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Notch mediates Wnt and BMP signals in the early separation of smooth muscle progenitors and blood/endothelial common progenitors

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During embryonic development in amniotes, the extraembryonic mesoderm, where the earliest hematopoiesis and vasculogenesis take place, also generates smooth muscle cells (SMCs). It is not well understood how the differentiation of SMCs is linked to that of blood (BCs) and endothelial (ECs) cells. Here we show that, in the chick embryo, the SMC lineage is marked by the expression of a bHLH transcription factor, *dHand*. Notch activity in nascent ventral mesoderm cells promotes SMC progenitor formation and mediates the separation of SMC and BC/EC common progenitors marked by another bHLH factor, *Scl*. This is achieved by crosstalk with the BMP and Wnt pathways, which are involved in mesoderm ventralization and SMC lineage induction, respectively. Our findings reveal a novel role of the Notch pathway in early ventral mesoderm differentiation, and suggest a stepwise separation among its three main lineages, first between SMC progenitors and BC/EC common progenitors, and then between BCs and ECs.

KEY WORDS: Chicken, Chick, Ventral mesoderm, Primitive streak, Smooth muscle cell, Blood cell, Endothelial cell, Notch, BMP, Wnt, dHAND, Scl, Tal1, Hemangioblast, Progenitor

INTRODUCTION

During primitive hematopoiesis, blood cells (BCs) and endothelial cells (ECs) are generated in the extraembryonic mesoderm from a common pool of progenitors (Baron, 2003; Lugus et al., 2005; Robertson et al., 1999; Vogeli et al., 2006; Weng et al., 2007), hereby called BC/EC progenitors. The extraembryonic mesoderm in birds and mammals is composed of somatic (adjacent to ectoderm) and splanchnic (adjacent to endoderm) parts, separated by the extraembryonic coelom (Downs, 2004; Duval, 1889; Jollie, 1990; Sabin, 1920) (Fig. 1A). The BC/EC progenitors aggregate to form blood islands within the extraembryonic splanchnic mesoderm, which contains in addition vascular SMCs (Kessel and Fabian, 1985; Murphy and Carlson, 1978; Sabin, 1920). The extraembryonic somatic mesoderm gives rise to mesothelial cells lining the ectoderm, from which the amnionic and chorionic membranes are derived (Adamstone, 1948; Oppenheim, 1966; Pierce, 1933; Romanoff, 1960; Wu et al., 2001). BC/EC progenitor markers, such as *Scl* (also known as *Tal1*) (Kallianpur et al., 1994), start to be expressed during early cell migration to populate the extraembryonic region (Minko et al., 2003). In the chick embryo, *Scl*-positive cells form soon after ventral mesoderm ingression at stage HH4. These cells aggregate to form blood islands at stage HH6, and the separation of BC and EC lineages starts with the initiation of globin gene expression in BCs at stage HH7 (Nakazawa et al., 2006). It is not well understood how the differentiation of BCs and ECs is linked to that of other cell types, mainly SMCs, in the ventral mesoderm population.

The roles of the Notch pathway in ventral mesoderm differentiation are not clear. Mouse mutant analyses of Notch receptors and other Notch pathway components have revealed a crucial role of the Notch pathway in angiogenic vascular remodeling and in artery/vein specification, but not in early hematopoiesis and vasculogenesis (Gridley, 2007). Its role in SMC differentiation has not been carefully studied in early development. Later in vivo and in vitro studies have shown a clear involvement of the Notch pathway in SMC differentiation, albeit with contradictory results (Doi et al., 2006; Doi et al., 2005; High et al., 2007; Morrow et al., 2005; Proweller et al., 2005).

In this work, we investigated the involvement of the Notch pathway in early extraembryonic mesoderm differentiation in the chick embryo. We show that the bHLH transcription factor *dHand* is a marker for both early and late SMC lineages. The segregation of SMC progenitors and BC/EC common progenitors, mediated by Notch activity, takes place soon after mesoderm ingression and before the separation of BCs and ECs. Furthermore, our data indicate that the primary function of the Notch pathway is to mediate the balance between SMCs and BC/ECs, instead of to induce SMC progenitors. Finally, we provide evidence that the Notch pathway functions in the context of two other main pathways (BMP and Wnt) that are also active during early ventral mesoderm differentiation.

