included in the *Hnf6* domain. Given the broad functional redundancy between OC factors (Jacquemin et al., 1999; Vanhorenbeeck et al., 2002; Clotman et al., 2005; Beaudry et al., 2006), these observations underline that these proteins may exert overlapping functions during MN development.

The OC transcription factors are dynamically and differentially expressed during maturation of MN. This suggests that specific combinations of OC might either define distinct subsets of MN or participate in specific steps of MN differentiation. Indeed, HNF-6 expression is initiated in a large majority of newly-born MN whereas the expression of OC-2 and of OC-3 is activated in more differentiated MN. OC-2 becomes predominant in MMC and HMC neurons at e14.5, whereas the expression of HNF-6 and OC-3 in these cells globally declines. PGC neurons predominantly express *Hnf6*, whereas *Oc2* and *Oc3* are only transiently detected at e12.5 in a very small portion of these cells. LMCm column is enriched in OC proteins as compared to the LMCl column. Furthermore, OC expression in each MN subset shows a dynamic temporal profile. In contrast, contrary to *Foxp1* expression (Dasen et al., 2008; Rousso et al., 2008), the distribution of OC factors does not seem to vary along the anteroposterior axis of the spinal cord. Hence, these observations suggest that OC factors could have multiple functions at several stages of MN development and/or in different subsets of differentiating MN. Moreover, OC proteins were detected in subsets of spinal
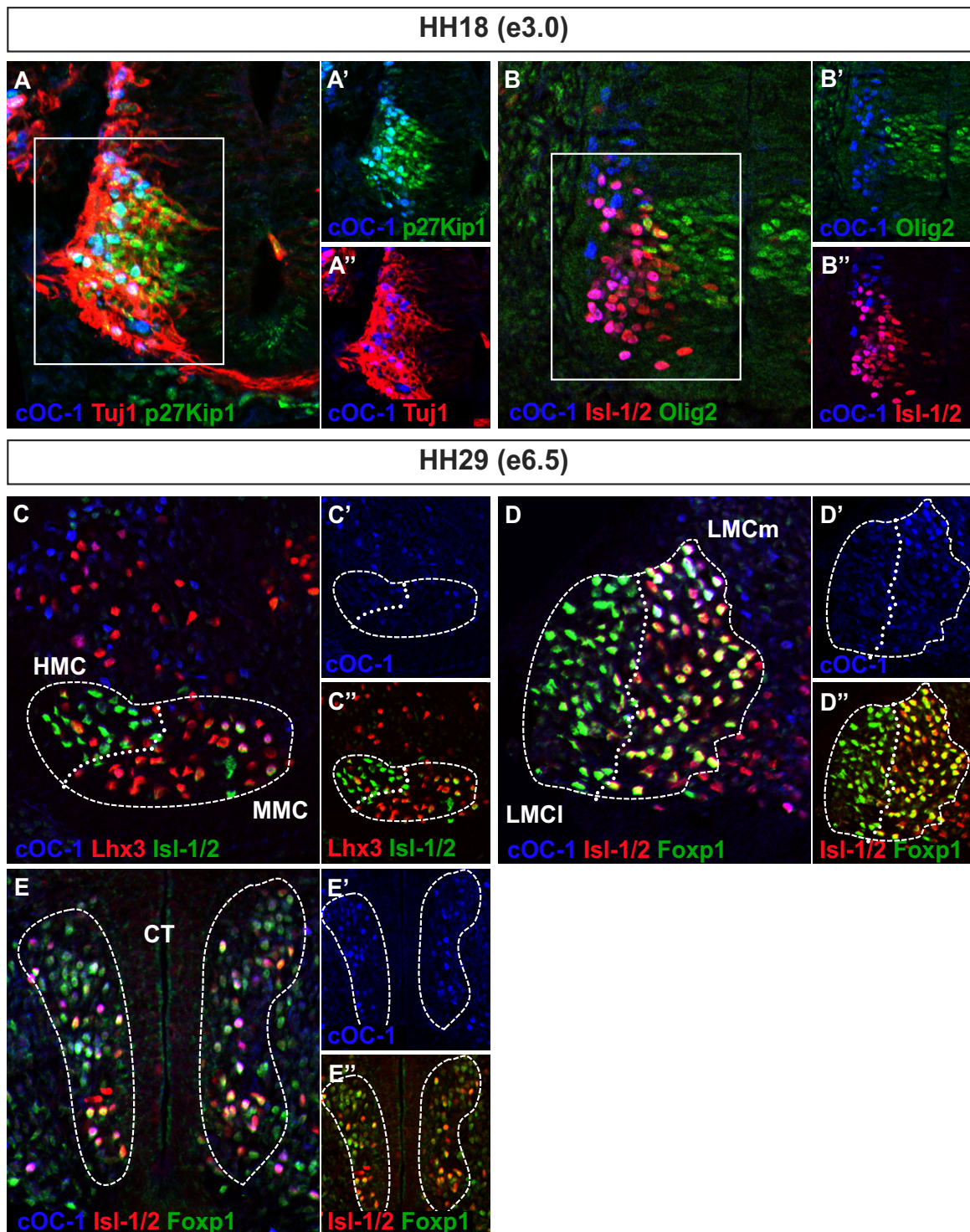
Fig. 8. Expression of the Onecut in the spinal MN is conserved between chick and mouse. (A–A") Immunolabelings on transverse sections of chick spinal cord at Hamburger–Hamilton 19 (HH19) stage, showed that cOC-1 (blue) was present in post-mitotic neurons that expressed the cell cycle inhibitor p27kip1 (green) and the neuronal marker β_{III}-tubulin (Tuj1 antibody, red). (B–B") cOC-1 (blue) was not expressed in Olig2$^+$ cells (green) but was present in Isl-1/2$^+$ MN (red), at HH19. (C–C") At HH29, cOC-1 was present in both HMC (green) and MMC (yellow), which were characterized by the expression of Isl-1/2 (green) or coexpression of Isl-1/2 and Lhx3 (red), respectively. HMC and MMC are delineated by white dashes. (D–D") cOC-1 (blue) was also broadly expressed in both LMCm (yellow) and LMCl (green) at HH29. MMC population is delineated by white dashes, the median and lateral columns being separated by white dots. (E–E") At HH29, cOC-1 (blue) was present in column of Terni (CT) neurons (equivalent to PGC neurons in mouse), which were characterized by coexpression of Isl-1 (red) and Foxp1 (green). CT are delineated by white dashes. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

