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STEM CELLS AND REGENERATION

RESEARCH ARTICLE

The zebrafish tailbud contains two independent populations of midline progenitor cells that maintain long-term germ layer plasticity and differentiate in response to local signaling cues

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ABSTRACT

Vertebrate body axis formation depends on a population of bipotential neuromesodermal cells along the posterior wall of the tailbud that make a germ layer decision after gastrulation to form spinal cord and mesoderm. Despite exhibiting germ layer plasticity, these cells never give rise to midline tissues of the notochord, floor plate and dorsal endoderm, raising the question of whether midline tissues also arise from basal posterior progenitors after gastrulation. We show in zebrafish that local posterior signals specify germ layer fate in two basal tailbud midline progenitor populations. Wnt signaling induces notochord within a population of notochord/floor plate bipotential cells through negative transcriptional regulation of *sox2*. Notch signaling, required for hypochord induction during gastrulation, continues to act in the tailbud to specify hypochord from a notochord/hypochord bipotential cell population. Our results lend strong support to a continuous allocation model of midline tissue formation in zebrafish, and provide an embryological basis for zebrafish and mouse bifurcated notochord phenotypes as well as the rare human congenital split notochord syndrome. We demonstrate developmental equivalency between the tailbud progenitor cell populations. Midline progenitors can be transfated from notochord to somite fate after gastrulation by ectopic expression of *msgn1*, a master regulator of paraxial mesoderm fate, or if transplanted into the bipotential progenitors that normally give rise to somites. Our results indicate that the entire non-epidermal posterior body is derived from discrete, basal tailbud cell populations. These cells remain receptive to extracellular cues after gastrulation and continue to make basic germ layer decisions.

KEY WORDS: Midline progenitor cells, Tailbud, Canonical Wnt, Notch, Posterior growth, Notochord, Floor plate, Hypochord, Mesogenin 1, MPC, PWPC, Neuromesodermal progenitors

INTRODUCTION

A major shift in the view of vertebrate germ layer induction is underway, with evidence from several species indicating that germ layer induction continues after gastrulation (Henrique et al., 2015; Kondoh and Takemoto, 2012). During body axis extension, the post-gastrula vertebrate embryo increases in length in large part due to the formation of new tissues at the posterior (or caudal) end (Benazeraf and Pourquie, 2013). Progenitor cells in a region called the tailbud, which is the posteriormost anatomical structure of all vertebrate embryos that forms after the completion of gastrulation, drive this process (Beck, 2015). Tailbud progenitor cells continuously exit and differentiate into elements of the growing body. Recent work demonstrated that the caudalmost progenitors within the tailbud, the posterior wall progenitor cells (PWPCs; sometimes referred to as neuromesodermal progenitors), are bipotential and continuously make neural/mesodermal fate decisions during embryo elongation. (Freese et al., 2014; Garriock et al., 2015; Gentsch et al., 2013; Gouti et al., 2014; Kondoh and Takemoto, 2012; Martin and Kimelman, 2012; Tzouanacou et al., 2009). These cells give rise to the growing spinal cord, somites and vasculature. Despite the basal plasticity of PWPCs, they never give rise to midline structures of the neural floor plate, mesodermal notochord and endodermal hypochord (Martin and Kimelman, 2012; Tzouanacou et al., 2009). Midline structures instead originate after gastrulation from a population of midline progenitor cells (MPCs), which are specified during gastrulation (Catala et al., 1996, 1995; Kinder et al., 2001; Melby et al., 1996; Schoenwolf and Sheard, 1990; Selleck and Stern, 1991; Shih and Fraser, 1996). After being specified these cells migrate with the organizer and eventually populate a region of the tailbud called the chordoneural hinge (CNH), immediately posterior to the end of the notochord (Wilson et al., 2009). During embryo elongation, MPCs continuously join growing tissues of the midline. In chicken embryos it is clear that different germ layer derivatives form from a single pool of MPCs, whereas in mouse embryos the notochord and floor plate progenitors are spatially distinct (Catala et al., 1996; Jeong and Epstein, 2003; Teillet et al., 1998). Whether MPC fate decisions are made solely during gastrulation or continuously during embryo elongation after gastrulation is unknown. The recent evidence of bipotential progenitors in the tailbud raised the possibility that MPCs could also maintain post-gastrula germ layer plasticity, similar to PWPCs (Beck, 2015; Wilson et al., 2009).

Midline tissues play essential patterning roles during development. The floor plate and notochord provide dorsal-ventral pattern to the spinal cord as well as mediolateral pattern to the adjacent somites, whereas the hypochord organizes the formation of midline blood vessels (Cleaver and Krieg, 1998; Placzek and Briscoe, 2005; Stemple, 2005). The notochord patterns adjacent tissues in part through the secretion of Hedgehog signals, which provide medial character to somites and ventral character to the spinal cord (Stemple, 2005). Floor plate forms just dorsal to the notochord in the ventralmost region of the spinal cord. It initially forms as a single row of cells of triangular cross-section (the medial floor plate) and expands to include adjacent cells of the ventral neural tube (lateral floor plate) (Placzek and Briscoe, 2005). The timing and location of floor plate induction vary across vertebrate species. Cell lineage analyses in chick embryos show that notochord and medial floor plate arise from a single pool of progenitors, whereas in mouse embryos sonic hedgehog-expressing notochord-

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fated cells are separated from future floor plate cells in the node after gastrulation (Catala et al., 1996; Jeong and Epstein, 2003; Schoenwolf and Sheard, 1990; Selleck and Stern, 1991). In the mouse, Hedgehog signaling from the notochord induces both the medial and lateral floor plate, whereas in zebrafish medial floor plate formation is independent of Hedgehog signaling (Chen et al., 2001; Chiang et al., 1996; Ding et al., 1998; Matise et al., 1998).

The least studied of the midline tissues is the hypochord, which is only present in anamniotes and serves to pattern nearby blood vessels by secreting VEGF (Cleaver and Krieg, 1998). The mesodermal or endodermal origin of the hypochord has been a matter of debate, but a consensus view between disparate anamniote species (including *Xenopus laevis*, axolotl and zebrafish) has emerged ascribing an endodermal character to this tissue (Cleaver et al., 2000; Eriksson and Lofberg, 2000; Lofberg and Collazo, 1997). Amniotes lack a hypochord but its function in patterning blood vessels may have been taken over by dorsal gut endoderm. This tissue forms adjacent to the same blood vessels and expresses VEGF (Dumont et al., 1995).

The common origin of midline tissues from MPCs in several vertebrate species, in addition to genetic and embryological data, has led to the allocation model of midline development, whereby local signals specify fate within MPCs (reviewed by Le Douarin and Halpern, 2000; Strahle et al., 2004). There is strong evidence for a conserved role of Notch signaling in regulating hypochord allocation during gastrulation, but the signals regulating medial floor plate allocation are less clear (Appel et al., 1999; Latimer and Appel, 2006; Latimer et al., 2002; Peyrot et al., 2011). Species-specific roles for Notch and Midkine signaling have been identified, but a conserved pathway regulating this process across all species has yet to be identified (Gray and Dale, 2010; Lopez et al., 2003, 2005; Peyrot et al., 2011; Schafer et al., 2005).