MATERIALS AND METHODS

DNA constructs and embryology

Fertilized hens' eggs, purchased from Shiroyama Farm (Kanagawa, Japan), were incubated to desired stages at 38.5°C, after which electroporation and ex vivo culture was carried out as described previously (Nakazawa et al., 2006). Fertilized quail eggs were purchased from Tokaiyuki (Toyohashi, Japan). dENotch1 (a gift from R. Kopan, Washington University, MS, USA) (Schroeter et al., 1998) with a 12×Myc-tag, DnSu(H) (a gift from Dr C. Kintner, Salk Institute, CA, USA) (Wettstein et al., 1997) with a 6×Myc-tag, dHAND (a gift from M. Howard, Medical University of Ohio, OH, USA) (Howard et al., 1999) with a 6×Myc-tag, and Scl (a gift from A. Chiba, University of Tokyo Hospital, Japan) (Kunisato et al., 2004) with a 6×Myc-tag were sub-cloned into the pCAGGS vector (a gift from H. Niwa, RIKEN, Japan) (Niwa et al., 1991), and a 3-4 μg/μl DNA concentration was used for electroporation. The CA-ALK6 expression construct was a gift from Dr H. Kondoh (Osaka University, Osaka, Japan) and Dr K. Miyazono (University of Tokyo, Tokyo, Japan). The CA-β-Catenin expression construct was a gift from Dr H. Kondoh (Osaka University, Osaka, Japan) and Dr A. Nagafuchi (Kumamoto University, Kumamoto, Japan). A 1.0-1.5 μg/μl final DNA concentration of a GFP-expression construct was used in cases where lineage distribution was revealed by co-electroporated GFP. The DNA constructs for

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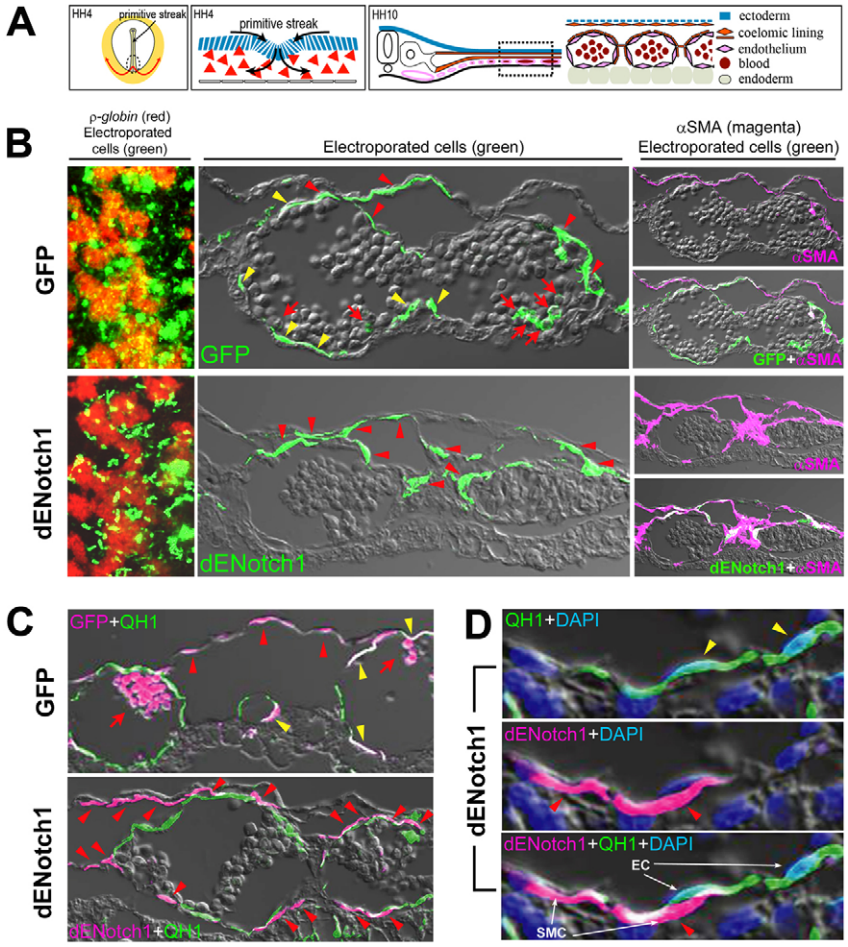

