Opposing FGF and retinoid pathways: a signalling switch that controls differentiation and patterning onset in the extending vertebrate body axis

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Summary

Construction of the trunk/caudal region of the vertebrate embryo involves a set of distinct molecules and processes whose relationships are just coming into focus. In addition to the subdivision of the embryo into head and trunk domains, this ''caudalisation'' process requires the establishment and maintenance of a stem zone. This sequentially generates caudal tissues over a long period which then undergo differentiation and patterning in the extending body axis. Here we review recent studies that show that changes in the signalling properties of the paraxial mesoderm act as a switch that controls onset of differentiation and pattern in the spinal cord. These findings identify distinct roles for different caudalising factors; in particular, Fibroblast Growth Factor (FGF) inhibits differentiation in the caudal stem zone, while Retinoic acid (RA) provided rostrally by somitic mesoderm is required for neuronal differentiation and establishment of ventral neural pattern. Furthermore, the mutual opposition of FGF and RA pathways controls not only neural differentiation but also mesoderm segmentation and might also underlie the progressive assignment of rostrocaudal identity by regulating Hox gene availability and activation. ***BioEssays*** **26:857–869, 2004. © 2004 Wiley Periodicals, Inc.**

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Abbreviations: BMP, Bone morphogenetic protein; CNS, Central nervous system; DN-FGFR, dominant negative FGF receptor; ES, embryonic stem; FGF, Fibroblast Growth Factor; FGFR, Fibroblast growth factor receptor; Ngn, neurogenin; RA, Retinoic acid; Raldh, retinaldehyde dehydrogenase; RAR/RXR, retinoic acid receptors; Shh, Sonic hedgehog; VAD, Vitamin A deficient.

Introduction

Unlike other regions of the central nervous system (CNS), the spinal cord is generated over a long period of time in a head-to-tail (rostrocaudal) sequence as the body axis extends. This is achieved by the activity of a caudally moving stem zone that gives rise to neural progenitors. As the spinal cord forms progressively, there is a spatial separation of the temporal events of neurogenesis along the rostrocaudal axis. This makes the forming spinal cord particularly amenable to analysis of the control and integration of neural differentiation and patterning. Such studies also inform our understanding of these processes in differentiating Embryonic Stem (ES) cells and in neural stem cells in vitro and help to devise strategies for generating specific neuronal cell types in this context.

In higher vertebrates, the neural plate forms in response to neural-inducing signals provided by the organiser (anterior primitive streak) and its precursors (e.g. in chick Fig. 1A,C and reviewed in Ref. 1). There is growing evidence that these signals are FGFs, which act in part by attenuating Bone Morphogenetic Protein (BMP) signalling that would otherwise promote formation of epidermis[2,3] (reviewed in Ref. 4). The early neural plate forms with a rostral (forebrain) character and more caudal regions of the CNS (midbrain, hindbrain and spinal cord) form in response to signals provided by newly formed mesodermal tissues that emerge from the primitive streak/marginal zone (reviewed in Ref. 5) (Fig. 1; Table 1). The caudalmost region, the spinal cord, is derived from the caudal part of the neural plate which lies either side of the primitive streak (and is also known as the caudal stem zone, see below, Fig. 1A, pink). In the chick embryo, cells in this region appear to be specified as neural, although at open

Table 1. Ability of different somitic/presomitic mesoderm populations and precursors (corresponding to red rectangle/brackets in Fig. 1) to promote/modulate caudal identities in different experimental assays

Origin of tissue (shown in Figure 1 corresponding to red rectangle/bracket in Figure 1)	Tissue grafted or cultured next to neural tissue	Caudal marker induced in response to tissue/signal (activation unless specified)	Signalling molecules that mimic mesoderm
①	Zebrafish marginal zone (equivalent to chick primitive streak)	*Krox-20, hoxb1b* (18,19) repression of otx2 (19,102)	
②	Hensen's node	En-2, Hoxc-6 (103) trunk morphology (104)	Fibroblast Growth Factor (FGF) (23)
③	Caudal presomitic mesoderm	*Pax3* (16,105)	FGF, Retinoic acid (RA), Wnt* (16,105)
④	Rostral presomitic mesoderm	*Hoxb8* (20)	FGF, Retinoic acid*+ Paraxial Mesoderm Caudalising activity (20)
⑤	Somites	Hoxb4 (9,10) (rhombomere identity) Change in motor neuron columnar identity (11)	Retinoic acid* large molecule (10)
⑥	Cervical level paraxial mesoderm (5–6 somite)	Modulation of Hox-c profile: promotes Hoxc5 (12)	Retinoic acid* (12)
	Cervical level paraxial mesoderm (14–15 somite)	Modulation of Hox-c profile: promotes Hoxc6 (12)	Retinoic acid* (12)
	Caudal thoracic level paraxial mesoderm (14s)	Modulation of Hox-c profile: (12) reduces Hoxc6 increases Hoxc9	FGF, Gdf11 (12)
	Rostral thoracic paraxial mesoderm (stage 12)	Reduces *Hoxd10* (13)	
⑦	Primitive streak/node	Hoxc6, 8, 9, 10 (12)	FGF*, Gdf11 (12)

These experiments involve either grafting the mesoderm near to neural tissue in the embryo or recombination with neural explants in culture. The signalling molecules that can mimic these activities and those that have also been shown to be required for these activities (*) are indicated. Experiments 5, 6, 7 are shown on a stage HH10 for simplicity; for the precise locations and stages (HH8–20s) of tissues used, consult the corresponding reference. References are in brackets.

neural plate stages some cells escape laterally and form epidermis and others close to the primitive streak can contribute to the mesoderm[6,7] (Fig. 1A, green dots). Here the activity of *Churchill*, a novel neural-specific transcription factor, may be particularly important for the stabilisation of neural cell fates.[3] *Churchill*, which is induced as a slow response to FGF signalling, inhibits expression of the early mesodermal gene *bra* as well as movement of cells through the primitive streak and may act at least in part via induction of *Sip1* that can both inhibit *bra* and modulate the BMP pathway.

Following the acquisition of neural fate, spinal cord progenitors give rise to a range of neuronal cell types that will then make appropriate connections within the CNS and with peripheral targets. This relies on neural progenitors acquiring particular identities at specific positions within the neural tube along both the dorsoventral and the rostrocaudal axes and again involves interactions with adjacent mesodermal tissues. In particular, Sonic hedgehog (Shh) from the axial mesoderm (notochord) regulates a cohort of genes which together define distinct progenitor domains that in turn give rise to particular neuronal subtypes in the dorsoventral axis (reviewed in Ref. 8). Rostrocaudal identity within the trunk is conveyed by Hox genes. These transcription factors are expressed in distinct domains along this axis and their onset in the CNS depends on signals from the primitive streak and paraxial mesoderm.[9–13] (Fig. 1A,C; Table 1). Here we review recent work focusing on the sequential generation of the spinal cord, which provides new insights into the mechanisms that control the onset of pattern and differentiation in the extending body axis. In particular, we address how the caudal stem zone is formed and maintained, how newly generated spinal cord progenitors progressively acquire dorsoventral and rostrocaudal pattern and how these events are integrated with the neuronal differentiation programme.