Zebrafish provide an ideal model with which to study the post-gastrulation regulation of cell fate. The ability to create genetic mosaic animals, along with the use of heat shock-inducible transgenes, allows for the precise manipulation of genes and signaling pathways specifically in individual MPCs after the completion of gastrulation. Using these methods we show that MPCs maintain germ layer plasticity during post-gastrulation stages of body axis formation. We present a model of the tailbud that is a hybrid of the historically opposed fixed fate and blastema models (Holmdahl, 1925; Pasteels, 1943), in which the tailbud consists of neither a uniform population of undifferentiated cells, nor small groups of fixed-lineage cells, but rather independent populations of multipotent progenitors that are held in specific signaling environments that dictate their cell fate.

RESULTS

The possibility that MPCs remain multipotent after gastrulation has been suspected for some time (Cambray and Wilson, 2002; Catala et al., 1995; Davis and Kirschner, 2000; Teillet et al., 1998). New reagents for inducible activation and repression of signaling pathways, combined with targeted cell transplantation, allowed us to study this question directly. We took advantage of a heterochronic cell transplantation strategy that maximizes the contribution of transplanted cells to the MPC pool (Fig. 1A) (Halpern et al., 1995). In most cases, control transplants contribute exclusively to notochord, medial floor plate and hypochord (Fig. 1B,B′). Transplanted cells remain undifferentiated after gastrulation for an extended period, as seen in Fig. 1C, where the indicated cell waits at the caudal end of the notochord for 4-5 h before contributing to the notochord (see also Movie 1).

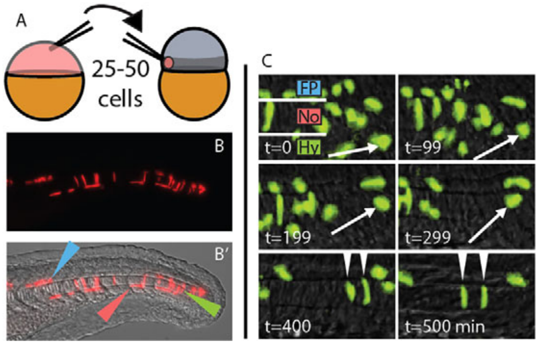

Fig. 1. A midline-directed transplant technique reveals long-lived MPCs. (A) A heterochronic cell transplantation scheme, from transgenic or transiently transgenic donors to wild-type zebrafish hosts, maximizes contribution to the MPCs. 25-50 cells are taken from a dye-injected sphere stage donor and placed in the prospective mesendoderm (darker gray band) of a 1000-cell stage host. (B,B′) At 24 h post fertilization (hpf), transplanted cells are observed exclusively in the derivatives of the MPCs. Green, red and blue arrowheads indicate notochord, hypochord and floor plate, respectively. (C) Transplanted cells can remain undifferentiated for an extended time, and can join the notochord, hypochord and floor plate of the neural tube. Arrow marks one cell that divides and differentiates into two notochord cells (arrowheads). *t*=0 is ∼18 hpf. Fp, floor plate; No, notochord; Hy, hypochord.

Canonical Wnt signaling and *sox2* manipulations affect notochord progenitors after gastrulation

In the posterior wall of the tailbud and in cell culture, Wnt signaling induces new mesoderm from PWPCs (Bouldin et al., 2015; Garriock et al., 2015; Gouti et al., 2014; Henrique et al., 2015; Jurberg et al., 2014; Martin and Kimelman, 2012; Tsakiridis et al., 2014). To determine whether Wnt signaling also induces new mesoderm formation within the tailbud MPCs, we used heat shock-inducible transgenic lines to temporally inhibit (*hsp70l:TCFΔC-GFP*) or activate (*hsp70l:β-Catenin-TFP*) Wnt signaling (Martin and Kimelman, 2012; Veldman et al., 2013). The T-box gene *ntla* (also known as *T, brachyury homolog a*) is expressed in differentiated notochord cells, notochord progenitors located just posterior to the differentiated notochord, and in posterior wall mesoderm progenitors (Martin and Kimelman, 2008; Schulte-Merker et al., 1994). Three hours after inducing a block in Wnt signaling there is a rapid loss of *ntla* expression in the notochord progenitor domain, but not in the differentiated notochord (Fig. 2B, outlined region). In the same time frame, activation of Wnt signaling causes an increase in *ntla* in the notochord progenitor region (Fig. 2C, outlined region). To confirm changes in notochord progenitors after Wnt manipulation, we examined the expression of *floating head* (*flh*, also known as *notochord homeobox*; a *Xnot* ortholog), which is expressed exclusively in notochord progenitors at this stage (Talbot et al., 1995). Expression of *flh* rapidly decreased after Wnt inhibition and increased within the MPCs following Wnt activation (Fig. 2F,G).

In the mouse tailbud, sustained ectopic expression of the transcription factor *Sox2* in tailbud PWPCs is sufficient to cause neural induction at the expense of paraxial mesoderm (Takemoto et al., 2011). In zebrafish, *sox2* is expressed in the region of the MPCs (Fig. 2I) and expands dramatically after Wnt signaling inhibition (Fig. 2J, arrowhead). Additionally, an endogenously tagged *sox2-p2a-sfGFP* reporter line (Shin et al., 2014) exhibits fluorescence in posterior notochord cells, which do not express *sox2* transcript or protein, indicating that at least some notochord cells were previously *sox2* positive (Fig. 2K,K′, arrowheads). These results suggest that the loss of notochord progenitor markers after

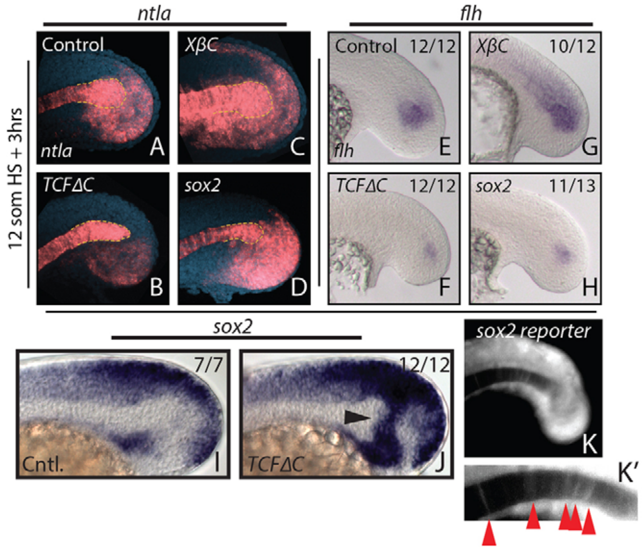

Fig. 2. Canonical Wnt signaling affects tailbud notochord progenitor fate through *sox2* repression. (A-H) Heat shock-inducible transgenic lines were used to manipulate canonical Wnt signaling or *sox2* expression after gastrulation at the 12-somite stage, and stained for *ntla* or *flh* expression 3 h after the heat shock. Loss of Wnt signaling causes a reduction in *ntla* expression specifically in the notochord progenitor domain (A,B, yellow dashed line indicates the progenitor domain), as well as a reduction in the notochord progenitor marker *flh* (E,F). Activation of Wnt signaling has the opposite effect on notochord progenitors (C,G). (I,J) *sox2* is normally expressed in regions directly adjacent to the notochord progenitor domain (I) and expands dramatically into the notochord progenitor domain 2 h after loss of Wnt signaling at the 12-somite stage (J, arrowhead). Heat shock induction of *sox2* expression phenocopies Wnt loss of function with respect to *ntla* (D, dashed yellow line) and *flh* (H) expression. A *sox2* reporter line shows weak fluorescence in notochord cells at the 16-somite stage (K,K′, arrowheads), indicating that notochord cells were once *sox2* positive. The number of embryos showing the illustrated phenotype among the total number examined is indicated.

