Specification and maintenance of the spinal cord stem zone

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Summary

Epiblast cells adjacent to the regressing primitive streak behave as a stem zone that progressively generates the entire spinal cord and also contributes to paraxial mesoderm. Despite this fundamental task, this cell population is poorly characterised, and the tissue interactions and signalling pathways that specify this unique region are unknown. Fibroblast growth factor (FGF) is implicated but it is unclear whether it is sufficient and/or directly required for stem zone specification. It is also not understood how establishment of the stem zone relates to the acquisition of spinal cord identity as indicated by expression of caudal Hox genes.

Here, we show that many cells in the chick stem zone express both early neural and mesodermal genes; however, stem zone-specific gene expression can be induced by signals from underlying paraxial mesoderm without concomitant induction of an ambivalent neural/mesodermal cell state. The stem zone is a site of FGF/MAPK signalling and we show that although FGF alone does not mimic paraxial mesoderm signals, it is directly required in epiblast cells for stem zone specification and maintenance. We further demonstrate that caudal Hox gene expression in the stem zone also depends on FGF and that neither stem zone specification nor caudal Hox gene onset requires retinoid signalling. These findings thus support a two step model for spinal cord generation – FGF-dependent establishment of the stem zone in which progressively more caudal Hox genes are expressed, followed by the retinoid-dependent assignment of spinal cord identity.

Key words: Stem zone, Stem cells, Spinal cord, FGF, MAPK, Hox genes, Chick

Introduction

The stem zone is a unique cell population set aside in the caudal part of the neural plate. These epiblast cells proliferate and re-arrange adjacent to the regressing primitive streak leaving behind progenitors that then generate the entire spinal cord (Brown and Storey, 2000; Schoenwolf, 1992) (reviewed by Diez del Corral and Storey, 2004). Cell labelling studies in the mouse suggest that self-renewing neural stem cells reside in this spinal cord primordium and divide at intervals to generate neural progenitors (Forlani et al., 2003; Mathis and Nicolas, 2000); such stem cells also exist in the chick embryo (Mathis et al., 2001) (S. E. Fraser, personal communication). There is evidence in frog, fish, chick and mouse embryos that this caudal-most part of the neural plate also contains cells that contribute to mesoderm and this mesoderm-forming potential persists in the neural/epiblast cells close to the anterior primitive streak through to tailbud stages (Brown and Storey, 2000; Cambray and Wilson, 2002; Davis and Kirschner, 2000; Kanki and Ho, 1997; Knezevic et al., 1998; Shih and Fraser, 1995). In higher vertebrates and in frogs, it remains unclear whether this indicates the presence of resident multipotent stem cells that can contribute to both neural and mesodermal layers, or whether different, but closely associated, cells in this region give rise to these different lineages as observed in zebrafish (Kanki and Ho, 1997; Shih and Fraser, 1995).

In chick and mouse, the stem zone first becomes molecularly distinct just prior to somitogenesis, when it expresses several transcription factors that distinguish it from the rest of the neural plate. These genes include the homeodomain-containing factor, *Sax1* (Schubert et al., 1995; Spann et al., 1994) (see Diez del Corral et al., 2002), and in the chick, the proneural gene homologue, *cash4* (Henrique et al., 1997). Epiblast cells close to the primitive streak express *Fgf8* (Crossley and Martin, 1995) and *bra* (Kispert et al., 1995; Kispert and Herrmann, 1994) and once caudal regression of the primitive streak is under way, expression of these genes spreads laterally into the morphologically defined open neural plate in both chick and mouse (Kispert and Herrmann, 1994; Kispert et al., 1995; Schmidt et al., 1997). This suggests that some cells in this region of the neuroepithelium co-express early mesodermal and neural genes.

Signals from the regressing node can induce both *cash4* and *Sax1* in the chick (Henrique et al., 1997) however, ablation of the node does not result in loss of *Sax1* expression (Spann et al., 1994), suggesting that other tissues share this property. At later stages, studies show that the paraxial mesoderm beneath the established stem zone is indeed required for maintenance of *cash4* and *Sax1* in the embryo (Diez del Corral et al., 2002). There is some evidence that FGF signalling accounts for this maintenance signal from the mesoderm. The anterior primitive streak expresses *Fgf2*, *Fgf3*, *Fgf4*, *Fgf8*, *Fgf12*, *Fgf13* and *Fgf18* (Boettger et al., 1999; Crossley and Martin, 1995;

Karabagli et al., 2002; Mahmood et al., 1995; Ohuchi et al., 2000; Riese et al., 1995; Shamim and Mason, 1999), and most of these factors persist in the regressing streak and are present in the stem zone itself (*Fgf2*, *Fgf3*, *Fgf8*, *Fgf18*) while *Fgf8*, *Fgf10* and *Fgf18* (Karabagli et al., 2002; Ohuchi et al., 1997) are also expressed by paraxial mesoderm. Furthermore, FGF4 or FGF8 can locally ectopically maintain expression of *cash4* and *Sax1* as the spinal cord develops (Bertrand et al., 2000; Diez del Corral et al., 2002). However, it is not known whether FGF acts directly on epiblast cells to specify or maintain the stem zone.

FGF signalling has long been implicated in the generation of the vertebrate body as disruption of this pathway results in failure to form this part of the embryo (e.g. Amaya et al., 1991; Draper et al., 2003; Griffin et al., 1995; Xu et al., 1999). The primary role of FGF signalling in mesoderm induction has made it difficult to assess its direct requirement for induction of tissue that depends on mesoderm derived signals (reviewed by Bottcher and Niehrs, 2005). However, this pathway has been shown to initiate neural development in the chick embryo, in mouse ES cells and most recently in the frog embryo (Delaune et al., 2005; Streit et al., 2000; Wilson et al., 2000; Ying et al., 2003) (reviewed by Stern, 2005). MAPK activation downstream of FGF signalling is implicated in this step in the chick (Eblaghie et al., 2003) and in the frog acts at least in part by interfering with BMP signal transduction by inactivating the BMP intermediary protein Smad1 (Delaune et al., 2005; Pera et al., 2003). MAPK signalling is also required for mesoderm induction (Saba-El-Leil et al., 2003; Umbhauer et al., 1995; Yao et al., 2003) and recent data suggest that low level FGF/MAPK may initiate neural development, while higher levels promote mesoderm formation (Delaune et al., 2005). Together, these studies indicate that serial FGF/MAPK mediated events may underpin stem zone formation and raise the possibility that prolonged exposure to such signalling is involved in specification and/or maintenance of this cell population.

FGF signalling not only mediates cell fate specification in the early embryo but also maintains an undifferentiated cell state in many cellular contexts (reviewed by Diez del Corral and Storey, 2004). During body axis extension, exposure to FGF inhibits neuronal differentiation (Diez del Corral et al., 2002) and onset of ventral patterning genes (Bertrand et al., 2000; Diez del Corral et al., 2003; Novitch et al., 2003). Furthermore, blocking FGF signalling also accelerates movement of cells out of the stem zone into the transition zone, which eventually forms the neural tube where neuronal differentiation commences (Mathis et al., 2001). These findings indicate a role for FGF signals in keeping cells in an undifferentiated, proliferative cell state and within the stem zone. Importantly, the maintenance of this undifferentiated state may prolong the period during which cells are able to respond to caudalising signals (Mathis et al., 2001; Vasiliauskas and Stern, 2001) and may thereby account for the expression of progressively more caudal Hox genes in the stem zone (Liu et al., 2001). These genes determine rostrocaudal character in the emerging body axis (reviewed by Deschamps et al., 1999), so, for example, *Hoxb8* expression identifies the spinal cord and is expressed in the neural tube caudal to somite 5 (Muhr et al., 1999). Interestingly, depending on context, many caudal Hox genes, including *Hoxb8* are induced by FGF or retinoic acid (RA) signalling (Bel-Vialar et al., 2002; Liu et al., 2001; Muhr et al., 1999; Oosterveen et al., 2003). However, recent work shows that FGF and retinoid signalling are mutually inhibitory in the extending body axis (Diez del Corral et al., 2003), and raises the possibility that initiation of caudal Hox gene expression in the stem zone under the influence of FGF switches to a dependency on somite-derived retinoids in differentiating tissues (reviewed by Diez del Corral and Storey, 2004).