Fig. 1. Notch active cells are biased to become SMCs in chick. (**A**) Schematics of ventral mesoderm cell ingression from the posterior primitive streak at stage HH4 and their contribution to three major lineages at HH10. (**B**) Cells expressing dENotch1 become SMCs at HH10 (lower panels), compared with GFP-expressing cells, which contribute to all three lineages (upper panels). (Left) Whole-mount views (red, *p-globin*; green, electroporated cells); (middle) sections showing the contribution of electroporated cells (red arrowheads, SMCs; yellow arrowheads, ECs; red arrows, BCs); (right) same sections as in middle panel co-stained with αSMA. (**C**) A similar SMC contribution of dENotch1-expressing cells is seen in quail embryos. Red arrowheads, SMCs; yellow arrowheads, ECs; red arrows, BCs; green, QH1 co-staining. (Upper panel) Control GFP-expressing cells contribute to all three lineages; (lower panel) dENotch1-expressing cells have a predominant SMC contribution. Most SMCs can be clearly distinguished from QH1-positive ECs. (**D**) Magnified view of a region in the lower panel of C. SMCs that are closely associated with the vasculature can still be distinguished from ECs under high magnification.

making *Notch1* and *Delta1* probes were kindly given by S. Yasugi (Tokyo Metropolitan University, Japan). The *Nrarp* probe corresponds to nucleotides 1-345 of NCBI # XM428951; the *Scl* probe to 717-1750 of NM205352; and the *dHand* probe to 167-1135 of BBSRC chicken EST contig #333817.4. The CA-FGFR2 expression construct, SU5402 treatment, and *Lmo2*, *Vegfr2* and *Ets1* probes have been previously described (Nakazawa et al., 2006). For the inhibition of the Notch pathway, DAPT (Calbiochem #565770) was dissolved in DMSO and added to albumin to give a final concentration of 50 μM (100 μM was used for the rescue of *Scl* expression in Notch-active cells). The dose effect graph in Fig. 6E indicates the relative concentration of the dENotch1 construct compared with the CA-ALK6 construct (e.g. 3.5× means 0.84 μg/μl of CA-ALK6 and 2.9 μg/μl of dENotch1). *P*-values for the statistical analyses were calculated by using a two-tailed *t*-test.

In situ hybridization and immunohistochemistry

Whole-mount in situ hybridization was carried out following a standard protocol (Stern, 1998; Streit and Stern, 2001). The Cy3 TSA plus system (PerkinElmer #NEL744) was used to reveal AP/fluorescent double in situ hybridization. The TSA system was also used for anti-pSmad1/5/8 detection. For immunohistochemistry, the following antibodies were used: QH1 (Developmental Studies Hybridoma Bank) for quail endothelial cells; anti-GFP (Molecular Probes #47894A and #40351A) for GFP protein; anti-MYC (Santa Cruz Biotechnology #sc-47694 and MBL #562) for dENotch1, dHAND and SCL; HRP-coupled secondary antibody (Santa Cruz Biotechnology #sc-2004); Alexa 488- or Alexa 568-coupled secondary antibodies (Invitrogen #48619A and #48029A); anti-rabbit Red Blood Cell (Rockland #103-4139); anti-αSMA (Abcam #ab5694) and anti-phospho-Smad1/5/8 (Cell Signaling #9511). Embryos were embedded in paraffin for 7- to 10-μm thin sectioning. Sections were mounted in MountQuick (DAIDO SANGYO #DM-01) or ProLongGold (Invitrogen #P49192A), and analyzed using an Olympus SZX12 or an Olympus FV1000 microscope.

RESULTS

Notch active cells contribute exclusively to the SMC lineage

To investigate roles of the Notch pathway in ventral mesoderm differentiation, we generated an expression construct for a constitutively active Notch, dENotch1. dENotch1 has the entire extracellular domain removed, but retains the transmembrane and intracellular domains, and thus causes ligand-binding-independent, but secretase-mediated cleavage-dependent, activation of the Notch pathway (Sato et al., 2008; Schroeter et al., 1998). Expression constructs were electroporated at stage HH3, when the majority of extraembryonic mesoderm-fated cells undergo ingression through the posterior primitive streak. The fate of electroporated cells was analyzed at stage HH10 when different lineages can be readily distinguished. Control GFP-expressing cells contributed to all three lineages (Fig. 1B), as reported previously (Nakazawa et al., 2006). dENotch1-expressing cells failed to contribute to either the BC or EC lineage, but instead contributed predominantly to the SMC lineage (Fig. 1B). Co-staining with alpha smooth muscle actin (αSMA) indicated that these dENotch1-positive cells initiated normal SMC differentiation (Fig. 1B). Similarly, when tested in the quail embryo, dENotch1-expressing cells lead to exclusive SMC contribution (Fig. 1C, red arrowheads in bottom panel), whereas control GFP-expressing cells contributed to all three lineages (Fig. 1C). Confocal microscopy analysis revealed that dENotch1-expressing cells adjacent to forming extraembryonic vessels are mutually exclusive with QH1-positive ECs (Fig. 1C,D). These data suggested that levels of Notch activity might influence the segregation of SMCs and BC/ECs.