Specifying the caudal hindbrain/spinal cord

Tissue recombination and grafting experiments in all model vertebrate embryos (fish, frog, chick and mouse) indicate that signals from paraxial or lateral mesoderm cell populations emerging from the primitive streak/marginal zone induce expression of caudal (non-forebrain) neural genes in the early neural plate[14–20] (see Fig. 1; Table 1). Three signalling pathways have been implicated in the acquisition of caudal neural identity (including caudal hindbrain and spinal cord identity): Fibroblast growth factor (FGF),[20–23] Wnts[24,25] and Retinoic acid (RA)[20,26,27] (see Fig. 1B). Nodal-related signals presented by non-axial mesoderm or its precursors have also been shown to be required for the acquisition of caudal

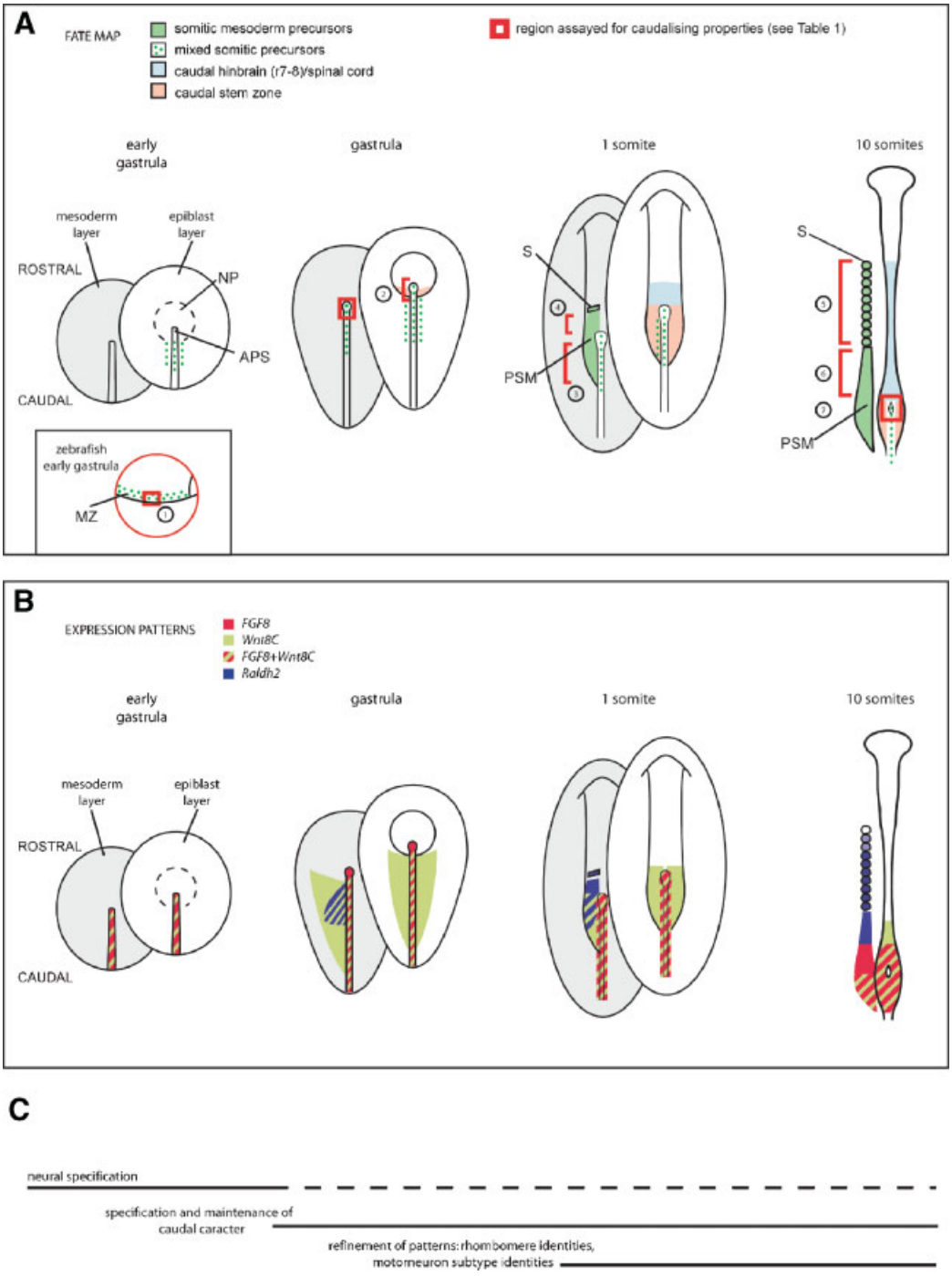

Figure 1. Summary fate map of paraxial mesoderm and caudal CNS and localisation of caudalising factors. **A:** Location of presomitic and somitic mesoderm and caudal hindbrain/spinal cord precursors at different stages of development in the chick mesoderm layer (grey in early gastrula to 1-somite stages) or epiblast.[7,96,97] Inset: Somitic mesoderm precursors in the zebrafish marginal zone at early gastrula stages.[98] Circled numbers and corresponding red rectangles/brackets indicate tissues used in experiments that assess the caudalising activity of somitic and presomitic mesoderm and its precursors (primitive streak and marginal zone) described in Table 1. MZ, marginal zone; NP, neural plate; APS, anterior primitive streak; PSM, presomitic mesoderm; S, somite. **B:** Expression patterns of major components of signalling pathways involved in caudalisation of the nervous system (*Fgf8, Raldh2* and *Wnt8C*) in the mesoderm layers and the epiblast.[23,49,99–101] **C:** Developmental processes taking place in neural tissue at the different stages. The dashed line indicates that this process is ongoing in the caudal stem zone but has stopped in other regions.

identity in whole embryos.[28,29] Some of these factors (FGFs, Wnts and Nodal) induce and/or act on caudal mesodermal tissue and there is some controversy as to whether they are also required in the neuroepithelium for its caudalisation (Wnts,[29,30] Nodal,[5] FGF[20,22]).