Here, we use a panel of neural, mesodermal and stem zone-specific marker genes to characterise the stem zone region. In vitro explant assays are used to identify tissues that specify this cell group and to assess whether stem zone specific gene expression can be induced independently of mesodermal gene expression. We further test whether FGF signalling is sufficient and/or directly required for specification and maintenance of this cell population. By assessing whether onset of stem zone and caudal Hox gene expression depends on the retinoid pathway, we also distinguish between the molecular mechanism underlying stem zone specification and maintenance, and that which assigns distinct rostrocaudal identities along the length of the spinal cord.

Materials and methods

Whole-mount in situ hybridisation

This was carried out using standard techniques (Wilkinson and Nieto, 1993) and a subset of embryos were cryosectioned at 15 μm.

Whole embryo treatment with inhibitors

Chick embryos of appropriate stages were placed on 1.2 μm Millipore filters (RTTP01300) in Opti.MEM (Gibco) supplemented with 5% calf serum ±SU5402 (Calbiochem) or PD184352 (a gift from P. Cohen, MRC PPU), and cultured in separate wells (NUNC 4 plates) for 3 hours in CO_2 (5%) at 38°C.

FGF beads

Heparin-coated beads soaked in 50 μg/ml murine FGF8B (R&D Systems) were grafted in New culture as described previously (Storey et al., 1998).

In vitro explant culture

Explants of epiblast or paraxial mesoderm from stages HH3-8 were cultured using standard techniques (Placzek and Dale, 1999). Two explants were taken from each embryo and comparisons were made between explant pairs (except where stated otherwise) following exposure of one of the pair to any of the following: mouse FGF8B, human FGF4 (R&D Systems) or SU5402 (concentrations in text); control explants were cultured in DMSO only. Explants were processed as described previously (Diez del Corral et al., 2002).

In vivo electroporation of dominant negative FGFR construct

A chick dnFGFR1 construct in which the truncated receptor (amino acids 1-425) is fused to *eYFP* in a Clontech vector (pEYFP-N1) (provided by C. J. Weijer) (Yang et al., 2002) or a control empty vector (pEYFP-N1) were introduced by standard techniques for in ovo electroporation or using a custom made chamber for transfection of embryos in EC culture (a gift from I. Mason, KCL, London) using an IntraCell T160 or a BTX ECM 830 pulse generator.

Vitamin A-deficient quail embryos

Vitamin A-deficient quail embryos were provided by E. Gale and M. Maden (KCL, London), and generated as described previously (Dersch and Zile, 1993).

Results

Cells in the stem zone express both early neural and mesodermal genes

To understand the context in which the stem zone is established, we characterised the expression profile of key marker genes in epiblast cells adjacent to the anterior primitive streak over a range of developmental stages (Fig. 1). Although the expression patterns of these genes have been described elsewhere, their presence in this cell population has not been systematically compared. At early primitive streak stages [Hamburger and Hamilton (HH) stage 3-3+] the pan neural gene *Sox3* is expressed in neural plate cells and overlaps medially with the early mesodermal marker gene *Brachyury* (*bra*), which is detected in the primitive streak and adjacent epiblast cells (Fig. 1A,A′,C,C′). In comparison with *bra*, *Fgf8* expression is more tightly restricted to the primitive streak at this stage (Fig. 1D,D′). By HH4, the neural plate has narrowed and epiblast cells located adjacent to the anterior primitive streak now express *Sox3, Sox2* and *bra* (Fig. 1E-H′). At this stage, *bra* expression in the epiblast is broadest below the node, where *Fgf8* transcripts are detected in the epiblast cells of the streak (Fig. 1H,H′).

Once the streak has begun to regress (HH6-7), *Sax1* expression begins and, at these stages, is a marker of the stem zone region of the neural plate (defined as the epiblast cell population that regresses caudally alongside the anterior primitive streak). Although the function of *Sax1* is not known, the restriction of this Nkx class transcription factor to open neural plate throughout axis extension suggests that it is indicative of the undifferentiated cell state characteristic of the stem zone. *Sax1* is first detected in epiblast cells close to the primitive streak in a domain that overlaps medially with that of *bra* (as well as *Sox2* and *Sox3*), while *Fgf8* transcripts (Fig. 1L,L′) are confined to streak epiblast at this time (compare Fig. 1I-L′ with M,M′). *Hoxb8* expression commences shortly after that of *Sax1*, and is initially located caudally in the primitive streak, but soon after appears in stem zone cells (Fig. 1N,N′). By HH8/9, *Sox3* expression has waned caudally, but *Sox2* is now strongly transcribed in epiblast cells adjacent to the anterior primitive streak, along with *bra*, *Fgf8*, *Sax1* and *Hoxb8* (Fig. 1O-T′). As development proceeds, *Sax1* and *Hoxb8* expression are maintained rostral to the regressing node in the forming transition zone, in which cells express only neural and stem zone genes (Fig. 1Y-Z′) [unlike *Sax1*, *Hoxb8* is additionally maintained in the differentiating neural tube and is therefore a persisting marker of spinal cord character (Muhr et al., 1999)]. Co-localisation of neural, mesodermal and stem