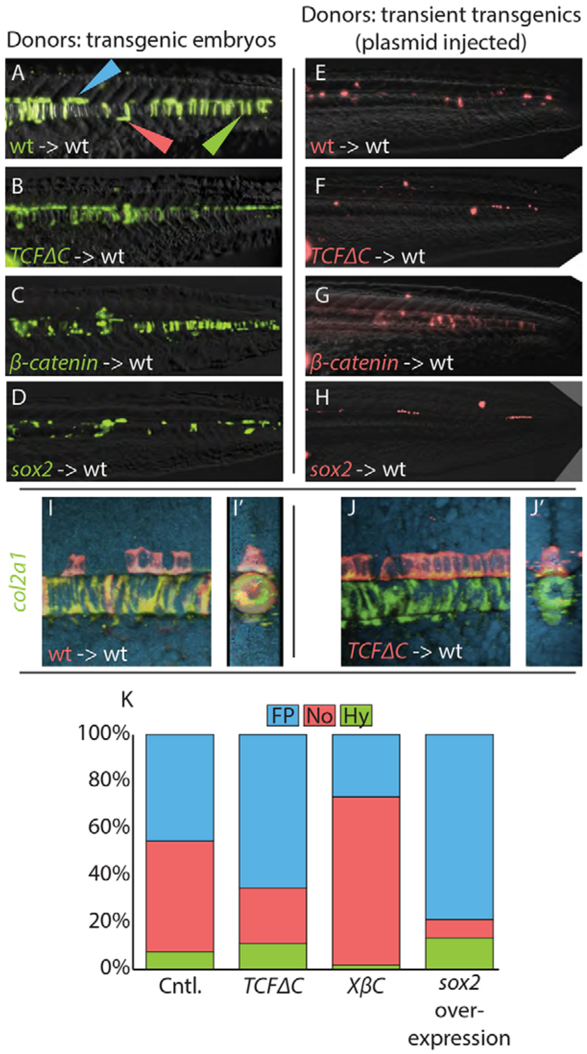

Fig. 3. Cell fate distributions are affected by changes in Wnt signaling or *sox2* overexpression. (A-H) Cells from stable transgenic donors (A-D) or from transiently transgenic donors (E-H) were transplanted into wild-type hosts and transgene expression induced after the completion of gastrulation (bud stage). (I-J′) In some cases, host embryos were stained by fluorescent *in situ* hybridization for *col2a1* expression (green) and imaged by confocal microscopy. Transplanted cells are in red. A maximum projection image (I,J) and digital transverse section (I′,J′) reveal the precise midline position of transplanted cells from control (I,I′) and *hsp70l:TCFΔC-GFP* (J,J′) transplanted cells. (K) The contribution of transient transgenic cells to floor plate, notochord and hypochord was quantitated (raw data and statistics are provided in Table 1). Blocking Wnt signaling (B,F,J,J′) expanded the floor plate contribution at the expense of notochord, and activating Wnt had the opposite effect (C,G). Overexpression of *sox2* (D,H) produced an effect very similar to blocking Wnt. Cell fate changes are statistically significant (see Table 1). Green, red and blue arrowheads indicate notochord, hypochord and floor plate, respectively.

Wnt signaling inhibition might be due to a failure to repress *sox2* in cells that would otherwise normally become notochord. In order to test this hypothesis directly we created a heat shock-inducible transgenic line to temporally overexpress *sox2* (*hsp70l:sox2-p2a-NLS kikGR*). Heat shock induction of *sox2* at the 12-somite stage phenocopied Wnt loss of function with respect to *ntla* and *flh* expression (Fig. 2D,H).

Wnt signaling induces notochord in bipotential floor plate/notochord progenitors by repressing *sox2* expression

To determine whether cell fate is affected by Wnt manipulations, we transplanted cells from the *hsp70l:TCFΔC-GFP* or *hsp70l:β-Catenin-TFP* transgenic lines into wild-type host embryos. This approach tests the ability of Wnt signaling to cell-autonomously specify fate in the MPCs after gastrulation has ended, in the context of an otherwise wild-type embryo. Wild-type cells predominantly join floor plate and notochord in approximately equal measure, with a minority of cells joining hypochord (Fig. 3A). A major advantage of this system is the ability to unambiguously identify cell fate based on position and morphology. We validated the use of widefield microscopy for analysis by using 3D confocal microscopy. The distinctive triangular cross-section of medial floor plate cells and circular cross-section of notochord cells can be seen, as well as their colocalization with expression of the midline marker *col2a1* (Fig. 3I,I′). Disruption of Wnt signaling at the end of gastrulation (bud stage) greatly enhanced the contribution of midline progenitors to floor plate and to a lesser extent to hypochord, at the expense of notochord (Fig. 3B,J,J′). Activated Wnt signaling greatly expanded notochord contribution at the expense of floor plate (Fig. 3C).

Initial experiments used donor embryos from stable transgenic lines (Martin and Kimelman, 2012; Veldman et al., 2013). To quantify tissue contribution an alternate transient transgenic approach was employed, using donors injected with plasmid DNA and integrated genomically with the *tol2* transposase system, which creates a mosaic scatter labeled embryo (Kikuta

Table 1. Raw data of tissue contribution from cell transplants

	Tissue contribution (cells)				Tissue contribution (%)			
Condition	FP	No	Hy	*P*-value	FP	No	Hy	Embryos counted
Control	43	47	8		43.9	48	8.2	9
Wnt loss of function (TCFΔC)	58	21	10	0.0024	65.2	23.6	11.2	21
Wnt gain of function (caβcat)	25	67	2	0.0029	26.6	71.3	2.1	16
sox2 overexpression	109	5	13	<0.001	85.8	3.9	10.2	18
Notch activation (NICD)	71	13	39	<0.001	57.7	10.6	31.7	10
Notch blocked [dnSu(H)]	44	60	0	0.0058	42.3	57.7	0	10

P-values were calculated by pairwise comparison of experimental and control conditions using Fisher's exact test as implemented in R (R Core Team, 2014). FP, floor plate; No, notochord; Hy, hypochord. caβcat, constitutively active β-catenin.

and Kawakami, 2009). We used the heat shock vector *hsp70l:p2a-NLS kikGR* to express our constructs of interest (see Materials and Methods) along with a nuclear label (this method was used for all subsequent cell fate quantitation with the exception of Wnt loss of function, which was performed with a heat shock-inducible *TCFΔC-cherry* fusion plasmid). Transient transgenic cells acted identically to cells from stable transgenic lines (Fig. 3E-G) and their tissue contribution is shown quantitatively in Fig. 3K and Table 1. Our results indicate that Wnt signaling is necessary and sufficient to induce notochord from MPCs that give rise to the floor plate and notochord.