Most of the above experiments that identify caudalising tissues and signals use caudal neural marker genes that are either expressed late (e.g. *Krox 20*) or expressed dynamically and in more than one region of the CNS and it is therefore difficult to relate these findings to the establishment of a generic caudal hindbrain/spinal cord identity that relies on a common mechanism. In particular, *Hoxb8* expression is present in the caudal stem zone (see below), it is also expressed in the differentiating spinal cord and has a late rostral domain that extends into the hindbrain.[20,31] *Hoxb8* can be induced in chick neural plate explants by rostral presomitic mesoderm, which does not express FGFs, but which provides RA and unknown signals from the paraxial mesoderm[20] (Fig. 1A,B; Table 1). As this induction requires RA and not FGF signalling,[20] this has lead to the suggestion that specification of spinal cord identity does not involve direct activation of the FGF pathway. However, recent reports indicate that exposure to FGFs promotes expression of such caudal Hox genes in newly generated spinal cord explants[12] and the early embryo[32] and that later *Hoxb8* expression in the hindbrain is under the control of RA.[31] This indicates that *Hoxb8* is regulated in different CNS regions by different "caudalising" signals and suggests that induction of *Hoxb8* by rostral presomitic mesoderm in vitro may represent the later domain of *Hoxb8* expression rather than exemplifying gene regulation in the stem zone (which is adjacent to FGF-expressing tissues and later comes to express *Fgf8* itself, see Fig. 1). These findings indicate that establishment of a generic spinal cord identity needs to be distinguished from the later assignment of distinct identities along the rostrocaudal axis of the caudal CNS and it may be that this first step is tied to the formation of the stem zone (see below).

Defining the caudal stem zone

As noted above, the cells of the caudal neural plate which regress alongside the primitive streak constitute the caudal stem zone and cells in this region give rise to neural progenitors, which are left behind by the zone and form the spinal cord[7,33] (Fig. 2). In the chick embryo, cells in the early caudal neural plate have a rough rostrocaudal order, with rostralmost cells leaving first and more caudally positioned cells giving rise to more caudal regions of the spinal cord.[7] Once caudal regression of the primitive streak is underway, neural precursors appear to be more tightly clustered around the anterior primitive streak and later still become integrated

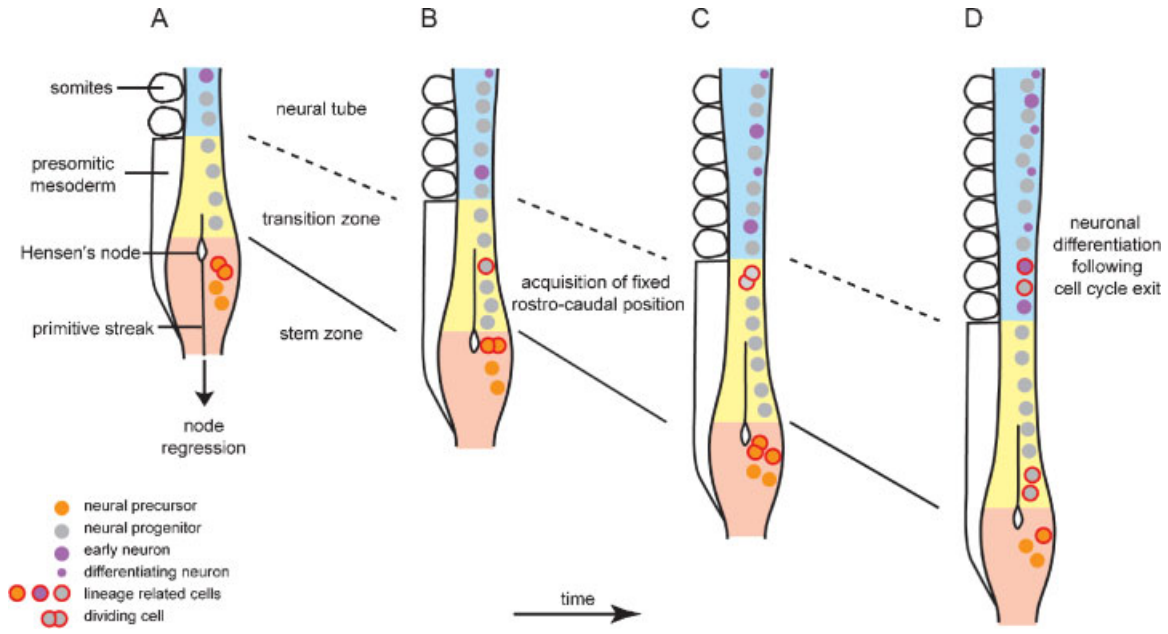

Figure 2. Progressive generation of the spinal cord by the stem zone. **A:** Neural precursor cells in the stem zone adjacent to primitive streak divide and **B:** can either leave the stem zone and become neural progenitors in the transition zone or remain resident in the stem zone. **C,D:** It is also possible that some sister cells born in the stem zone leave together. Once cells enter the transition zone they acquire a fixed rostrocaudal position and when somites form adjacent to the neural tube they can undergo neuronal differentiation. Progenitor cells may divide to produce two progenitors, two neurons or a neuron and a progenitor. Whether a cell remains a neural progenitor or differentiates is regulated by lateral inhibition (see text). This generalised scheme is based on work in chick and mouse.[7,33,34]

into the tailbud to give rise to the caudalmost spinal cord. Although, single cells in the caudal neural plate have yet to be shown to follow an asymmetric stem cell mode of division (which generates a resident neural stem cell and daughter neural progenitor cell that enters the differentiation pathway), S. Fraser's group have demonstrated that some cells are resident in the caudal neural plate, while others leave this region and it is therefore considered a stem zone[33] (see Fig. 2). Further, clonal analysis in the mouse embryo does support the existence of resident neural stem cells in a caudally regressing stem zone[34] and such cells may yet be discovered in the chick.

Some experiments have been carried out in the chick to examine the induction of the stem zone genes, *cash4* and *Sax1*, which are markers of this cell population during caudal regression.[35,36] These show that signals from the regressing anterior primitive streak promote expression of *cash4* and *Sax1* and that these signals can be mimicked by FGF.[23,36] However, as noted above, FGF induces mesoderm and neural tissue and a requirement for FGF signalling in the neuroepithelium for induction of stem zone genes has yet to be assessed. There is, however, growing evidence that FGF signalling is directly required for the maintenance of the stem zone. Forced expression of a dominant negative variant of FGF Receptor1 (FGFR1) induces precocious movement of cells out of the stem zone and into the neural tube where they are able to differentiate further[33] (see Fig. 2). FGFs can also maintain the expression of *cash4* and *Sax1*.[37,38] Furthermore, removal of the presomitic mesoderm (an important source of FGFs) underlying caudal neural tissue leads to loss of these stem zone markers.[39] As we discuss in the following section, FGF signalling not only maintains the integrity and character of the stem zone but it also represses neuronal differentiation and ventral patterning[37–39] and thus ensures the maintenance of an undifferentiated caudal precursor pool/stem zone able to give rise to the entire spinal cord.