Notch pathway is active during early ventral mesoderm differentiation

We next investigated the timing of Notch function during SMC differentiation from stage HH3 to stage HH10. In situ hybridization analyses revealed that *Notch1*, *Delta1*, and the Notch pathway components *Hairy2*, *Herp2* and *Lunatic-fringe* (*L-fringe*; Fig. 2A,D; data not shown) are expressed from stage HH3 in the posterior primitive streak where ventral mesoderm cells are being generated. Some of these expression patterns had been reported previously (Caprioli et al., 2002; Jouve et al., 2002). Furthermore, a Notch activity-regulated target gene, *Nrarp* (Notch-regulated ankyrin repeat protein) (Krebs et al., 2001; Lamar et al., 2001), is also strongly expressed in the posterior primitive streak (Fig. 2A,D), suggesting that the Notch pathway is active during ventral mesoderm formation. Sections revealed a prominent non-uniform distribution of *Delta1* and *L-fringe* (Fig. 2B), and to a lesser degree of other pathway members (data not shown), in the epiblast and nascent ventral mesoderm cells. Confirming the ability of the dENotch1 construct to activate the Notch pathway, ectopic expression of dENotch1 resulted in ectopic expression of *Nrarp* (Fig. 2C; see also Fig. S1A in the supplementary material) and *L-fringe* (see Fig. S1B in the supplementary material). Treatment of embryos from stage HH3 to stage HH4 with DAPT, a Notch pathway inhibitor (Dovey et al., 2001), resulted in the reduction of both endogenous (Fig. 2C,D) and induced (Fig. 2C; Fig. S1A in the supplementary material) Notch activity in the posterior primitive streak. This was in contrast to Notch activity in the neuroectoderm, which did not show a prominent reduction with DAPT treatment (Fig. 2D). After ingression, ventral mesoderm cells migrate extensively to populate the extraembryonic territory and initiate cellular differentiation. During these processes, however, the Notch pathway does not seem to be active, as indicated by a complete lack of expression of *Delta1*, *L-fringe* and *Nrarp*, and weak expression of *Notch1*, *Herp2* and *Hairy2* in the extraembryonic regions at stages HH6-HH7 and HH10, even after an extended period of in situ staining (see Fig. S1C in the supplementary material).