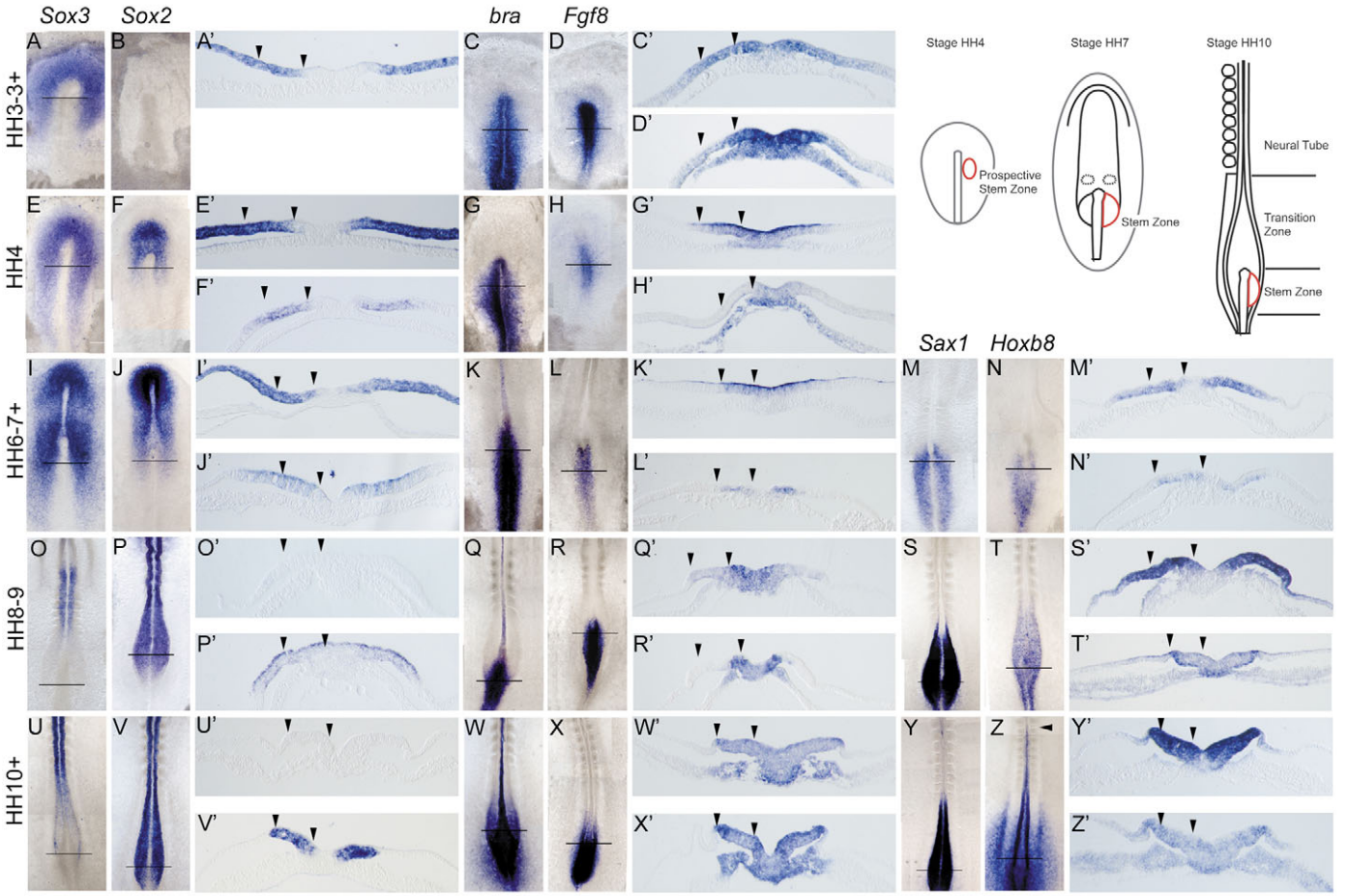

Fig. 1. Comparison of early neural, mesodermal and stem zone-specific gene expression adjacent to the primitive streak during body axis extension. Expression of *Sox3*, *Sox2*, *bra* and *Fgf8* at HH3-3+ (A-D′), HH4 (E-H′) and, additionally, *Sax1* and *Hoxb8* at HH6-7 (M-N′), HH8+-9 (S-T′) and HH10+ (U-Z′). Levels of transverse sections are indicated with a black bar and epiblast adjacent to the primitive streak lies between arrowheads. Arrowhead in Z indicates somite 5. Diagrams show the positions of the stem zone from HH stages 4-10.

zone genes (*Sox2*, *bra*, *Fgf8*, *Hoxb8* and *Sax1*), however, persists in the stem zone and in the margins of the anterior primitive streak at HH10+ (Fig. 1V-Z).

These gene expression patterns identify an enduring region of overlap between neural and mesodermal genes in the epiblast adjacent to the anterior primitive streak, which suggests that cells in this position have the potential to form mesodermal as well as neural tissue. The onset of *Sax1* in epiblast flanking the streak at HH6-7 also raises the possibility that creation of an ambivalent neural/mesodermal cell state is a prerequisite for stem zone specification.

The spinal cord stem zone is specified by late primitive streak stages

To determine when the stem zone is specified, explants of HH3 epiblast [3c explants (Muhr et al., 1999); Fig. 2A] were cultured in isolation and screened for expression of genes characteristic of the stem zone. *Sax1* and *Hoxb8* are barely detected in 3c explants after 24 hours and then in only a few cases (3/25; 2/10; respectively; Fig. 2B,C). However, the early mesodermal gene *bra* (8/13) and the pan neural markers *Sox2* and *Sox3* (11/15, 7/7 respectively) are detected in 3c explants after 18-24 hours (Fig. 2D,E; data not shown). This indicates that 3c epiblast has early neural and mesodermal cell characteristics, but has yet to receive signals that specify the stem zone. We therefore next assessed explants of epiblast from the same position (adjacent to the anterior primitive streak) at a later stage, HH4 (Fig. 2F). These 4c explants express *Sax1* (11/18) and *Hoxb8* (13/16) (Fig. 2G,H) within 24 hours, indicating that signals that specify the stem zone have been received by the late primitive streak stage.

Expression of stem zone specific genes is confined to neural plate also expressing early mesodermal genes

Importantly, like 3c epiblast, 4c explants express both the early mesodermal marker gene *bra* (9/10 cases) and the neural marker *Sox2* (12/12) (Fig. 2I,J). A previous study has reported that explants of epiblast adjacent to the primitive streak do not express *bra* (Muhr et al., 1999). This might be explained if explants in these two studies were taken from slightly different regions. To address this possibility, we assessed marker gene expression after 24 hours culture in HH4 epiblast explants taken either adjacent to the primitive streak explant (4a) or lateral to this region (4b) (Fig. 2K). Expression of all marker genes is detected in 4a explants (*Sax1*, 7/13; *Hoxb8*, 8/11; *bra*, 8/8; *Sox2*, 6/7) (Fig. 4L-O), but only the pan-neural gene *Sox2* is consistently expressed in 4b explants (*Sax1*, 2/32; *Hoxb8* 0/10; *bra*, 3/30; *Sox2*, 23/26) (Fig. 4P-S). This may explain the difference between our findings and those of Muhr et al. (Muhr et al., 1999), and indicates that markers of the stem zone are detected only in epiblast that expresses early mesodermal and neural genes, and/or that inclusion of prospective paraxial mesoderm (as indicated by *bra* expression) is required for *Sax1* induction.

Signals from caudal paraxial mesoderm specify the stem zone, but do not induce an ambivalent cell state

To assess whether the paraxial mesoderm is a source of signals that specify the stem zone, we combined 3c explants with