Changing signalling properties of paraxial mesoderm regulate onset of neuronal differentiation and establishment of the ventral patterning system

Once cells leave the stem zone they enter a transition region in which a few cells are poised to differentiate (Fig. 2), but neuronal differentiation and ventral patterning (see below) only commence in the forming neural tube as it becomes flanked by somites[38–41] (Figs. 2, 3C–E). Here, expression of proneural genes (i.e. *Neurogenins* (*Ngn*)*1* and *2*) promotes neuronal differentiation and triggers the cell selection mechanism known as lateral inhibition, which ensures that not all cells differentiate into neurons at the same time.[42,43] As cells become neurons, they also acquire particular subtype identities and, in the ventral spinal cord, motor neurons and

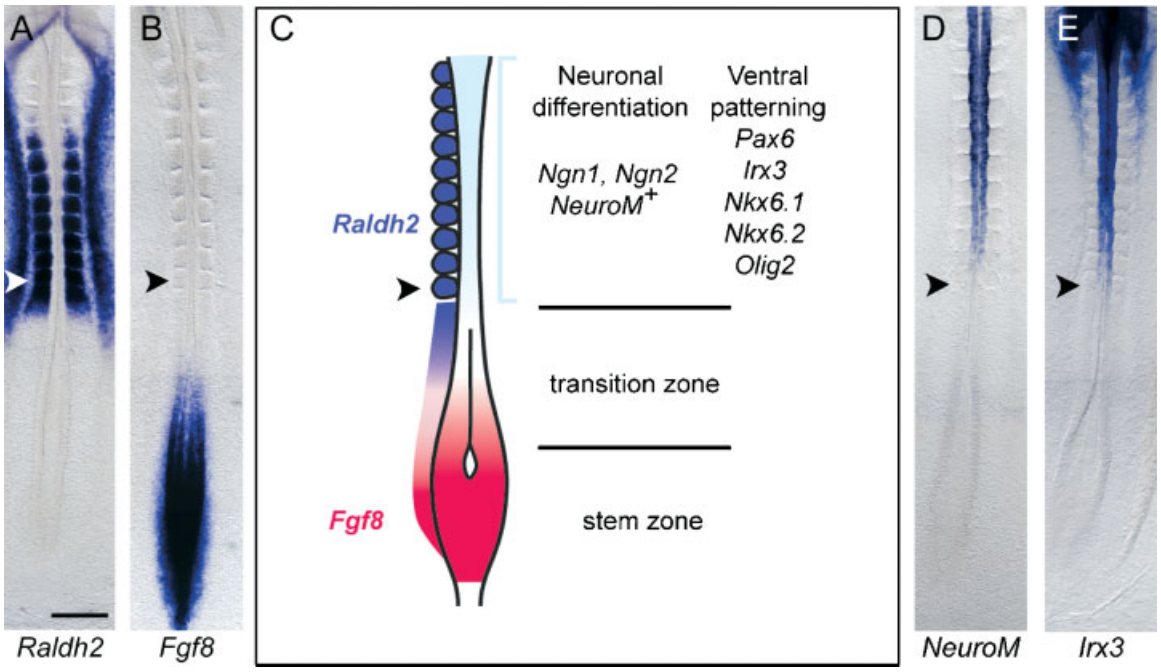

Figure 3. Expression patterns of key genes in the extending body axis. **A,B, D,E:** Expression of *Raldh2*, *Fgf8*, *NeuroM* and *cIrx3* in stage 10–13 somites embryos. Arrowhead, most-recent somite. Scale, 200μm. **C:** Rostrocaudal restriction of *Fgf8* and *Raldh2* in the paraxial mesoderm and expression of *Fgf8* and transcription factors involved in neuronal differentiation and ventral neural patterning.

different types of interneurons are specified in precise dorsoventral positions by expression of specific combinations of homeodomain and bHLH factors (reviewed in Ref. 8). Indeed, the neurogenic and ventral patterning programmes are linked by cross-regulatory interactions between these classes of genes (Fig. 4) (e.g. Refs. 44,45; reviewed in Ref. 43). The conjoint onset of these ventral patterning and neuronal differentiation genes in the spinal cord (e.g. from early somite stages, *Pax6*, *Irx3*, *Nkx6.1* and *Nkx6.2*; and later stages, *Olig2, Dbx1* and *Dbx2*[38,41,46,47]) (Fig. 3) further suggests that they are regulated by the same mechanism(s). This could involve either an activator provided by the somites or a caudal repressor and as is often the case in biology, the answer is a bit of both.

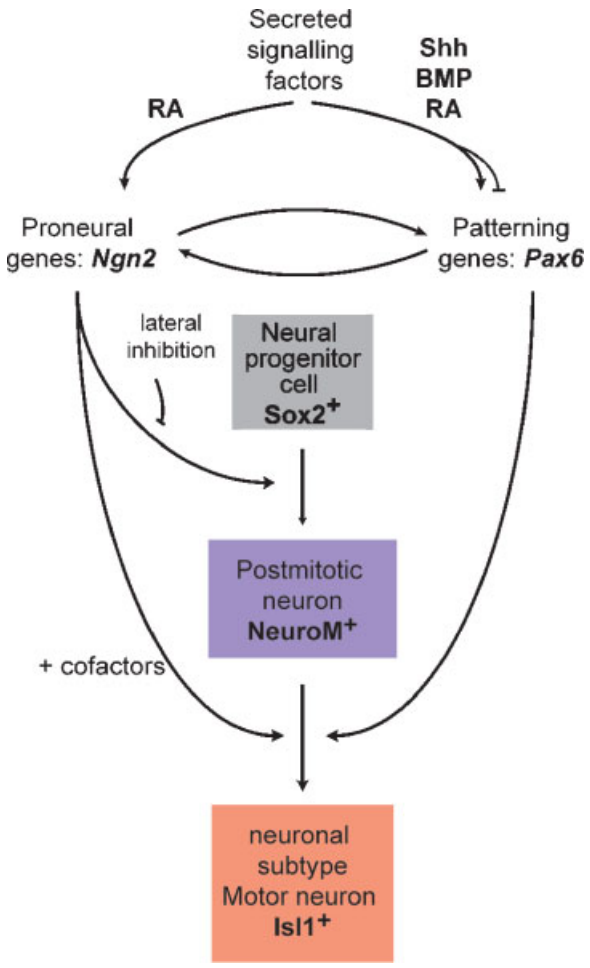

Figure 4. Regulatory relationships between neurogenic and patterning genes in the developing neural tube. Patterning genes and neuronal differentiation genes are expressed in restricted domains in response to extrinsic secreted factors (e.g. Shh, BMP and RA) in precise spatiotemporal domains. Both bHLH (e.g. Ngn2) and homeodomain-containing transcription factors (e.g. Pax6) have roles in promoting neuronal differentiation and neuronal subtype specification (e.g. motor neuron) (see Ref. 43).