We also performed transplants as before using cells from stable (Fig. 3D) or transient transgenic (Fig. 3H) *hsp70l:sox2-p2a-NLS kikGR* donor embryos transplanted into wild-type host embryos. After heat shock induction of *sox2*, almost all transgenic cells joined the floor plate at the expense of notochord (Fig. 3D,H,K, Table 1). These results indicate that a key function of Wnt signaling required for notochord induction is the transcriptional repression of *sox2*, and provide a mechanistic understanding of how Wnt signaling can induce mesoderm from bipotential neural/mesodermal tailbud progenitor cells. Based on prior studies, we hypothesize that the same mechanism also functions during mesoderm induction in PWPCs (Jurberg et al., 2014; Martin and Kimelman, 2012).

Endoderm induction continues after gastrulation

Although Wnt and Sox2 manipulations clearly showed that germ layer fate decisions continue to be made within the MPCs after gastrulation to generate neural and mesodermal tissues, the effect on endodermal hypochord fate was very subtle. To provide more conclusive evidence that endoderm continues to be induced in the tailbud MPCs after gastrulation ends, we tested for continued responsiveness to Notch signaling, which is required for hypochord induction during gastrulation (Appel et al., 1999; Latimer and Appel, 2006; Latimer et al., 2002; Peyrot et al., 2011). We designed heat shock-inducible vectors in the same manner as the *β-catenin* and *sox2* constructs, to allow inducible expression of the Notch intracellular domain (NICD) or a dominant-negative *Suppressor of Hairless* [*dnSu(H)*] (Wettstein et al., 1997), allowing temporal cell-autonomous activation or inhibition of the Notch signaling pathway in transplanted cells, respectively.

Activated Notch signaling resulted in a dramatic increase in the contribution of MPCs to the hypochord at the expense of notochord (Fig. 4A,C, Table 1). Disrupted Notch signaling completely blocked MPC contribution to hypochord, whereas contribution to notochord was increased (Fig. 4B,C, Table 1). These results indicate that Notch signaling acts continuously on MPC fate determination throughout body formation, and provide the first evidence that endoderm continues to be induced from a multipotent progenitor population after gastrulation ends. The inhibition of Notch signaling had no effect on floor plate contribution, whereas Notch activation caused a slight increase in the percentage of floor plate cells. These results are consistent with the previously reported gastrula stage role of Notch signaling in promoting floor plate cell proliferation, but not affecting floor plate specification (Latimer and Appel, 2006).

Two independent populations of MPCs in the zebrafish tailbud

Our results suggest that the generation of notochord through active Wnt signaling and the generation of notochord due to the absence of Notch signaling are independent processes, operating in two separate MPC populations. The first population generates notochord through positive Wnt signaling in bipotential notochord/floor plate progenitors and is independent of Notch signaling, whereas the second population generates notochord through the absence of Notch signaling within hypochord/notochord progenitors, possibly independently of Wnt signaling. An alternative hypothesis is that a single population of pluripotent cells gives rise to all three cell types, and that both the presence of Wnt and the absence of Notch are required in the same cells for notochord induction. Wnt and Notch signaling commonly interact with each other at the molecular level in an antagonistic manner during fate specification, where Wnt inhibits Notch signaling and vice versa (reviewed by Hayward et al., 2008). In this scenario, notochord induction after positive Wnt signaling would require subsequent Notch inhibition, or notochord induction after Notch inhibition would require Wnt activation. To distinguish between these two models, we performed an analysis of combined loss of Wnt and Notch signaling in whole embryos to determine if notochord tissue is rescued.

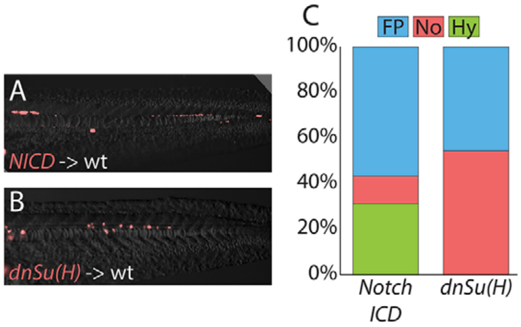

Fig. 4. MPCs remain responsive to changes in Notch signals after gastrulation. Transiently transgenic cells were transplanted into wild-type host embryos, and transgene expression activated after gastrulation had completed. Activated Notch signaling cell-autonomously promotes hypochord and floor plate fates at the expense of notochord (A), and Notch activity is required for cells to adopt a hypochord fate (B), with changes quantitated (C; raw data and statistics are provided in Table 1). Cell fate changes are statistically significant (see Table 1).

DAPT, a small-molecule inhibitor of Notch signaling, was used in combination with genetic manipulations of Wnt and Sox2 to test for notochord rescue. To visualize notochord, we used a *col2a1* probe, which also labels the floor plate and hypochord (Yan et al., 1995). After inhibition of Wnt signaling or activation of *sox2*, *col2a1* expression is lost from the notochord domain and a thin stripe of expression persists (Fig. 5C,E), which is likely to represent the expanded floor plate based on *foxa2* expression (Fig. 5G,I,K). No rescue of *col2a1* expression in notochord was observed when DAPT treatment was combined with other manipulations (Fig. 5A-F). However, we found that Notch inhibition rescued the enhanced expression of the floor plate marker *foxa2* caused by loss of Wnt or overexpression of *sox2* (Fig. 5G-L), consistent with the role of Notch in promoting floor plate proliferation (Latimer and Appel, 2006). Additionally, we found that expanded *sox2* expression resulting from Wnt inhibition did not depend on Notch function (Fig. 5M-P). Together, these results suggest that Wnt and Notch function independently during notochord specification from two different progenitor pools.

To further confirm the existence of separate MPC pools we created a fate map of the MPCs. We marked the starting positions and eventual tissue contribution of clearly distinct MPCs in 18 embryos (75 cells). We found that MPCs initially located dorsal to the notochord contributed only to notochord or floor plate, whereas ventral MPCs contributed to hypochord or notochord (Fig. 5Q). Cells caudal to the posterior end of the notochord were only observed joining the notochord in wild-type embryos. These results provide further evidence of independent MPC populations in the tailbud.