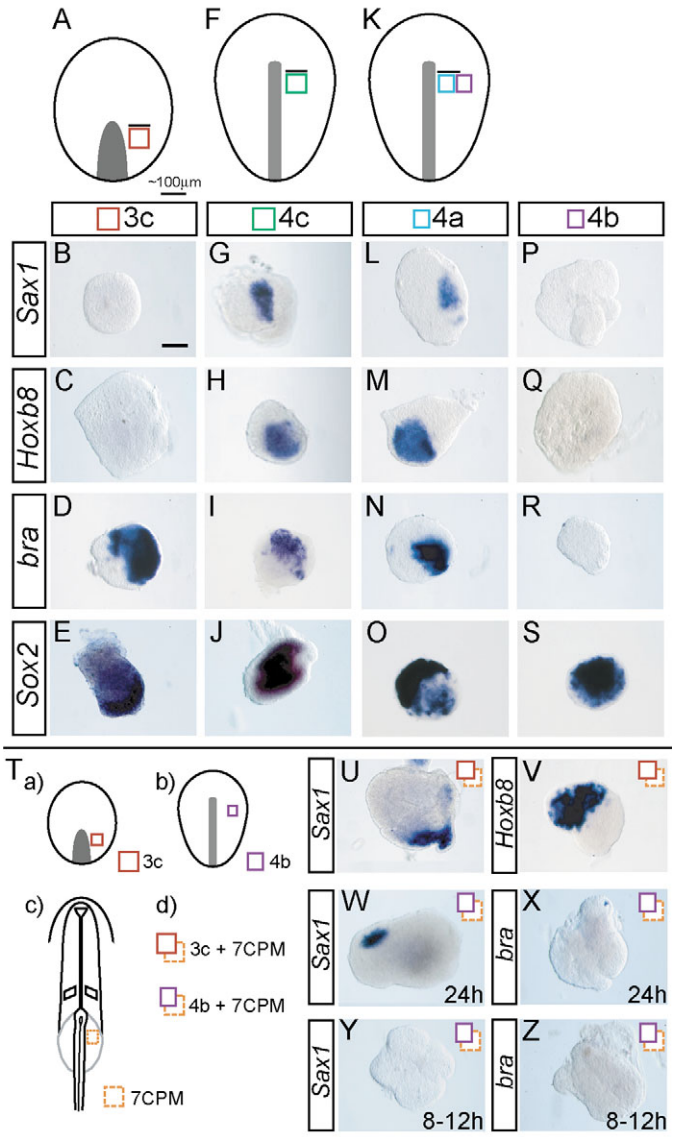

Fig. 2. Timing and tissue interactions underlying stem zone specification. 3c epiblast explants (A) do not express *Sax1* (B) or *Hoxb8* (C), but do express *bra* (D) and *Sox2* (E). 4c explants (F) express *Sax1* (G), *Hoxb8* (H), *bra* (I) and *Sox2* (J). 4a and 4b explants each include a region medial (a) or lateral (b) to 4c (K). 4a explants express *Sax1* (L), *Hoxb8* (M), *bra* (N) and *Sox2* (O). 4b explants do not express *Sax1* (P), *Hoxb8* (Q) or *bra* (R), but are *Sox2* positive (S). All explants assessed after 24 hours. (T) Explants 3c, 4b, 7CPM (caudal paraxial mesoderm) and combination experiments (a,b,c and d, respectively). Stage 7 CPM induces expression of *Sax1* (U) and *Hoxb8* (V) in 3c explants. In 4b explants, the CPM induces *Sax1* (W) but not *bra* (X) after 24 hours. Expression of *Sax1* (Y) and *bra* (Z) is also undetected after 8-12 hours. Scale bar: 100 μm.

caudal paraxial mesoderm (CPM) from beneath the established stem zone at HH7 (7CPM; Fig. 2T). 3c explants cultured with stage 7CPM express *Sax1* (10/10) and *Hoxb8* (13/13) (Fig. 2U,V), indicating that this mesoderm provides signals that can specify the stem zone. Significantly, 4b explants (which express *Sox2* but neither *Sax1* nor *bra* after 24 hours of culture, see above) can also be induced to express

Sax1 by this paraxial mesoderm after 24 hours in some cases (7/19; Fig. 2W). Importantly, *Sax1* is induced in 4b tissue in the absence of *bra* expression (only 1/26 *bra* positive cases after 24 hours incubation) (Fig. 2X), suggesting that it is possible to specify the stem zone without also inducing early mesodermal gene expression. One explanation for the lack of *bra* in these combinations could be that it is induced prior to *Sax1* in the epiblast (as observed in the embryo, see Fig. 1) but is present only transiently in this assay and therefore absent at the 24-hour time point. To address this possibility, we assessed gene expression in 4b/7CPM combinations after a shorter period and found, after 8-12 hours, little *Sax1* (1/10) and no *bra* expression (9/9) (Fig. 2Y,Z). These findings contrast with the detection of both *bra* and *Sax1* in control 4a explants taken from the same embryos and processed in parallel (24 hours *Sax1*, 16/22; *bra*, 28/38; 12 hours *Sax1*, 2/18; *bra*, 14/17) and indicate that *bra* is not a prelude to *Sax1* expression in the 4b/7CPM assay. Together, these data demonstrate that stem zone specification, as indicated by *Sax1*, is mediated by paraxial mesoderm signals and show that this step need not involve creation of an ambivalent neural/mesodermal cell state.

FGF signalling is not sufficient to specify the stem zone

We next tested whether FGF signalling is sufficient to mimic the signal(s) provided by the paraxial mesoderm. FGF4 induces mesodermal genes in extra-embryonic epiblast, but expression of stem zone genes takes place many hours later, suggesting that FGF4 indirectly promotes formation of the stem zone (Storey et al., 1998). However, FGF4 induces *Fgf8* in this assay (Storey et al., 1998) and FGF8 has been shown to be a rapid inducer of neural tissue (Streit et al., 2000) and so one possibility is that FGF8 works directly to induce stem zone genes. Beads soaked in FGF8 or control PBS washed beads were therefore grafted in contact with HH3 extra-embryonic epiblast (Fig. 3A) and ectopic gene expression assessed at intervals. However, we find that FGF8, like FGF4, first elicits expression of the mesodermal marker *bra* (5/9) after 4 hours (Fig. 3B,C), and only somewhat later do we detect ectopic expression of *Sax1* (10/11) (10-12 hours) and *cash4* (2/16) (16-18 hours) (Fig. 3D; data not shown). Using in vitro explant methods, we also assessed the ability of FGF4 to induce *Sax1* expression in 3c epiblast. These explants do not express *Sax1* after 24 hours of culture (22/25 as above) and addition of FGF4 does not elicit *Sax1* expression (8/8) at 10 ng/ml (data not shown). We also tested whether exposure to FGF4 or FGF8 promotes *Sax1* expression in 4b explants. In nearly all cases, we found that neither FGF4 nor FGF8 is able to elicit *Sax1* in these 4b explants, although increases in *bra* expression are found in control 4a explants and in a few 4b explants in response to high concentrations of these factors, after 24 hours (see Table 1; and Fig. 3E-L). These findings therefore demonstrate that FGF4 and FGF8 can promote mesodermal gene expression in these assays but indicate that they are insufficient for induction of stem zone marker genes. However, this does not exclude the possibility that in addition to promotion of mesodermal character FGFs act on the epiblast as necessary co-factors with other later mesoderm derived signal(s) to specify the stem zone.

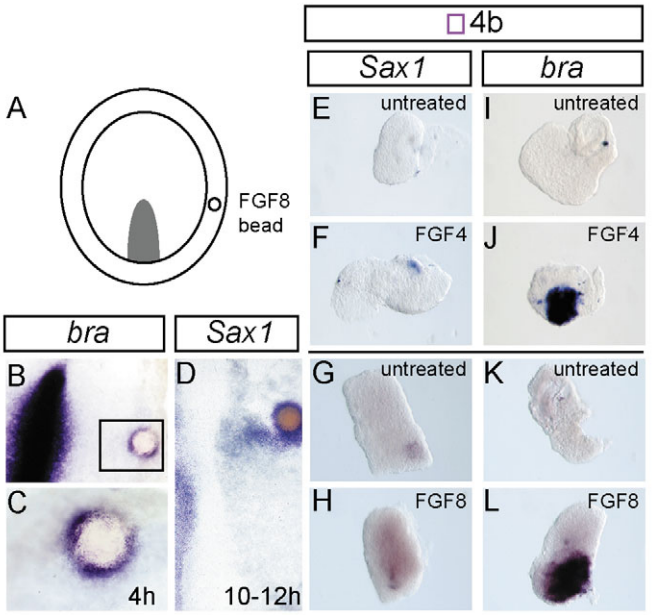

Fig. 3. FGF signalling is insufficient for stem zone specification. FGF8 soaked bead in extra-embryonic epiblast at HH3 (A). *bra* is induced by FGF8 within 4 hours (B) (high magnification in C), but stem zone gene *Sax1* (D) is only detected after 10 hours. 4b explants do not express *Sax1* following exposure to 200 ng/ml FGF4 (E,F) or to 250 ng/ml FGF8 (G,H), but can express *bra* following exposure to 200 ng/ml FGF4 (I,J) or to 250 ng/ml FGF8 (K,L). Scale bar: 100 μm.