A caudal repressor activity provided by the presomitic mesoderm was first described by F. Pituello's group, who showed that removal of presomitic mesoderm results in the precocious onset of the ventral patterning gene *Pax6*.[37] The signal responsible was identified as FGF, which is produced by presomitic mesoderm cells (Fig. 3B,C) and is able to repress *Pax6*. More recent findings have shown that repression by presomitic mesoderm and, in particular, by FGF is a general mechanism that represses neuronal differentiation (e.g. expression of the neuronal marker *NeuroM*) and the whole cohort of ventral patterning genes described above and consequently restrains differentiation at the caudal end of the developing spinal cord.[38,39] Conversely, upregulation of *Pax6* and *Irx3* is observed in stem zone explants and in the embryo following suppression of the FGF pathway (by treatment with SU5402 or electroporation with Dominant Negative (DN) FGFR1), indicating that FGF is required to repress *Pax6* and *Irx3*.[37,38] *NeuroM* expression, however, is not promoted by blockade of FGFR signalling in stem zone explants indicating that, as suggested above, downregulation of FGF is not the sole requirement for expression of these genes and that "activating" factors are also involved. Indeed, impairment of signalling between somitic tissue and neural tube (by insertion of a piece of membrane or removal of recently formed somites) results in a decrease in *Pax6*, *Irx3* and *NeuroM*,[37,39] indicating that a signal from the somite normally activates their expression. This is further confirmed by the ability of somitic tissue to induce *Pax6* and *NeuroM* in stem zone explants.[39,41]

The somite-derived activator appears to be retinoic acid. The production of this small signalling molecule is most likely catalysed by Raldh2, an enzyme present at somitic stages in rostral presomitic mesoderm and somites[48,49] but absent in more caudal regions (Figs. 1B, 3A,C). Treatment of stem zone explants with RA or a retinoic acid receptor (RAR) agonist induces the expression of the neuronal marker *NeuroM* whereas interference with the retinoid pathway (either by inhibiting aldehyde-dehydrogenases or with RAR/RXR antagonists) blocks the ability of somites to promote neuronal differentiation.[38] The requirement for retinoids in the CNS is also clear from the analysis of different experimental conditions where the retinoid pathway has been attenuated (e.g. Vitamin A (retinoid) Deficient (VAD) quails, *Raldh2*$^{-/-}$ mutant mice, forced expression in the chick of a dominant negative variant of *RAR* and of *Cyp26*, an enzyme that degrades RA).[38,47,50] VAD embryos have been well characterised with respect to their abnormal hindbrain patterning[51] and also display dramatically abnormal development of the spinal cord as indicated by reduced neural tube size, neuron number, expression of proneural (*Ngn1* and *Ngn2*) and ventral patterning transcription factors (*Olig2*, *Pax6*, *Irx3*, *Nkx6.2*).[38,50] Retinoid signalling is also required in neural explants for expression of

Dbx1 and *Dbx2*, two further transcription factors that pattern the ventral progenitor domains.[46,47] Changes in expression of these ventral genes have dramatic consequences, as they are involved with or required for specification of interneuron subtypes (V0, V1, V2) and motor neurons. Furthermore, as recently shown by T. Jessell's group, RA is additionally required for subsequent steps leading to motor neuron differentiation[47] and later, when Raldh2 and other Raldhs[52,53] are expressed within spinal cord itself, it also mediates specification of motor neuron subtypes.[54,55]

So, while FGF provided by presomitic mesoderm and present in the stem zone itself represses neuronal differentiation and establishment of the ventral patterning system, RA provided by somites promotes these steps (Fig. 5A,C). FGF and RA have been shown to have these opposite actions on neuronal differentiation in many different contexts both in vivo and in vitro (e.g. FGF;[56–59] RA[26,60–63]), however, the forming spinal cord is the first developmental context in which these signalling pathways have been shown to have opposing actions on the same cell population.

Mutual inhibition between FGF and RA pathways controls differentiation and segmentation during body axis extension

It turns out that these opposite activities of FGF and RA are due in part to mutual inhibition between these pathways (Fig. 5A,C). While caudally supplied FGF8 represses *Raldh2* expression and hence RA synthesis in the paraxial mesoderm, RA attenuates *Fgf8* levels in both the stem zone and presomitic mesoderm[38]—this may involve either or both repression of *Fgf8* transcription or acceleration of *Fgf8* message decay.[64] This mutual inhibition controls the speed of a caudalward travelling wave of *Raldh2* expression and a complementary decline in *Fgf8* levels, instigated by the caudal movement of the primitive streak, a likely source of *Fgf8*-inducing signals (Fig. 5A,C). Not only are these regulatory relationships observed in chick explanted tissues but, in vitamin A-deficient embryos, the *Fgf8* domain is expanded rostrally in both paraxial mesoderm and caudal neural tissue indicating its slowed downregulation in the absence of RA.[38] Conversely, *Fgf8* transcripts are absent in mice exposed to excess RA due to lack of the RA degrading enzyme *Cyp26*, which is normally expressed in caudal regions.[65]

Importantly, the ability of RA to promote neuronal differentiation involves more than just downregulation of *Fgf8*; as blocking FGF signalling is not sufficient to induce neurons in stem zone explants. Furthermore, neuronal differentiation and many ventral patterning genes fail to be expressed in retinoid-deficient spinal cord long after ectopic FGF has declined.[38] Conversely, FGF does not just block neurogenesis by repressing *Raldh2* in the mesoderm, but can also inhibit neuronal differentiation and ventral patterning genes in isolated neural tube.[38,47] So, RA and FGF pathways do not only mutually interfere with each other's signal production, but they also have opposite activities within the neuroepithelium.