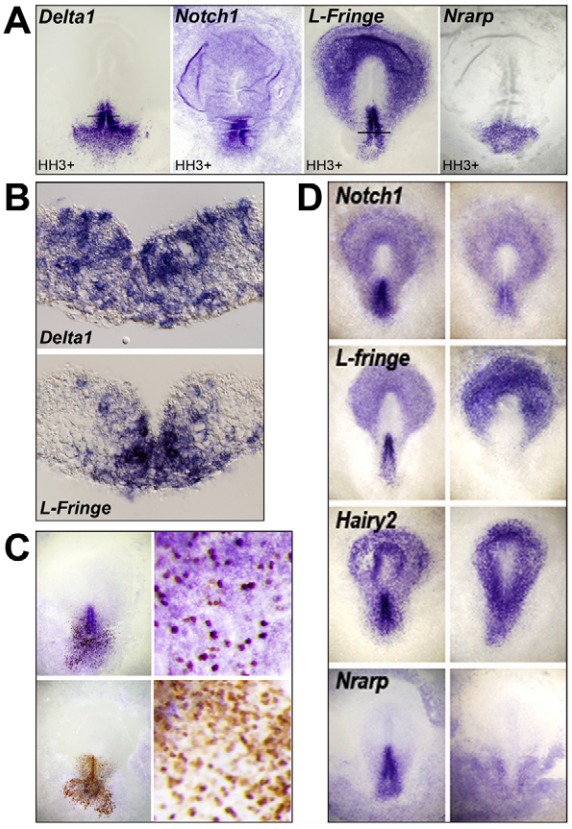

Fig. 2. The Notch pathway is active during early extraembryonic mesoderm generation. (**A**) Expression of *Delta1*, *Notch1*, *Lunatic-fringe* (*L-fringe*) and *Nrarp* at HH3+. Lines indicate section levels shown in B. (**B**) Sections showing *Delta1* and *L-fringe* expression at the posterior primitive streak level indicated in A. Salt-and-pepper positive staining for *Delta1* (in both the epiblast and newly ingressed cells) and *L-fringe* (weakly in the epiblast cells and strongly in the newly ingressed cells) can be seen. (**C**) dENotch1 can induce *Nrarp* cell-autonomously, and both induced and endogenous *Nrarp* expression can be repressed by DAPT. (Top-left panel) DMSO treatment after dENotch1 electroporation; (bottom-left panel) DAPT treatment after dENotch1 electroporation. Right panels show magnified views near the posterior primitive streak of embryo shown on the left. (**D**) Inhibition of the Notch pathway by DAPT results in a specific reduction (right) of Notch pathway genes in the posterior primitive streak (left). The neural plate expression of *Notch1*, *L-fringe* and *Hairy2*, however, is not affected.

dHAND is a marker for early and late SMCs

Because the Notch pathway is active during early ventral mesoderm formation and because dENotch1-expressing cells are biased to become SMCs, we investigated whether a simple binary choice between progenitors for SMCs and BC/ECs is being made based on Notch activity. No SMC progenitor-specific marker has been described so far in the literature. We therefore searched for early SMC markers through an in situ-based screen and found *dHand*, a bHLH transcription factor, to be an ideal early SMC marker (Fig. 3A). Although no detailed expression analysis has been reported for *dHand* during extraembryonic mesoderm differentiation in amniotes, mice with a *dHand*-null mutation have severe defects in yolk sac vasculature that are due to a failure of vascular SMC progenitors to differentiate and form proper contacts with ECs (Srivastava et al., 1997; Yamagishi et al., 2000). In the chick embryo, *dHand* is expressed at stage HH10 in both the vascular smooth muscle layer and the extraembryonic somatopleural mesoderm layer (Fig. 3A,B), with both being co-positive for αSMA (Fig. 3C). These two smooth muscle layers line the extraembryonic coelom and have been variably called mesothelial layers or coelom linings. In this work, they are referred to as the somatic SMC layer (upper) and the vascular SMC layer (lower). During early ventral mesoderm formation, *dHand* was seen to be expressed in a salt-and-pepper pattern in nascent extraembryonic mesoderm populations (Fig. 3D; see also Fig. S2C in the supplementary material). A similar salt-and-pepper pattern of expression was observed for the BC/EC marker *Scl* (see Fig. S2A,B in the supplementary material). Double in situ analysis for *dHand* and *Scl*, however, revealed mutually exclusive patterns of *dHand* and *Scl* at stage HH4-HH5 (Fig. 3D; see also Fig. S3D in the supplementary material), suggesting that *dHand*-positive SMC progenitors and *Scl*-positive BC/EC progenitors are being segregated soon after ingression.

dHAND and Scl are mutually inhibitory and act after Notch mediated segregation

We then wished to determine whether Notch activity plays a role in the mutual exclusion of *dHand* and *Scl* expression. When the dENotch1-expression construct was electroporated into posterior streak cells at stage HH3 and analyzed at stage HH4-HH5, most dENotch1-positive cells were seen to be co-positive for *dHand* expression; dENotch1 staining was excluded from *Scl*-positive cells