Specification of the stem zone depends on FGF signalling

To assess whether FGF signalling is required for specification of the stem zone, we blocked FGFR activation in combinations of 3c epiblast and stage 7 caudal paraxial mesoderm (Fig. 4A) using the soluble FGFR inhibitor SU5402 (10 μM) (Mohammadi et al., 1997). Blocking FGF signalling inhibits induction of *Sax1* and *Hoxb8* expression compared with DMSO-only-treated contralateral pairs (24 hours of culture; *Sax1* 8/10, Fig. 4B,C; *Hoxb8* 7/13, Fig. 4D,E). This experiment establishes that FGF signalling is required for this specification step. However, it does not indicate whether FGF acts in the mesoderm, in the epiblast or in both tissues, nor does it separate a requirement for FGF signalling for maintenance of neural character (see Streit et al., 2000), from a specific requirement for stem zone gene expression.

To address the direct involvement of FGF signalling for stem zone specification, we therefore introduced constructs that

Table 1. FGF signalling can induce *bra* but not *Sax1* expression in HH stage 4 neural plate explants

	*Sax1**		*bra**	
Treatment	4a	4b	4a	4b
FGF4 (10 ng/ml)	4/4	0/4	4/4	0/4
FGF4 (200 ng/ml)	1/3	0/3	2/2	2/2
FGF8 (50 ng/ml)	3/5	0/5	4/4	1/4
FGF8 (250 ng/ml)	2/3	1/6	2/3	1/4

*Upregulation of expression observed in treated versus contralateral untreated explants.

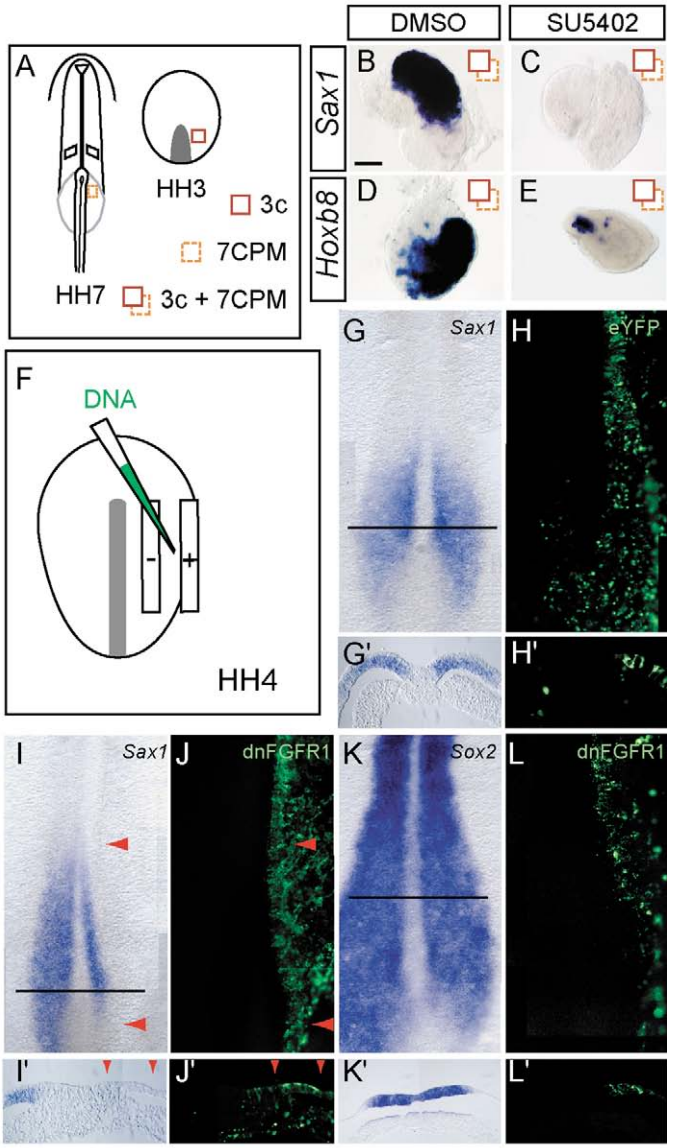

Fig. 4. FGF signalling is required for stem zone specification. 3c and stage 7 CPM explant combination experiments (A). The ability of 7CPM to induce *Sax1* (B,C) and *Hoxb8* (D,E) is inhibited by exposure to SU5402 but not DMSO. Electroporation scheme at HH4 (F). Mis-expression of GFP only does not alter *Sax1* expression (G,H), but that of DnFGFR1 inhibits *Sax1* onset (red arrowheads, I-J′), while not reducing *Sox2* (K-L′).

interfere with FGFR signalling into the caudal neural plate at stage HH4 (Fig. 4F). At this later stage, the neural plate expresses *Sox2* and retains neural character in vitro (Darnell et al., 1999), and may therefore allow us to test the requirement for FGF signalling for onset of stem zone genes, without loss of neural character. Here, we have focussed on onset of *Sax1* expression. Introduction of a control EYFP construct has no effect on *Sax1* expression (6 cases, Fig. 4G-H′) while dominant-negative (dn)FGFR1-EYFP inhibits onset of *Sax1* (five out of five cases) assessed after 14 hours (Fig. 4I-J′). Mis-expression of dnFGFR1-EYFP does not, however, affect *Sox2* expression levels (five cases) (Fig. 4K-L′), suggesting that FGF signalling is specifically required for *Sax1* expression in this tissue. Together, these experiments indicate that in addition to previously demonstrated requirements for FGF signalling in induction and maintenance of early neural and mesodermal character, this pathway is also directly required in epiblast cells for onset of stem zone specific gene expression.

FGF/MAPK signalling is directly required to maintain the stem zone

The persistence of active MAPK signalling in the regressing stem zone (data not shown) suggests that this cell population has a continuing requirement for FGF signalling. To assess this possibility, we introduced the dnFGFR1-EYFP or a control EYFP construct into the established stem zone at HH9+-10 and cultured embryos for 6-7 hours (Fig. 5A). In embryos expressing EYFP alone no reduction of *Sax1* expression was observed (six cases) (Fig. 5B-C′). By contrast, mis-expression of dnFGFR1-EYFP leads to a dramatic loss of *Sax1* (5/5 cases) (Fig. 5D-E′). To assess the specificity of dnFGFR1 repression of *Sax1*, expression of the pan neural marker *Sox2* was also examined in cells expressing dnFGFR1. *Sox2* expression appears unaffected in dnFGFR1 expressing embryos (4/4 cases) (Fig. 5F,G′). These findings demonstrate that FGF signalling is directly required in stem zone cells to maintain *Sax1* expression.