MN at post-natal stages. Consequently, they could exert around birth additional functions that may differ from those exerted during embryonic development.

The OC factors exhibit an original expression pattern when compared to other transcription factors such as Isl-1, Isl-2, Hb9, Lhx1/5 and Foxp1. Indeed, OC proteins are not detected in all the cells of a particular column, but are rather present in subsets of MN in different columns. This is in contrast to Isl-2, Hb9 and Lhx1/5 which are specifically expressed in all the LMCl neurons and to Isl-1 which is present in LMCm but not in LMCl, or to Foxp1 which is detected in all the PGC and LMC neurons. Hence, OC factors are not markers of specific columns, but may rather be instrumental in defining further subdivisions within the MN subpopulations. However, we were not able to correlate these subdivisions with specific properties of the OC$^+$ cells nor with expression patterns of other MN markers, even in the LMC where these subdivisions do not correspond to the pools of MN (Dasen et al., 2005; Dalla Torre di Sanguinetto et al., 2008). Similarly, OC proteins are present in subsets of ventral interneurons and may define specific interneuron subpopulations.

Finally, our observations establish that the HNF-6/cOC-1 expression pattern in the developing spinal MN is conserved between chick and mouse embryo. This suggests a probable conservation of OC functions in MN differentiation. However, in the mouse spinal cord, HNF-6 expression is initiated around 22S simultaneously to Isl-1, while cOC-1 begins to be expressed around HH18 (30S) in the chick. Consequently, cOC-1 expression is delayed when compared to that of Lhx3 and Isl-1, as observed for *Oc2* and *Oc3* in the mouse. These results suggest that the function of chick cOC-1 and of murine HNF-6 at early stages of MN differentiation might be different. Whether other OC are expressed earlier than cOC-1 in the chick embryonic spinal cord remains to be investigated.

CONCLUSION

In this study, we report for the first time a qualitative and quantitative analysis of OC expression during development of the MN. We show that OC factors are dynamically, differentially and transiently expressed at different stages of MN differentiation and exhibit a partially overlapping expression pattern. Together, our data suggest that OC transcription factors may participate in the regulatory networks that control the early steps of MN differentiation, and could exert later functions in subsets of the described MN subpopulations. Finally, they provide evidence that the OC expression pattern in developing MN is conserved in chick and mouse.

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APPENDIX

Supplementary data