Levels of FGF signalling are also crucial for the process of somitogenesis itself. A fall in FGF below a threshold in the presomitic mesoderm defines the "determination wavefront" that positions the future somite boundary.[66,67] The maintenance of high FGF signalling blocks segment formation and its downregulation is therefore essential for the development of the embryo. Although it is assumed that *Fgf8* downregulation is due to the caudal movement of the streak, the critical importance of this decline in FGF signalling for somite production suggests that this should be a more tightly regulated event. The ability of RA to attenuate *Fgf8* in the presomitic mesoderm (and FGF8 to repress *Raldh2*) thus provides a mechanism that may facilitate a discrete drop in FGF signalling in the rostral presomitic mesoderm and importantly links this event to the maturation of the mesoderm. In support of this role for RA signalling in regulating somite size, VAD animals not only have an expanded *Fgf8* domain in the presomitic mesoderm, but also have smaller somites.[38] This reduction in somite size is consistent with a model in which excess FGF leads to fewer cells falling below a threshold of FGF signalling within the period of one oscillation of the segmentation clock.[66] This action of RA on somite boundary position has also just been confirmed in frogs, where RA attenuates FGF signalling by promoting expression of a MAPK phosphatase in the presomitic mesoderm and where conversely FGF is required for expression of *Cyp26* in the caudal region.[68]

The opposition of FGF and RA signalling is a recurrent theme in cellular differentiation. Although clearly context dependent, RA is generally viewed as promoting differentiation while FGF elicits proliferation in primary and transformed cell cultures and in embryonic stem (ES) cells. FGFs act as mitogens in several types of neural progenitor cell in vitro (e.g.[57]) and in ES cells.[58,59] Conversely, RA promotes neural and neuronal differentiation in embryonic carcinoma cells (e.g. P19[60]) and ES cells.[62,63] Although it is not clear how these factors act to prevent/promote neuronal differentiation, several studies in carcinoma cell lines, where FGF and RA have these opposing activities indicate that these pathways can interfere with each other at various levels. For example, high levels of FGF4 characteristic of male germ-cell cancers are reduced in embryonic carcinoma lines exposed to RA.[69] In carcinoma cell lines, RA can also repress *FGFRs* and *FGF-binding protein*[70,71] and can induce a switch to a less-active *Fgf8* isoform, promoting the preferential binding of RARα to an RARE in the *Fgf8* promoter.[72] In turn, activation of Erk/MAPK (a pathway stimulated by FGF signalling) inhibits RA activity in NIH3T3 cells.[73] In vivo, FGF also inhibits *Raldh2* and *RARβ* in the extending limb bud,[74] which FGF signalling from the isthmus opposes the activity of RA in the anterior hindbrain[75] and this may help preserve