Cells mis-expressing dnFGFR1 tend to move out the stem zone, a phenomenon that appears characteristic of cells lacking FGF signalling (Mathis et al., 2001) and which makes it difficult to target large groups of cells in this region. We therefore also assessed expression of the *Sax1* following a brief (3 hour) exposure to the FGFR antagonist SU5402 or the highly specific MEK antagonist PD184352 (Davies et al., 2000) as this might provide a snapshot of FGF/MAPK requirement in this tissue. In all cases, control DMSO and inhibitor treated embryos were processed in parallel and reacted for the same period of time. Exposure of embryos at HH9+-10 to SU5402 (60 μM) leads to loss of *Sprouty2* expression (a known target of FGF signalling) and decreases *Sax1* expression, while *Sox2* expression is not reduced (Fig. 5H-M; Table 2). Similarly, *Sprouty2* and *Sax1* expression are depleted after exposure to 60 μM PD184352 (Fig. 5N,O; Table 2), while again *Sox2* expression is not lost (Fig. 5P-S; Table 2). Together, these different experimental approaches indicate that FGF/MAPK signalling is directly and specifically required in the epiblast to maintain the stem zone.

Establishment of the stem zone is distinct from acquisition of spinal cord identity

We have shown above that tissue level regulation of *Hoxb8* onset is very similar to that of *Sax1*, a stem zone-specific gene, and that the ability of caudal paraxial mesoderm to induce *Hoxb8* in epiblast explants is sensitive to FGF signalling. However, previous work has indicated that *Hoxb8* induction is dependent on retinoid signalling provided by more rostral paraxial mesoderm (Muhr et al.1999). To clarify this apparent switch in caudal Hox gene regulation from FGF in the stem zone to retinoic acid in the differentiating spinal cord, we tested whether FGF signalling is required for the maintenance of *Hoxb8* in the stem zone and whether retinoid signalling is required for stem zone specification and for *Hoxb8* expression in this domain. Brief exposure of whole embryos to SU5402 at HH 9+-10 (as above) inhibits *Hoxb8*

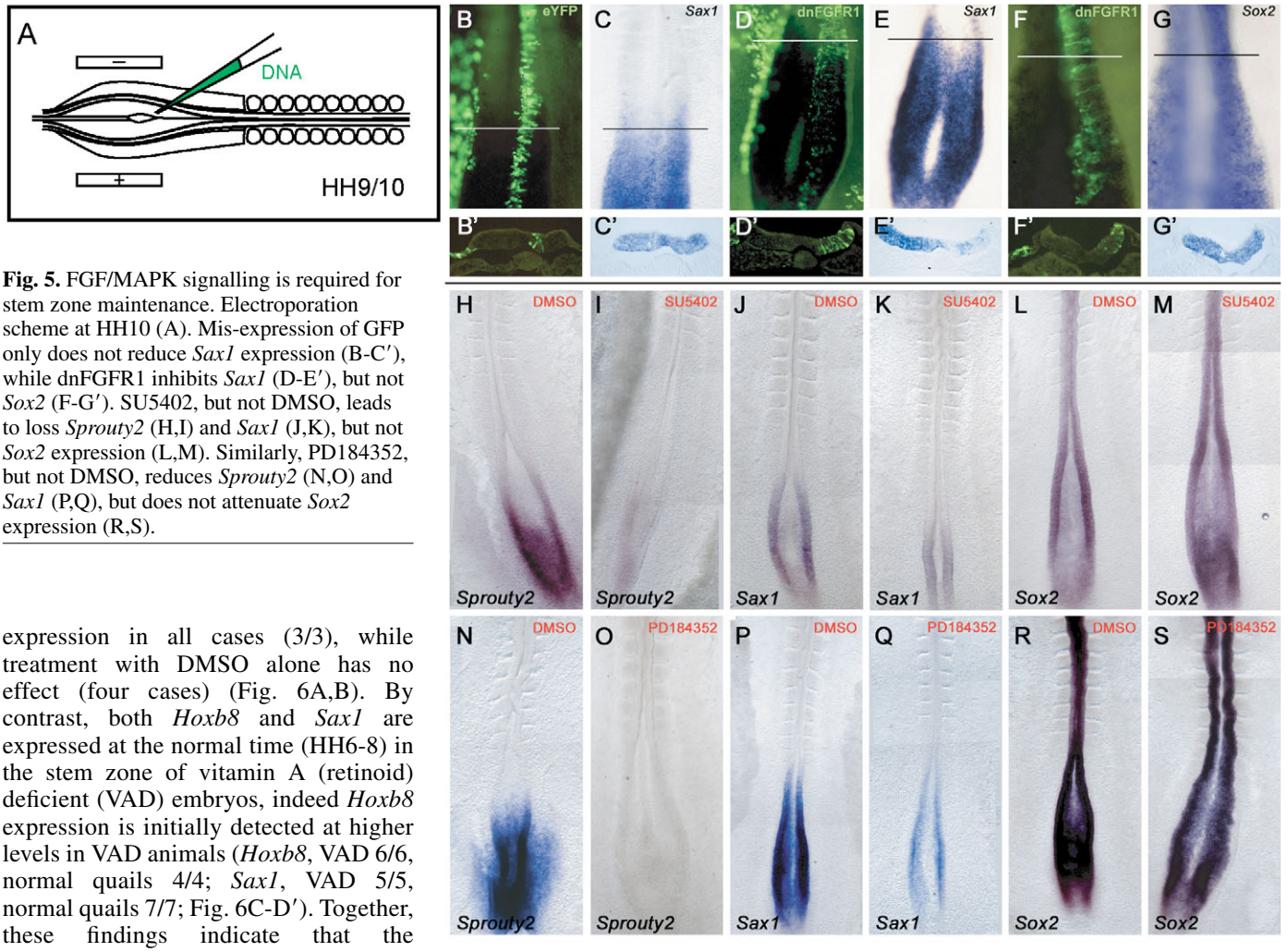

Fig. 5. FGF/MAPK signalling is required for stem zone maintenance. Electroporation scheme at HH10 (A). Mis-expression of GFP only does not reduce *Sax1* expression (B-C′), while dnFGFR1 inhibits *Sax1* (D-E′), but not *Sox2* (F-G′). SU5402, but not DMSO, leads to loss *Sprouty2* (H,I) and *Sax1* (J,K), but not *Sox2* expression (L,M). Similarly, PD184352, but not DMSO, reduces *Sprouty2* (N,O) and *Sax1* (P,Q), but does not attenuate *Sox2* expression (R,S).

expression in all cases (3/3), while treatment with DMSO alone has no effect (four cases) (Fig. 6A,B). By contrast, both *Hoxb8* and *Sax1* are expressed at the normal time (HH6-8) in the stem zone of vitamin A (retinoid) deficient (VAD) embryos, indeed *Hoxb8* expression is initially detected at higher levels in VAD animals (*Hoxb8*, VAD 6/6, normal quails 4/4; *Sax1*, VAD 5/5, normal quails 7/7; Fig. 6C-D′). Together, these findings indicate that the expression of *Hoxb8* in the stem zone depends on the FGF pathway and that establishment of this region and onset of this caudal Hox gene are independent of retinoid signals.

Discussion

We report four main findings: (1) the stem zone contains numerous cells that express both early neural and mesodermal genes; (2) stem zone specification takes place by late primitive streak stages, is mediated by signals from the underlying paraxial mesoderm, but, importantly, need not be prefigured by creation of an ambivalent mesodermal/neural cell state; (3) although high levels of FGF signalling can induce mesodermal gene expression in neural plate explants, this is not sufficient to induce stem zone-specific gene expression, which, although dependent on FGF signalling in epiblast cells, requires additional signals from paraxial mesoderm; and (4) caudal Hox gene expression in the stem zone is also FGF dependent and neither establishment of the stem zone nor onset of caudal Hox gene expression requires retinoid signalling. This contrasts with the retinoid-dependent expression of such Hox genes in the differentiating neural tube and demonstrates that distinct molecular mechanisms operate to regulate gene expression in the stem zone and to assign fixed rostrocaudal identity in the extending body axis.