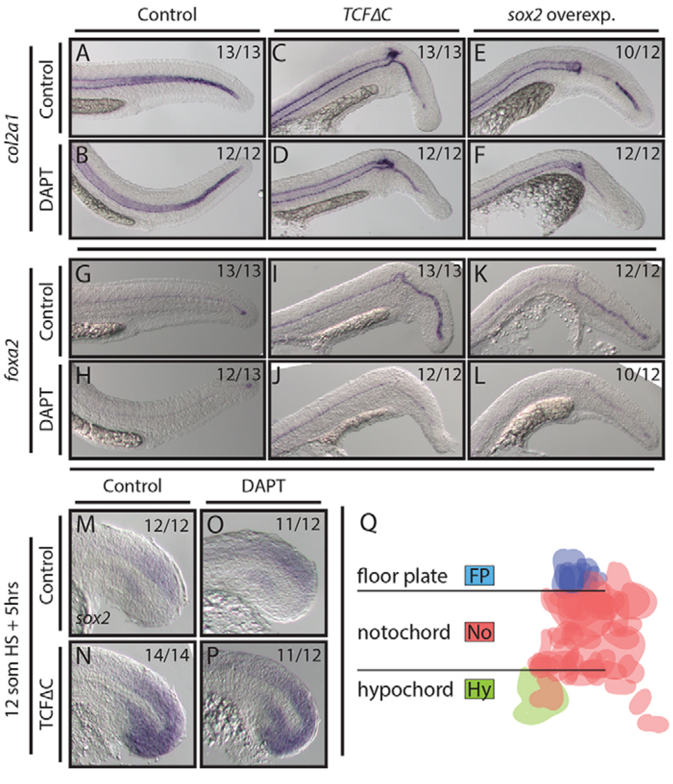

Fig. 5. Wnt and Sox2 pattern midline tissues independently of Notch activity. (A-L) Loss of Wnt signaling and overexpression of *sox2* using stable transgenic lines causes the notochord domain of *col2a1* expression to be lost in the tail (A,C,E), whereas the floor plate marker *foxa2* shows enhanced expression (G,I,K). Combining DAPT treatment with these conditions immediately after the heat shock has no effect on notochord patterning (B,D,F) but does reduce *foxa2* expression (H,J,L). (M-P) Blocking Wnt signaling results in ectopic *sox2* expression throughout the tailbud (M,N). This effect is independent of active Notch signaling (O,P). (Q) A fate map of MPCs (see text for details) indicates that floor plate cells are exclusively derived from a dorsal MPC population (blue), whereas hypochord (green) is derived only from a ventral MPC population. Cells are false colored based on fate; horizontal lines indicate the boundary of the notochord.

Dorsal and ventral MPCs fail to integrate properly in embryos with ectopic tails

The loss of BMP signaling during late gastrulation stages of zebrafish development results in the formation of a single ectopic tail on the ventral side of the primary tail (Connors et al., 1999; Pyati et al., 2005). The ectopic tail frequently contains notochord cells (Gebruers et al., 2013; Pyati et al., 2005; Yang and Thorpe, 2011). We reasoned that this might represent a splitting of the dorsal and ventral MPC populations, which would provide further evidence for the existence of the two MPC populations that we identified by genetic and lineage tracing-based methods. If ectopic tails represent the failure of the dorsal and ventral MPCs to properly integrate, the primary tail posterior to the ectopic tail should include floor plate and notochord but never hypochord, whereas the ectopic tail should always have notochord and hypochord.

In order to visualize notochord in live embryos, we developed a new transgenic reporter line that expresses the photoconvertible *kaede* coding sequence under the control of a 1 kb fragment of the *ntla* promoter. This line exhibits *kaede* mRNA expression specifically in notochord progenitors, and the Kaede protein perdures in the notochord and is absent from the floor plate and hypochord (Fig. 6A,B). In *pntl:kaede* embryos treated with the BMP inhibitor DMH1 at 75-85% epiboly (mid- to late gastrulation), we observed that notochord is always present in both the primary and ectopic tail, indicating that the notochord has split when ectopic tails are present (Fig. 6D,D′; 100%, *n*=24).

To visualize floor plate and hypochord, we used the *sox2-p2a-sfGFP* reporter line, which expresses *sfGFP* in the spinal cord

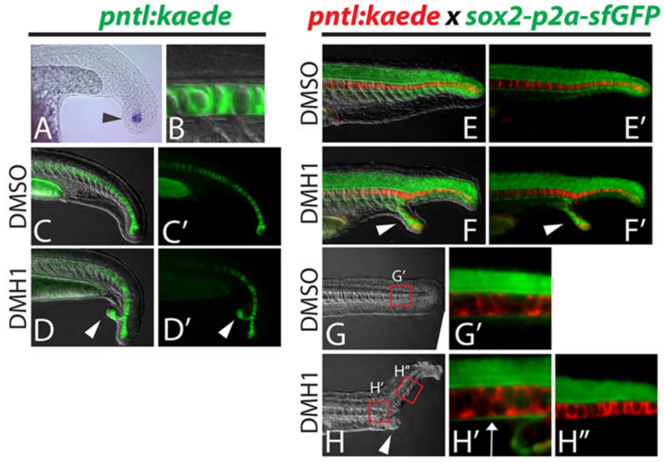

Fig. 6. Dorsal and ventral MPCs separate in embryos with ectopic tails. (A-D′) A newly created transgenic reporter line (*pntl:kaede*) exhibits *kaede* mRNA expression in notochord progenitor cells (A, arrowhead) and Kaede protein perdures in the notochord only (B). Ectopic tails always contain notochord cells (D,D′, arrowheads). (E-H″) Notochord and hypochord were visualized simultaneously by crossing the *pntl:kaede* line to the *sox2-p2a-sfGFP* line and photoconverting the Kaede from green to red. Analysis of ectopic tails at 24 hpf revealed that the hypochord is diverted from the primary midline into the ectopic tail (F,F′, arrowheads). At 36 hpf it is clear that the hypochord is present anterior to the ectopic tail (ectopic tail is pictured in H, arrowhead) as labeled by sfGFP driven by the *sox2* locus (H′, arrow), but is absent in the primary midline posterior to the ectopic tail (H″).

including the floor plate, as well as the hypochord. The *sox2-p2a-sfGFP* reporter line was crossed to the *pntl:kaede* reporter and the Kaede was photoconverted from green to red to allow simultaneous imaging of the notochord, hypochord and floor plate (Fig. 6E,E′). In embryos with ectopic tails, the hypochord extends into the ectopic tail (along with some notochord), and is completely absent from the midline in the primary tail in regions posterior to the ectopic tail (Fig. 6F,F′,H-H″). By contrast, floor plate is continuous in the primary tail anterior and posterior to the ectopic tail (Fig. S1), and previous work has demonstrated that neural tissue, including floor plate, is never found in the ectopic tail (Gebruers et al., 2013; Pyati et al., 2005; Yang and Thorpe, 2011). These results indicate that embryos with ectopic tails undergo a separation of the dorsal and ventral MPC populations, and that ventral MPC derivatives (notochord and hypochord) populate the ectopic tail and dorsal MPC derivatives (notochord and floor plate) reside in the primary tail.

Tailbud notochord progenitors are competent to become paraxial mesoderm

PWPCs give rise to somitic and endothelial mesoderm, but never become notochord (Martin and Kimelman, 2012; Tzouanacou et al., 2009), despite being in close proximity to the MPCs (Kanki and Ho, 1997). Our results demonstrating a Wnt-mediated fate decision between a neural or mesodermal fate for MPCs mirror the results for PWPCs. We hypothesized that MPCs might be at least partially equivalent to PWPCs and adopt different fates in response to slight differences in their locations and environments. To test whether MPCs could be directed to join somites rather than midline tissues, we created a heat shock-inducible *msgn1* transgenic line (*hsp70l:msgn1-p2a-NLS kikGR*). Msgn1 was recently shown in the mouse to be a master regulator of paraxial mesoderm fate, and is normally absent from notochord progenitor cells (Chalamalasetty et al., 2014; Yoo et al., 2003). We asked whether misexpression of *msgn1* in notochord progenitors of the zebrafish tailbud is sufficient to cause them to adopt a paraxial mesoderm fate. When *msgn1* is misexpressed throughout the whole embryo at the start of gastrulation, embryos fail to form a notochord and somites develop across the midline in its place (Fig. 7B,B′). Embryos also appear shorter than their wild-type siblings, which is likely to be due to the ability of *msgn1* to promote the differentiation rather than maintenance of PWPCs, which prematurely exhausts this progenitor pool (Fior et al., 2012; Yabe and Takada, 2012). When *msgn1* is induced throughout the embryo at the end of gastrulation (bud stage), the notochord ends abruptly and there is a transition to midline somite formation (Fig. 7D,D′, arrowhead). The change in fate of midline mesoderm was confirmed by examining the expression of *myoD* (*myod1*), a marker expressed in skeletal muscle of the somite (Halpern et al., 1995; Weinberg et al., 1996), and *col2a1*, which is expressed in the notochord (Yan et al., 1995). In embryos in which *msgn1* was activated at bud stage, there is an expansion of *myoD* expression across the midline (Fig. 7G,G′,H) and a loss of midline *col2a1* expression (Fig. 7J,J′).