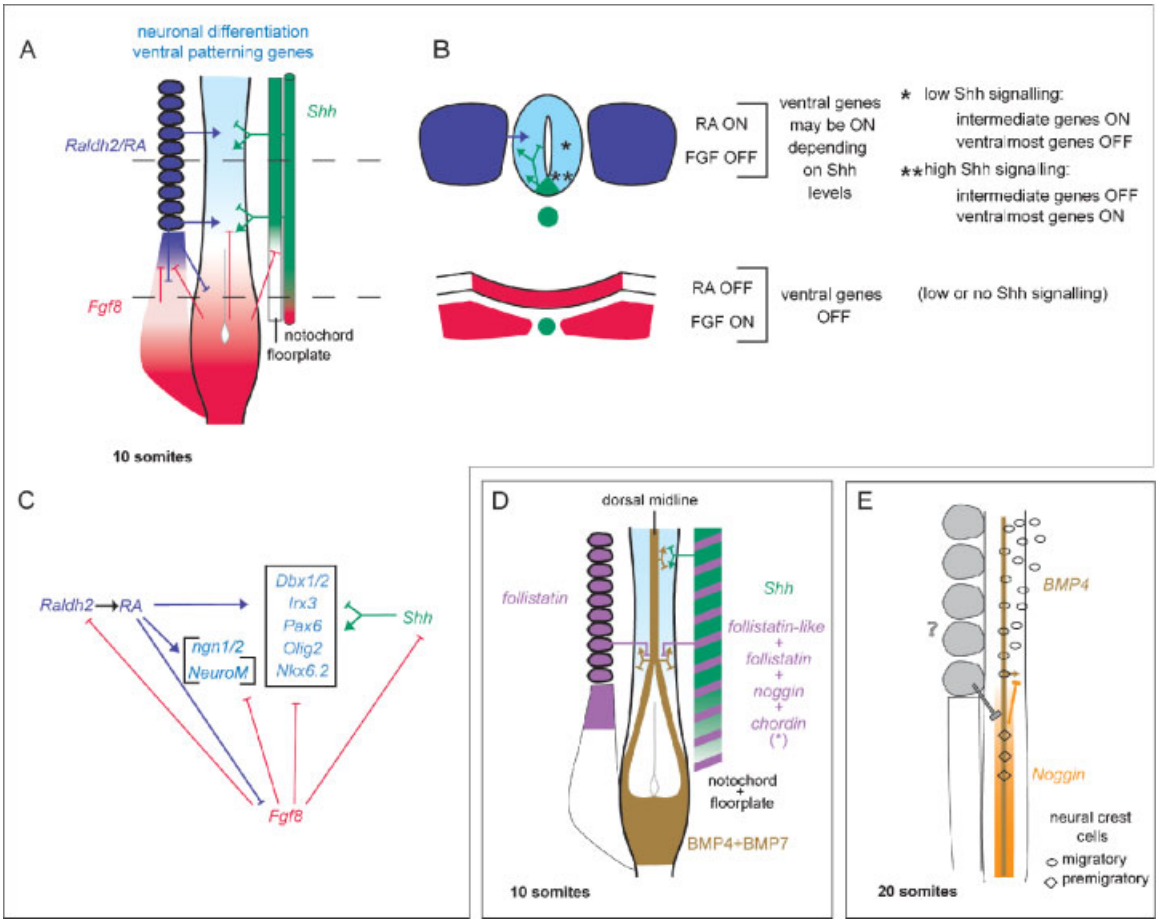

Figure 5. Somite signalling and the integration of pathways regulating maturation in the forming neural tube. **A:** Neuronal differentiation and ventral patterning genes (light blue) are regulated by signals from the paraxial (left) and axial (right) mesoderm. FGF from the caudal neural plate/stem zone and caudal paraxial mesoderm represses neuronal differentiation and most ventral neural genes. Retinoic acid, synthesised by Raldh2 in the somites is required for the expression of some of these genes (see text). Shh from the notochord/floorplate activates or represses ventral patterning genes in a concentration-dependent manner. These three signalling pathways interact with each other at different levels; specifically FGF represses both *Raldh2* in paraxial mesoderm and *Shh* in the floorplate and RA attenuates *Fgf8* in presomitic mesoderm and in caudal stem zone. Downregulation of FGF and upregulation of RA drives the progressive activation of patterning and differentiation genes within a ventral domain that is defined by Shh and BMP (not shown) signalling. **B:** Cross sections at the level of the somites and the presomitic mesoderm. Ventral gene expression is repressed when FGF signalling is on, even in the presence of low Shh that might allow expression of intermediate genes. At somitic levels, where RA is present and FGF signalling has ceased, ventral gene expression is possible and is restricted along the dorsoventral axis depending on the levels of Shh signalling. **C:** Gene regulatory network controlling the onset of neurogenesis and ventral patterning in the extending spinal cord. **D:** BMP from the stem zone and the dorsal neural tube opposes Shh activity and also regulates gene expression in the neural tube. Other secreted factors (BMP antagonists) expressed by paraxial and axial mesoderm modulate BMP signalling from the stem zone and the dorsal neural tube. *: *for a precise description of expression of each BMP antagonists at the ventral midline see*.[85] **E:** BMP signalling promotes neural crest cell migration but is antagonised caudally by *Noggin* present in the dorsal transition zone. An unknown somite-derived signal represses *Noggin* and thereby regulates the progressive onset of neural crest migration in the neural tube.[91] Activating and repressive arrows are deduced from changes in gene expression following addition or removal of the signalling factors. They do not represent direct gene regulation.

rhombomere1 as a highly proliferate region which gives rise to cerebellum. The opposition of FGF and RA pathways thus appears to be a fundamental and conserved mechanism for regulating differentiation.

An 'opposing signal' model for colinear expression of Hox genes

A current idea is that continued FGF signalling in the stem zone not only keeps cells undifferentiated but allows them to

respond to further caudalising signals.[33,76] Some support for this proposal comes from recent analysis of the progressive onset of Hox genes, which are expressed in the paraxial mesoderm and the caudal hindbrain and spinal cord and act to confer positional identity in the rostrocaudal axis (reviewed in[77] and see[78]). These transcription factors are organised into clusters on four chromosomes (*Hoxa–Hoxd*) and 3′ genes are expressed first and in the rostral CNS while more 5′ genes appear progressively later in caudal regions as they form; a phenomenon known as colinearity (reviewed by[79]). In many contexts, exposure to RA has been shown to be required for expression of 3′ Hox genes, such as *Hoxb4* in the developing hindbrain[10] and *Hoxc5* in the cervical spinal cord,[12] while expression of more 5′ Hox genes in the spinal cord requires FGF.[12,80] However, depending on context, some Hox genes can be induced by FGF and RA in the developing CNS (e.g.,[12,20,31,32] Fig. 1A, Table 1), indicating more complex patterns of regulation. Indeed, during extension of the body axis, which involves onset of progressively more 5′ Hox genes (e.g. *Hoxc6–Hoxc10*) FGF and RA appear to have distinct roles in *Hox* gene regulation. Onset of Hox genes c6–10 in newly generated chick spinal cord requires FGF signalling and exposure of such explants to increasing FGF concentrations leads to expression of progressively more 5′ genes.[12] This might relate to the apparent increase in caudal *Fgf8* levels as development proceeds or may reflect a longer period of exposure to FGF experienced by neural precursors that remain in the stem zone.[12] Further, exposing the early chick embryo to FGF leads to rostral expansion of 5′ *Hox* gene domains and this has suggested a model for progressive onset of 5′ *Hox* genes under the influence of caudal FGF.[32] Our recent finding that RA provided by somites attenuates caudal FGF signalling thus suggests a further role for RA in the regulation of Hox gene expression, as exposure to retinoids may prevent the expression of further 5′ *Hox* genes in cells leaving the stem/transition zone. Indeed, exposure to somitic mesoderm (RA source) inhibits onset of *Hoxd10*[13] and RA also blocks onset of 5′ *Hox* genes in transition zone explants.[12] Thus, the combination of Hox genes expressed by nascent spinal cord may be set as cells experience RA and consequently lose the influence of FGF in the extending axis (Fig. 6). Further, as noted above, the ability of FGF to repress *Raldh2* helps to protect stem zone cells from RA, thereby allowing expression of progressively more 5′ *Hox* genes in this cell population.

This new 'opposing signal' model for the colinear activation of caudal *Hox* genes in the forming CNS is also consistent with observations of *Hox* gene regulation in the emerging paraxial mesoderm. Here initial expression of *Hox* genes in the primitive streak under the influence of FGF is followed by a later step that fixes the Hox code as somitogenesis takes place.[66,81] However, the rostral limits of *Hox* gene expression in the CNS differ from those in the paraxial mesoderm, where the assignment of the Hox code is also linked to the segmentation clock[79,82] suggesting some differences in the mechanisms operating in these tissues.

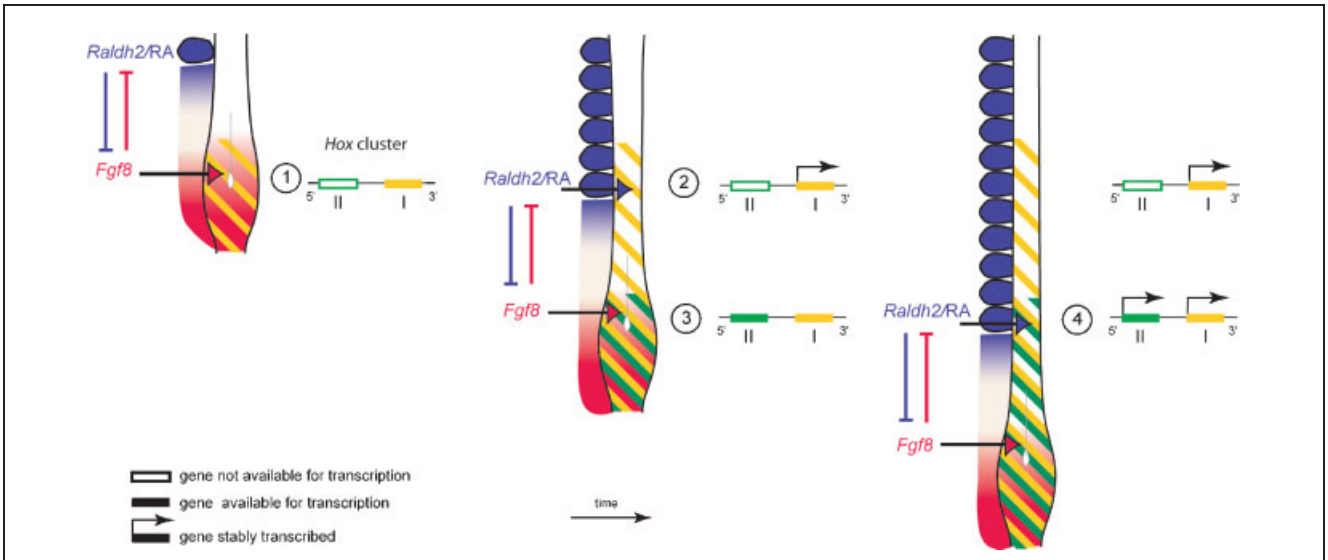

Figure 6. Opposing signal model for colinear expression of *Hox* genes. Progressive onset of 5′ *Hox* genes during caudal extension of the body axis: (step 1) *Hox* gene I (yellow box) becomes available under the influence of FGF signalling; (step 2) some cells expressing *Hox* gene I leave the stem zone, FGF signalling is attenuated by RA and so no further 5′ *Hox* genes become available. RA now promotes stable transcription of the available *Hox* gene I; (step 3) FGF promotes availability of the next 5′ *Hox* gene, II (green box) and (step 4) when such cells leave the stem zone and encounter RA both Hox I and II genes will be stably transcribed. (A later step where some caudal Hox genes can repress more rostral ones has not been included.[78])