The stem zone contains an ambivalent cell population

Analysis of the dynamics of marker gene expression in epiblast cells adjacent to the primitive streak in the context of fate-mapping data provides a detailed description of the stem zone and identifies an ambivalent cell population within this region. We reveal an overlap between mesodermal and pan neural gene expression at early streak stages in epiblast cells adjacent to the primitive streak. In the mouse embryo single cell labelling has identified epiblast cells at equivalent stages that contribute to both neural and mesodermal lineages (Forlani et al., 2003). By late streak stages in the chick, cell movement towards the anterior primitive streak is much reduced (Joubin and Stern, 1999), but this overlap between mesodermal and neural genes persists in epiblast close to the anterior streak in the region where *Sax1* expression commences. Furthermore, cell labelling in the chick at HH4 and HH6-7+ reveals that some cells remain in this region as it regresses caudally and that these cells can contribute to mesodermal as well as neural lineages (Brown and Storey, 2000). Clearly, although single cell labelling is required to determine whether these resident cells are a mixture of mesodermal and neural precursors or a bipotent cell population, the overlap of *bra*, *Sox2* and *Sax1* in this region supports the existence of an ambivalent cell population. Furthermore, HH6-7 stem zone explants cultured for a long

Table 2. Inhibition of FGF/MAPK signalling downregulates *Sax1* expression

	Treatment*					
	DMSO	SU5402 60 μM	DMSO	PD184352 20 μM	DMSO	PD184352 60 μM
Sprouty2	0/5	5/5	0/3	2/2	0/1	1/1
Sax1	0/6	5/5	0/2	0/2	0/4	6/6
Sox2	0/2	0/1	0/4	0/4	0/1	0/1

*Observed downregulation of expression.

period (48 hours) contain cells expressing *paraxis*, a marker of differentiating paraxial mesoderm (Diez del Corral et al., 2002), suggesting that *bra* expression in the stem zone is indeed indicative of mesodermal potential and not just a marker of epiblast cells close to the primitive streak.

At the ten-somite stage *bra*, *Sox2* and *Sax1* are expressed in the stem zone (see Charrier et al., 1999), while more rostrally transition zone cells express just *Sox2* and *Sax1* (see Fig. 7). This distinction is consistent with cell labelling experiments in the vicinity of the node at HH10, which show that here cells generate only neural progeny (Mathis et al., 2001). Significantly, expression of *Sip1*, which directly represses both *bra* and the epidermal gene *E-cadherin* (Papin et al., 2002; Sheng et al., 2003; Van de Putte et al., 2003), is absent from the stem zone but is detected in the transition zone at HH10 (Sheng et al., 2003) (K.G.S., unpublished), an observation that further supports the multipotency of cells in the stem zone. The evidence for neural stem cells in the mouse stem zone comes from an experiment in which only cells expressing a reporter gene under control of a neural specific promoter are labelled (Mathis and Nicolas, 2000) and so clonal contribution to both neural and non-neural tissue has yet to be determined. It is therefore currently not known whether generation of the spinal cord depends on stem cells that give rise to mesodermal and neural progenitors located in the stem zone or if this relies on a distinct neural stem cell population. An interesting possibility is that ambivalent stem zone cells may be part of the self-renewing cell group identified in the mouse tail bud at the junction between the node and anterior primitive streak, which gives rise to both neural and paraxial mesodermal derivatives, although these cells also retain the ability to form notochord (Cambray and Wilson, 2002).

Specification of the stem zone

Identification of an ambivalent cell population in the stem zone led us to examine the specification and regulation of this region, and to assess whether this specification step involves the creation of an ambivalent cell state. We found that the stem zone, as indicated by expression of *Sax1* and *Hoxb8*, is specified by late primitive streak stages via signals presented by caudal paraxial tissue. This differs from a previous report that *Hoxb8* is induced by rostral paraxial tissue taken from above the level of the primitive streak (Muhr et al., 1999), but this probably reflects differences in *Hoxb8* regulation in distinct domains (see below). Using lateral neural plate (4b) explants, which express *Sox2* but neither *Sax1* nor *bra*, we found that caudal paraxial mesoderm could induce stem zone character without concomitant expression of *bra*. This strongly suggests that it is possible to establish stem zone character without first passing through an ambivalent mesodermal/neural cell state. However, expression of *Sox2* and *Sax1* is indicative of transition zone cells and as noted above, we have yet to ascertain whether a purely neural cell population is sufficient to generate the entire spinal cord or whether bipotent cells in the stem zone underlie this activity.

FGF and caudal paraxial mesoderm signals specify and maintain the stem zone

FGF signalling has long been known to underlie the generation of caudal tissues, but its direct involvement in the different steps underlying this process has not been fully elucidated. We show using both in vivo and in vitro assays that signalling provided by either FGF4 or FGF8 is not sufficient to induce stem zone-specific gene expression. However, this does not rule out the possibility that FGF signalling is a necessary cofactor for stem zone specification. To distinguish between the necessity for FGF signalling for expression of early neural genes and for stem zone specification we mis-expressed dnFGFR1 in the established neural plate at HH4. These experiments demonstrate that onset of *Sax1*, but not maintenance of *Sox2*, depends on FGF signalling and thus identify a third FGF requiring step, which takes place after neural and mesodermal induction, during the generation of caudal neural tissue. We further show that this is a continuing requirement in the extending body axis, as maintenance of *Sax1* also depends on FGF/MAPK signalling in epiblast cells.

As FGF signalling is not sufficient for stem zone specification other signals provided by the caudal paraxial mesoderm must be involved. These include WNT proteins (Nordstrom et al., 2002) and TGFβ family members (Liu et al.,

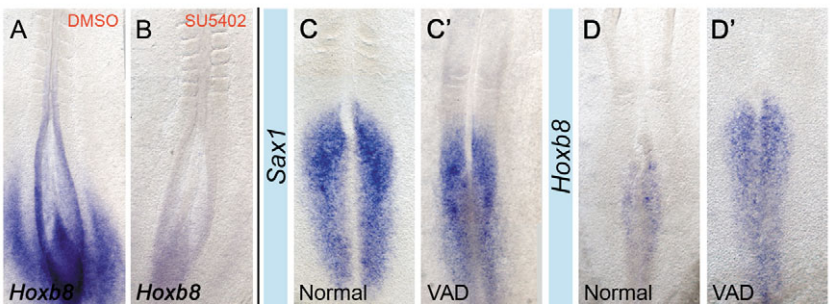

Fig. 6. Stem zone gene expression in FGF- and retinoid-deficient embryos. SU5402, but not DMSO, leads to loss of *Hoxb8* expression (A,B). Vitamin A-deficient (VAD) quails commence expression of *Sax1* (C,C′) and *Hoxb8* (D,D′) in the stem zone as in normal quail embryos.