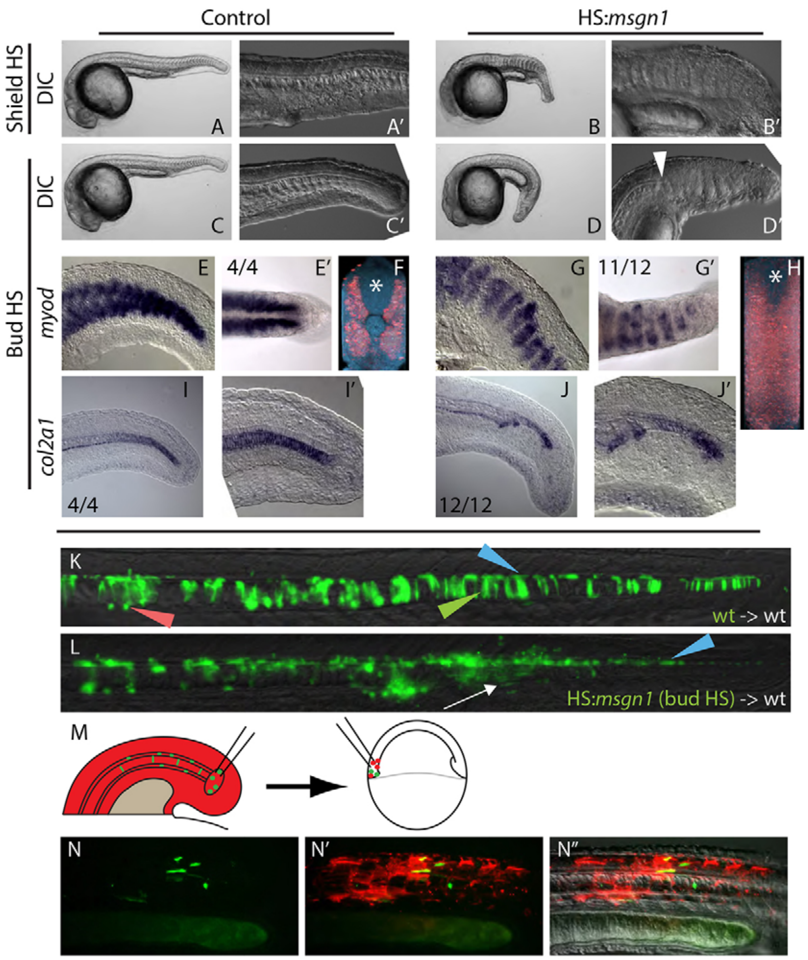

Fig. 7. Local signals regulate MPC versus PWPC fate. (A-B′) Heat shock induction of *msgn1* at the start of gastrulation (shield stage) results in the absence of notochord and the ectopic expansion of somites across the midline at 24 hpf. (C-D′) Heat shock induction of *msgn1* after gastrulation (bud stage) causes the notochord to end abruptly, followed posteriorly by the expansion of somites at the midline at 24 hpf. The arrowhead indicates the transition from notochord to somite at the midline. (E-J′) In embryos heat shocked at bud stage, *myoD* expression at 24 hpf is present in posterior regions of the embryo (E,G) and, when viewed dorsally, can be seen extending across the midline (E′,G′). Fluorescent *in situ* hybridization of *myoD* expression reveals (by digital transverse section) the absence of notochord and presence of muscle across the midline (F,H, asterisk marks the position of the spinal cord). Expression of *col2a1* indicates that the notochord is truncated (I-J′). (K,L) Ectopic *msgn1* expression at bud stage cell-autonomously converts some MPCs to a somitic fate, as seen in transplanted cells. Control donor cells only contribute to floor plate, notochord and hypochord (K); somite contribution was observed in 1/16 embryos. By contrast, *HS:msgn1* donor cells transition from notochord contribution to somite contribution (L, white arrow; notochord to somite switch was observed in 12/16 embryos) without affecting floor plate contribution. Green, red and blue arrowheads indicate notochord, hypochord and floor plate, respectively. (M-N″) A serial transplantation scheme (M) was used to target MPCs to the PWPCs. MPCs (green) from the 12-somite stage tailbud are able to contribute to skeletal muscle tissue when analyzed at 24 hpf (N-N″).

In order to determine whether *msgn1* acts cell-autonomously to induce a fate change from notochord to paraxial mesoderm, we transplanted *hsp70l:msgn1-p2a-NLS kikGR* cells into the MPC population of wild-type host embryos and heat shocked the hosts at bud stage. In embryos in which there is only midline contribution before the heat shock, misexpression of *msgn1* causes a cell-autonomous fate change from notochord to somite, based on the absence of notochord cells and presence of somite cells, but does not inhibit floor plate formation (Fig. 7L). These results are similar to previous reports of the effects of ectopic *msgn1* expression in zebrafish during gastrulation, which is sufficient to repress midline fates and induce presomitic mesoderm fates (Yabe and Takada, 2012).

We performed a novel transplant strategy to further confirm that it is the physical location and local signaling environment that direct MPCs to a notochord instead of a somite fate, rather than an inherent difference in potential. This method was developed to transplant MPCs into the PWPC population in order to determine if the MPCs would contribute to somites if they resided in the PWPC signaling environment (Fig. 7M). We were successful in transplanting cells from the tailbud ten times, with three transplants containing MPCs. Of these, two were able to contribute to somites and form muscle (Fig. 7N-N″). This result further suggests that MPCs are competent to respond to the PWPC signaling environment and become somite tissue.

DISCUSSION

The entire vertebrate posterior body is derived from multipotent progenitor cell populations

The major tissues of the posterior body of vertebrate embryos, including the somites, spinal cord, vasculature, notochord and hypochord (dorsal endoderm), are derived from the tailbud (Beck, 2015; Wilson et al., 2009). Prior work demonstrated that the PWPCs have germ layer plasticity and give rise to the somites, vasculature and spinal cord (Martin and Kimelman, 2012; Takemoto et al., 2011; Tzouanacou et al., 2009). It can also be assumed that in zebrafish the fin mesenchyme, which originates solely from presomitic mesoderm, is derived from PWPCs of the tailbud, as this is the major source of posterior paraxial mesoderm (Lee et al., 2013). Here we show that the remainder of the posterior body is derived from two populations of germ layer plastic MPCs. A dorsal MPC population gives rise to floor plate and notochord, and a ventral MPC population becomes notochord and hypochord (Fig. 8).