In addition to attenuating FGF, RA may also stabilise/activate *Hox* gene expression, as retinoid receptors form complexes with chromatin-remodelling enzymes and liganded RA receptors bind co-factors that in turn recruit proteins with histone acetyltransferase (HAT) activity. These enzymes promote a relaxed chromatin conformation that facilitates transcriptional activity (reviewed by[83]). So, while cells in the caudal stem zone experience FGF and thereby express progressively more 5′ *Hox* genes, RA attenuates FGF just rostral to the stem zone and may thereby locally restrict the Hox code and may then also activate stable transcription of the available subset of *Hox* genes (Fig. 6).

A somite-mediated signalling switch regulates neural tube maturation

The transition from an FGF to an RA environment in the extending body axis thus appears to constitute a switch that promotes differentiation and patterning at the level of the forming somites. Here, cells now also encounter further signals that modulate dorsoventral patterning, a process that is regulated by ventrally supplied Sonic hedgehog (Shh) and dorsally produced BMP signalling (reviewed in[8]). As the somites form, *Shh* appears in the floorplate of the neural tube (in addition to the notochord) (Fig. 5A–C) and BMP antagonists (*follistatin*, *follistatin-like*, *chordin* and *noggin*), which sensitise neural cells to Shh signalling, are produced by somites and/or notochord (Fig. 5D).[84–86]

A key question then is to understand how FGF and RA interact with other signalling pathways either side of this switch point and how they are integrated within cells to control a common cohort of target genes that mediate dorsoventral patterning and neuronal differentiation. Recent advances have provided some insight into how FGF and Shh pathways may interact in this context (but see Ref. 87). Ventral patterning genes have been classed into two groups depending on their response to high Shh levels: class II (i.e *Nkx2.2* and *Nkx6.1* and *Nkx6.2*) are activated and therefore expressed ventrally, while class I genes (i.e. *Pax6*, *Irx3*, *Dbx*) are repressed and are thus expressed in the intermediate region of the neural tube (Fig. 5B).[88] This pattern of regulation makes it difficult to see how FGF repression of both Class I and II genes could be achieved by simply interfering with Shh signalling. However, Class I genes, such as *Irx3* and *Pax6*, can be upregulated by low-level Shh[63,89] and so FGF could act on both classes of genes by blocking the Shh pathway. Interestingly, *Shh* expression in the floorplate is also repressed by high levels of FGF[38] and this might explain why *Shh* and consequently its target genes are not present at more caudal levels in the embryo. However, FGF does not simply act by reducing *Shh* transcription as it represses *Irx3* expression even in explants that have been exposed to Shh protein, but do not contain *Shh* transcripts.[38] Similarly, mis-expression of a DNFGFR1 construct also leads to local upregulation of *Irx3* further supporting the idea that FGF can repress *Irx3* independently of effects on *Shh* transcription.[38] One mechanism that integrates FGF and Shh signalling may be regulation of common intracellular components (e.g. *Gli* genes[90]) that control ventral gene expression. *Shh* transcription can depend on Shh transduction[86] so FGF interference with downstream components of Shh signalling might also explain the repression of *Shh* by FGF. Alternatively, inputs from the Shh and FGF signalling pathways could act in parallel, regulating distinct transcriptional activators and/or repressors that bind to regulatory regions near each target gene.

The complex gene regulatory network that governs ventral patterning is not yet completely elucidated but it is tempting to propose that regulation by caudal FGF ensures the establishment of the correct combinatorial code of ventral patterning genes that underlies cell type specification. This idea springs from the observation that genes expressed in response to low Shh concentrations (i.e. *Pax6*, *Irx3*) are strongly repressed by FGF signalling, while those that require high-level Shh (i.e. *Nkx2.2*, *Nkx6.1*) are less affected.[38] FGF signalling may therefore prevent the expression of genes such as *Pax6* and *Irx3* in regions with initially low *Shh* signalling such as the ventral midline in caudal regions (Fig. 5B) where they may interfere with the later activation and/or function of genes such as *Nkx2.2*, *Nkx6.1*. and thereby alter cell type specification.[88]

As discussed above, while FGF represses Class I Shh-responsive genes, RA, conversely promotes their expression. Retinoic acid binds to RAR/RXRs which function as transcriptional activators and are expressed homogenously within the neural tube.[38] In principle, these receptors could drive expression of Class I patterning genes throughout the dorsoventral axis. Transcriptional repressors induced in response to high Shh signalling might then restrict expression of Class I to a particular domain. In this way, Shh signalling could pattern the response of neuroepithelial cells to systemic RA.

Concluding remarks

Opposition of FGF and RA pathways is emerging as a pivotal event that may act to integrate dorsoventral and rostrocaudal patterning systems within the developing spinal cord and to coordinate them with the differentiation of neural progenitors. This ensures the generation of the correct number of neurons with specific subtype identities. An unidentified somite signal has also been shown at later stages to promote neural crest migration by downregulating *Noggin* transcription at the dorsal midline[91,92] (Fig. 5E) and it will be interesting to assess whether retinoic acid plays a role in this step too. Signalling from the paraxial mesoderm to the neuroepithelium also serves to coordinate the differentiation of these two tissues and may even help match neurons to their eventual target tissues (see[78]). Retinoic acid provided by the mesoderm also patterns the developing gut[93] and so might orchestrate

differentiation of all three germ layers as they are laid down in the extending body axis. Key future experiments should identify how FGF and RA pathways interact and how they generate opposing outcomes as well as how other signalling pathways collude to control this differentiation switch. Finally, it is interesting to speculate that this opposing signal mechanism may have been conserved during evolution and might therefore also operate in invertebrates such as short-germ-band insects and spiders in which the body axis is generated sequentially.[94,95]

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