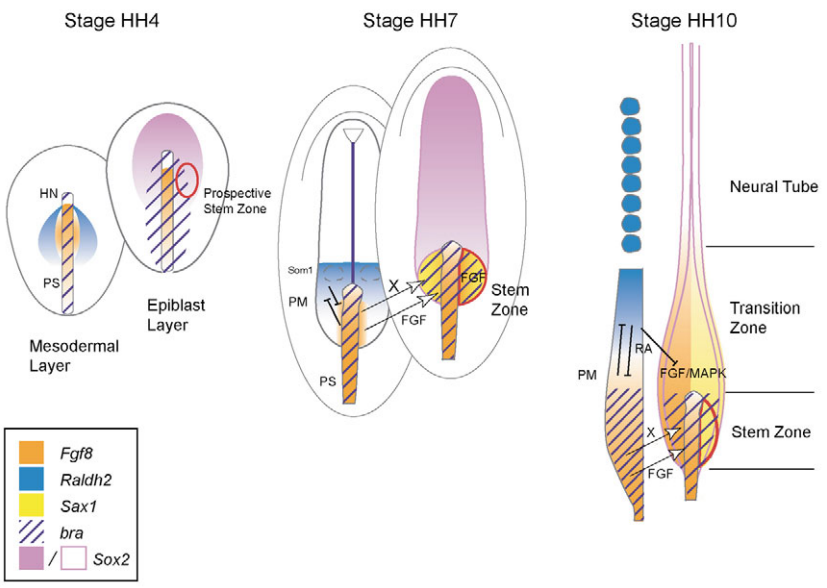

Fig. 7. Steps in stem zone specification and maintenance. At HH4, an overlap between pan-neural (*Sox2*) and early mesodermal gene (*bra*) expression is observed in epiblast cells close to the anterior primitive streak. Underlying paraxial mesoderm begins to express *Raldh2*, while *Fgf8* expression is restricted to the primitive streak. From HH7, stem zone-specific gene expression (*Sax1*) commences in epiblast cells close to the primitive streak. *Sax1* onset requires signals from the paraxial mesoderm and an intact FGF pathway, which works at least in part by inhibiting the retinoid pathway (see text). At this time, *Raldh2* is rostrally restricted by FGF8 in newly emerged paraxial mesoderm (Diez del Corral et al., 2003). Retinoic acid can also attenuate *Fgf8* levels (Diez del Corral et al., 2003) and so a fine balance between FGF and retinoid signalling is required for formation of the stem zone. *Sax1* induction can take place without prior creation of an ambivalent mesodermal/neural cell state. At HH10, *Sax1* and *Fgf8* expression are also present in the transition zone and *Sax1* is maintained by FGF/MAPK signalling. There is a persisting overlap of neural and mesodermal genes in the stem zone, which may be the location of bipotent stem cells.

2001). Here, we have focussed on the retinoid pathway, as the retinoid synthesising enzyme *Raldh2* is initially expressed in the caudal paraxial mesoderm (Swindell et al., 1999) just prior to *Sax1* onset. However, we find that onset of both *Sax1* and *Hoxb8* appear at the normal time in vitamin A-deficient embryos, indicating that retinoic acid is not necessary for stem zone specification. By contrast, during normal development, the *Sax1* expression domain appears to expand rostrally, coincident with restriction of *Raldh2* expression to rostral paraxial mesoderm. This rostral retreat of *Raldh2* reflects the persisting expression of *Fgf8* and *bra* in the stem zone and in paraxial mesoderm cells emerging from the primitive streak and is driven by the ability of FGF signalling to inhibit onset of *Raldh2* (Diez del Corral et al., 2003).

Interestingly, this apparent rostral expansion of *Fgf8/bra/Sax1* coincides with the onset of the retinoic acid catabolising enzyme *Cyp26a* in the stem zone (Blentic et al., 2003; Swindell et al., 1999), which in the frog requires FGF signalling (Moreno and Kintner, 2004). This may further help to create a retinoid-free region at the caudal end of embryo and, consistent with this, *Cyp26a* knockout mice exhibit a truncated phenotype (Abu-Abed et al., 2001; Sakai et al., 2001) also seen following exposure to retinoic acid (Kessel, 1992), which suggests that excess retinoid signals drive premature differentiation of the stem zone. Stem zone specification thus depends on unknown signals from the paraxial mesoderm and FGF-mediated activity, which works at least in part by establishing a retinoid-free region (summarised in Fig. 7).

FGF signalling declines as cells move from the stem zone to transition zone and is driven by the ability of retinoic acid to attenuate *Fgf8* levels (Diez del Corral et al., 2003). In the frog, high FGF signalling induces mesoderm, while lower levels promote neural tissue (Delaune et al., 2005) and a similar conclusion can be inferred from data in the chick (Eblaghie et al., 2003; Storey et al., 1998; Streit et al., 2000) (this paper). This finding fits nicely with the loss of *bra* expression as cells leave the stem zone and encounter retinoic acid. Furthermore, ectopic caudal neural tissue forms at the expense of mesoderm in *Cyp26a* mutant mice and following application of exogenous retinoic acid (Abu-Abed et al., 2001; Sakai et al., 2001) (reviewed by Maden, 2002) and this phenotype is also observed in embryos lacking FGF signalling (Ciruna et al., 1997). Consistent with this, retinoid deficient embryos have a strikingly narrow neural tube, suggesting that fewer cells are assigned to a neural fate when retinoid levels are low (Diez del Corral et al., 2003; Maden et al., 1996; Molotkova et al., 2005; Wilson et al., 2003). Retinoic acid, by controlling *Fgf8* levels, may thus also help to resolve mesodermal versus neural cell fates in the extending body axis.

Stem zone specification and maintenance is distinct from assignment of spinal cord character

Previous work has analysed the regulation of *Hoxb8* as a marker of spinal cord identity (Muhr et al., 1999). This involved examination of the signalling pathways required for regulation of *Hoxb8* by rostral, but not caudal, paraxial mesoderm. This rostral mesoderm does not express FGFs, but synthesises retinoic acid (Berggren et al., 1999; Swindell et al., 1999), and Muhr and colleagues demonstrated a requirement for the retinoid pathway, but not FGF signalling, for induction of *Hoxb8* by rostral paraxial mesoderm. *Hoxb8* is first expressed in the stem zone at HH8+ (Fig. 1) and, unlike *Sax1*, persists in the neural tube, defining neural tissue caudal to somite 5. It also later extends rostrally into the posterior hindbrain where it again relies on retinoic acid (Oosterveen et al., 2003). However, exposure of the early embryo or explanted caudal neural tissue to FGF promotes *Hoxb8* expression (Bel-Vialar et al., 2002; Dasen et al., 2003; Liu et al., 2001), suggesting that an initial phase of *Hoxb8* expression is responsive to FGF signalling. We have shown here that *Hoxb8* induction by caudal paraxial mesoderm is dependent on FGF signalling and that brief exposure of the whole embryo to an FGFR inhibitor attenuates *Hoxb8* expression in caudal regions. Furthermore, as noted above, onset of *Hoxb8* in the stem zone does not require retinoid signalling. These findings demonstrate that distinct molecular mechanisms underlie the

specification and maintenance of the stem zone and assignment of a fixed spinal cord identity and support a two step model for spinal cord generation, based on the opposition of FGF and retinoid signalling pathways in the extending body axis (Diez del Corral and Storey, 2004). This involves the production of new tissue in the stem zone under the influence of FGF signalling, where progressively more caudal Hox genes are expressed. This is followed by somite-derived retinoid signals, which attenuate FGF signalling as cells leave the stem zone and enter the forming neural tube. Retinoid signalling then drives differentiation, including assignment of rostrocaudal identity, as the progressive onset of more caudal Hox genes ceases when cells form the neural tube.

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