Based on our work, along with the previous analysis of PWPCs, we provide a hybrid view of two historically disparate models of the tailbud. The ‘blastema’ model proposed that the tailbud is a mass of undifferentiated cells that can be induced to form any tissue type of the elongating body axis, whereas the ‘fixed fate’ model suggested that the tailbud is a mosaic of lineage-specific progenitor cells (Holmdahl, 1925; Pasteels, 1943). Our work supports a model situated between the two extremes, in which the zebrafish tailbud is composed entirely of basal progenitor cells capable of giving rise to multiple germ layers, but which reside in specific physical or molecular compartments that prevent cells within a particular compartment from joining all of the tissues of the axis (Fig. 8). For example, we showed that *msgn1* must be excluded from MPCs in order for mesodermal descendants to join the notochord rather than somites. Expression of *msgn1* in cells that will form somites involves positive inputs from T-box transcription factors, canonical Wnt signaling, and FGF signaling, which may only occur at the correct levels in the region of the PWPCs (Fior et al., 2012; Wittler et al., 2007; Yabe and Takada, 2012). Alternatively, the unique signaling environment of the MPCs can turn on the specific expression of transcriptional repressors such as *flh*, which may inhibit *msgn1* expression, similar to its normal role in inhibiting *tbx16* (also known as *spadetail*) expression (Amacher and Kimmel, 1998). Importantly, the loss of *flh* function causes a fate change from notochord to somite, suggesting that the MPCs are in a unique environment that prevents them from adopting PWPC fates (Halpern et al., 1995; Talbot et al., 1995).

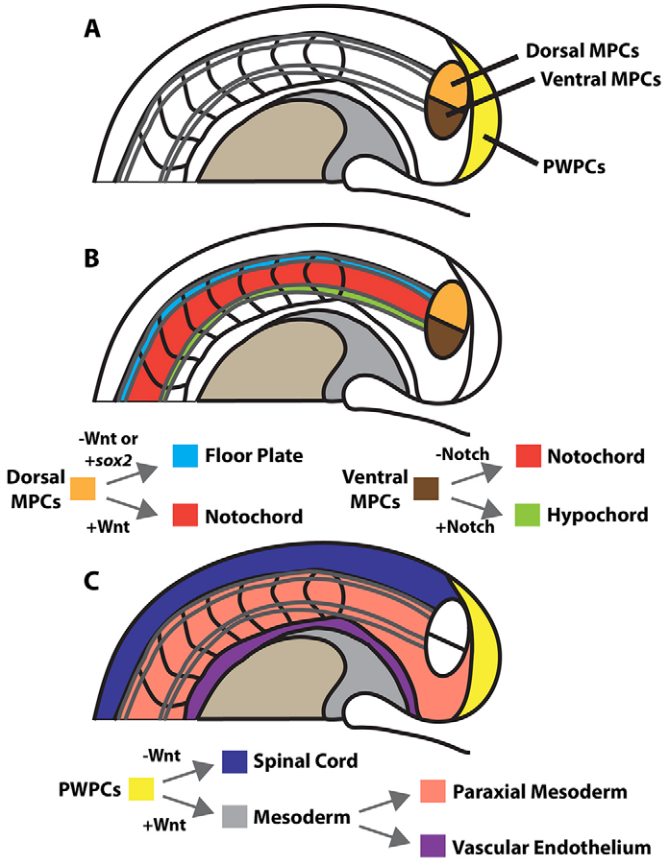

Fig. 8. Model of the progenitor populations in the zebrafish tailbud and the signals that allocate cell fate. (A) The entire posterior body is derived from three distinct germ layer plastic progenitor cell populations. Midline tissues of the medial floor plate, notochord and hypochord originate from two MPC populations. (B) The dorsal population (orange) gives rise to notochord and floor plate depending on canonical Wnt signaling-mediated repression of *sox2* expression. The ventral population (brown) gives rise to notochord and hypochord depending on the presence or absence of Notch signaling. (C) A model of the PWPCs modified from that of Martin and Kimelman (2012), in which PWPCs (yellow) generate spinal cord and mesoderm, and the mesoderm subsequently gives rise to paraxial mesoderm and vascular endothelium. The neural ectoderm/mesoderm fate decision in both the PWPCs and dorsal MPCs is mediated by canonical Wnt signaling (B,C).

Two populations of MPCs in the tailbud

A surprising finding of this study is that the tailbud contains not one, but two, MPC populations. The ventral population contributes to hypochord and notochord, and the dorsal population gives rise to notochord and floor plate. Previous studies in zebrafish and mouse have indicated that the notochord may be derived from multiple sources. In zebrafish, cells of Kupffer’s vesicle, which is a ciliated organ of asymmetry, originate from dorsal forerunner cells, which are distinct from shield-derived axial mesoderm (Melby et al., 1996). During tailbud stages, Kupffer's vesicle collapses and some of these cells join the posterior notochord but never join the floor plate or other spinal cord fates (Melby et al., 1996). In mouse embryos, a specialized group of cells at the periphery of the node, called the node crown cells, give rise exclusively to tail notochord but not floor plate, and appear to be distinct from other node-derived notochord cells (Cambray and Wilson, 2002; Yamanaka et al., 2007). Therefore, two populations of cells giving rise to posterior notochord might be a common feature of vertebrate development.

The two populations of MPCs that we observed in the tailbud provide an embryological basis for posterior bifurcated notochord phenotypes in zebrafish and mouse, as well as the rare split notochord syndrome (SNS) birth defect observed in humans. In zebrafish, loss of BMP signaling during late gastrulation or early somitogenesis stages results in a partially penetrant phenotype of an ectopic tail on the ventral side of the posterior embryo (Pyati et al., 2005; Stickney et al., 2007; Yang and Thorpe, 2011). The same phenotype occurs after loss of non-canonical Wnt signaling, and in both cases the posterior notochord is bifurcated along the dorsal-ventral axis, with one part in the normal notochord domain and the other extending into the ectopic ventral tail (Gebruers et al., 2013; Pyati et al., 2005; Yang and Thorpe, 2011). This phenotype arises from defective cell migration (Yang and Thorpe, 2011). We showed that embryos with ectopic ventral tails exhibit a separation of the two MPC populations, with the ectopic tail containing descendants of the ventral MPCs, including hypochord and notochord, whereas the primary midline posterior to the ectopic tail contains only descendants of the dorsal MPCs (floor plate and notochord). We hypothesize that BMP and non-canonical Wnt signaling are required for the proper migration and/or coalescence of the two independent MPC populations. Ectopic tails also contain somitic tissue (Pyati et al., 2005; Yang and Thorpe, 2011), which may be due to the organizer activity of the tailbud, as observed in frog embryos (Gont et al., 1993). Alternatively, this might be due to respecification of some of the ventral MPCs as they enter a different signaling environment, similar to that observed upon expanding *msgn1* expression into the tailbud MPCs.

During mouse development, loss of EphA2 function causes a very similar dorsal-ventral bifurcation of the posterior notochord (Naruse-Nakajima et al., 2001), indicating that proper morphogenesis in the mammalian tailbud is also likely to be required for the integration of two separate notochord (or possibly MPC) populations. Our work, as well as that of others, provides a molecular and embryological framework with which to investigate the etiology of the rare congenital defect of SNS, in which the posterior notochord is bifurcated, leading to abnormal patterning of the posterior trunk (Yazici et al., 2014).

Canonical Wnt signaling is a conserved regulator of all neural-mesodermal fate decisions in the zebrafish tailbud

Our finding that changes in Wnt signaling after the completion of gastrulation can alter the fate distribution of MPCs provides evidence that these cells continue to make germ layer decisions during post-gastrula stages. Combined with results from PWPCs in zebrafish, mouse and cell culture (Bouldin et al., 2015; Garriock et al., 2015; Gouti et al., 2014; Henrique et al., 2015; Jurberg et al., 2014; Martin and Kimelman, 2012; Tsakiridis et al., 2014), our data indicate that Wnt signaling has a conserved role in the induction of all tailbud mesoderm from discrete bipotential neuromesodermal progenitors. As in PWPCs, Wnt induces mesodermal fate through repression of *sox2* expression. In PWPCs, Wnt-mediated repression of *sox2* is accomplished by the repressor activity of the Wnt target T-box transcription factors *tbx16* in zebrafish and *Tbx6* in mouse (Bouldin et al., 2015; Takemoto et al., 2011). The identity of the intermediate effector of Wnt-mediated repression of *sox2* in the MPCs is unclear. If it is also a T-box transcription factor, *tbx2b* is a likely candidate in zebrafish based on its spatiotemporal expression pattern and role in notochord formation (Dheen et al., 1999).

During mammalian development, all floor plate is induced by the underlying notochord through Hedgehog signaling (Chiang et al., 1996; Ding et al., 1998; Matise et al., 1998; Placzek and Briscoe, 2005), making it unclear whether a similar Wnt-mediated allocation mechanism between notochord and floor plate also functions in mammals. Floor plate formation in zebrafish involves both Hedgehog-dependent and -independent processes. The lateral floor plate is induced by notochord-derived Hedgehog signaling, but the medial floor plate originates from a common notochord/floor plate progenitor that does not depend on Hedgehog signaling (Charrier et al., 2002; Chen et al., 2001; Odenthal et al., 2000; Strahle et al., 2004). Medial floor plate formation instead depends on the proper allocation from a common notochord and floor plate progenitor (Halpern et al., 1997). Based on fate-mapping studies and genetic manipulations, the allocation model of medial floor plate formation is predicted to occur in other species as well, such as frog and chick (Le Douarin and Halpern, 2000). Future work is required to determine whether Wnt repression of *sox2* is a common mechanism of notochord/floor plate determination from MPCs in other vertebrates.

Conclusions

Our work highlights the strengths of zebrafish as a model organism, including the ability to temporally manipulate signaling in individual cells, allowing for the precise determination of factors regulating cell fate at the cell-autonomous level. These methods allowed us to show that three midline embryonic structures with key patterning roles, namely the hypochord, notochord and floor plate, are continuously generated after gastrulation from basal MPCs located within the tailbud. This indicates, along with prior work, that all non-epidermal tailbud-derived tissues in zebrafish are generated from discrete basal progenitor populations capable of giving rise to at least two different germ layers (Martin and Kimelman, 2012; Tzouanacou et al., 2009). Our results show the presence of two distinct MPC populations in the tailbud: one that contributes to hypochord and notochord and a second that gives rise to notochord and floor plate. This work will provide insight into poorly understood human diseases associated with defective notochord development, including SNS and the rare but highly lethal cancer chordoma (Nibu et al., 2013; Yazici et al., 2014). Chordoma is an aggressive tumor type found along the axis of the body, and is thought to be derived from notochord remnants that persist from embryonic stages into adulthood (Nibu et al., 2013).

MATERIALS AND METHODS

Generation of *hsp70l:sox2-p2a-NLS kikGR*, *hsp70l:msgn1-p2a-NLS kikGR* and *pntl:kaede* transgenic zebrafish

All zebrafish procedures were performed in accordance with and approved by the Stony Brook University Institutional Animal Care and Use Committee (IACUC). *sox2* and *msgn1* were inserted without their stop codon into the *hsp70l:p2a-NLS kikGR* vector (Bouldin et al., 2014) to create *hsp70l:sox2-p2a-NLS kikGR* and *hsp70l:msgn1-p2a-NLS kikGR*. The plasmid constructs were co-injected with *in vitro* transcribed *tol2* transposase mRNA to create stable transgenic lines *Tg(hsp70l:sox2-p2a-NLS kikGR)* and *Tg(hsp70l:msgn1-p2a-NLS kikGR)* with allele designation SB100 and SB101, respectively (Kawakami, 2004). A 1 kb fragment of the *ntla* promoter (*pntl*) directly upstream of the start codon was cloned upstream of the *kaede* coding sequence. A stable transgenic line was made using *tol2*-based methods.

Induced expression in transgenic and transiently transgenic embryos

Transgenic embryos were incubated for 30 min in prewarmed embryo medium at 40°C (41°C for *hsp70l:β-Catenin-TFP* embryos) to induce transgene expression. Transient transgenic embryos were created by co-injecting 25 pg *tol2* transposase mRNA with 25 pg of one of the following plasmids: *hsp70:TCFΔC-cherry*; *hsp70l:β-Catenin-p2a-NLS kikGR*; *hsp70l:sox2-p2a-NLS kikGR*; *hsp70l:NICD-p2a-NLS kikGR*; *hsp70l:dnSu(H)-p2a-NLS kikGR*; or the *hsp70l:p2a-NLS kikGR* vector without insert. Expression in transient transgenic cells was induced by 39°C heat shock. Heat shocks were performed on bud stage embryos unless otherwise specified.

In situ hybridization and small-molecule treatment

Standard alkaline phosphatase and fluorescent *in situ* hybridization reactions were performed as previously described (Griffin et al., 1995; Lauter et al., 2011). The small-molecule inhibitors DMH1 or DAPT (both Selleck Chemicals) were used at 10 µM or 100 µM, respectively.

Cell transplantation

Donor embryos were injected with 1% Rhodamine-dextran alone or in combination with plasmid DNA and *tol2* transposase mRNA. 25-50 cells from sphere stage (mid-blastula) donor embryos were transplanted into the margin of 1000-cell stage hosts using a CellTram (Eppendorf). Donor cells could be taken from any part of the embryo without affecting the tissue to which they contributed; cells were taken from the animal pole for convenience. This strategy maximizes the contribution of transplanted cells to the midline progenitor zone (Halpern et al., 1995).

MPC to PWPC transplantation

Donor embryos were injected with 1% Fluorescein-dextran and host embryos were injected with 1% Rhodamine-dextran. Transplants to target the MPC population were performed as above. Host embryos containing donor cells only within the midline or MPC populations were isolated at the 12-somite stage and used as MPC donors. Using forceps, the epidermis was stripped from the tailbud to facilitate cell removal. Cells were transplanted from the tailbud to the ventral margin of unlabeled wild-type host embryos, where PWPCs originate.

Microscopy and image analysis

Time-lapse imaging and imaging of fluorescent embryos were performed on either a DMI6000B microscope equipped with a DFC360 FX camera (Leica) or a spinning disk confocal microscope (Zeiss AxioImager with Yokogawa CSU-10 scanner unit). Bright-field images were obtained using a M165FC microscope (Leica) equipped with an Infinity 3 camera (Lumenera). Images were prepared using Photoshop (Adobe) or ImageJ (Schneider et al., 2012). Time-lapse images were aligned using the ImageJ plugin 'linear stack alignment with SIFT' (Lowe, 2004) and, in some cases, the 'straighten' command was used for ease of analysis.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

S.R.T. and H.G. performed experiments; R.H.R. and B.L.M. performed experiments, analyzed the data and wrote the manuscript.

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Supplementary information

Supplementary information available online at
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