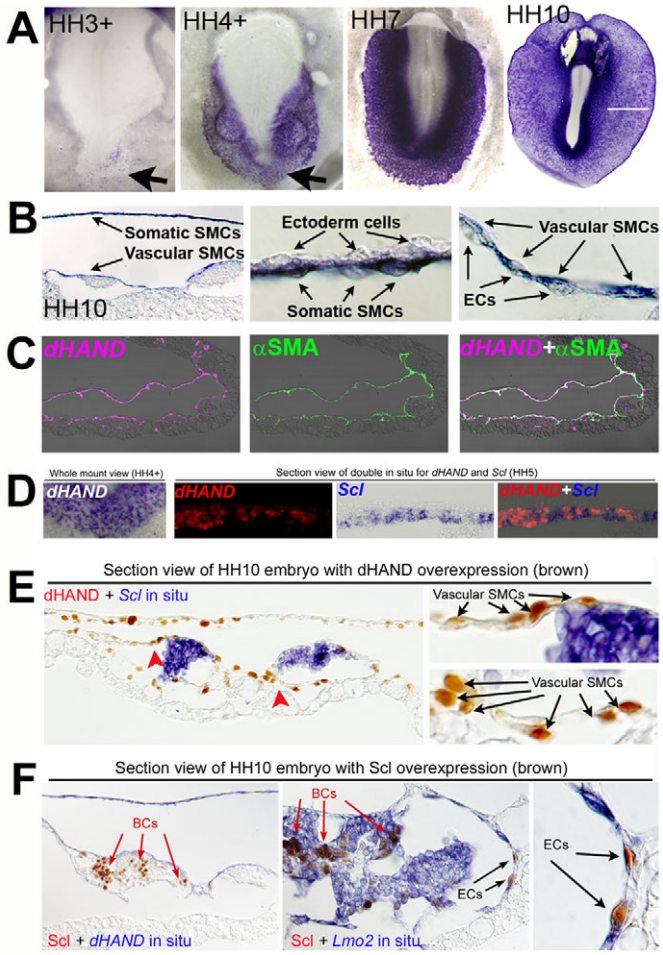

Fig. 3. Mutual antagonism of dHAND and Scl. (**A**) *dHand* expression from HH3 to HH10 in whole-mount views. Arrows indicate nascent extraembryonic mesoderm expressing *dHand*, which marks the SMC lineage. (**B**) Sections of an HH10 embryo, indicating *dHand* expression in both somatic (left and middle panels) and vascular (left and right panels) SMCs. (**C**) *dHand*-expressing cells are co-positive for αSMA. (**D**) *dHand* and *Scl* are expressed in non-overlapping cells during early extraembryonic mesoderm generation (HH4-HH5). (**E**) dHAND-overexpressing cells are *Scl* negative, and become SMCs. Red arrowheads indicate regions magnified in right panels. (**F**) Scl-overexpressing cells are *dHand* negative, and become mainly BCs with minor EC contribution.

(see Fig. S3A,B and Fig. S4B,D in the supplementary material). This restrictive localization of dENotch1-expressing cells in the *dHand*-positive lineage was maintained afterwards (HH7; see Fig. S3C in the supplementary material). When electroporated embryos were cultured with DAPT, dENotch1-expressing cells were seen in both *Scl*-positive and *Scl*-negative populations (see Fig. S4C in the supplementary material), similar to controls (see Fig. S4A in the supplementary material). Although the Notch pathway is active only during early phases of ventral mesoderm formation, both *dHand* and *Scl* are expressed in their respective lineages continuously, suggesting that *dHand*- and *Scl*-expressing cells might reinforce an early fate choice without further input from the Notch pathway. To test this, we introduced ectopic dHAND or Scl into early extraembryonic fated mesoderm cells at stage HH3. When analyzed at stage HH5, dHAND ectopic-expressing cells were *Scl* negative, and vice versa (see Fig. S4E,F in the supplementary material). At stage HH10, dHAND ectopic expression resulted primarily in an SMC lineage contribution (n=3; Fig. 3E), whereas Scl ectopic expression gave rise to the BC/EC lineages (n=3; Fig. 3F). When Scl and dENotch1 were co-introduced, the exclusive SMC distribution caused by dENotch1 was completely reversed when analyzed either at stage HH10 (see Fig. S5A-D in the supplementary material) or at stage HH7 (see Fig. S5E,F in the supplementary material), suggesting that Scl and dHAND mediated respective lineage specification acts after Notch-mediated lineage segregation.

Cells with high Notch activity are strongly biased to become SMCs, but cells with low Notch activity show only a minor bias for BC/ECs

If levels of Notch activity determine the choice of SMCs or BC/ECs in the ventral mesoderm population, experimental reduction of Notch activity should lead to a strong BC/EC contribution at the expense of SMCs. When embryos were cultured from stage HH3 to stage HH10 with DAPT, which can lead to a strong reduction of Notch activity in the ventral mesoderm population, as shown in Fig. 2, all three cell types were still present when analyzed with the relevant markers (*dHand*, αSMA, *Lmo2* and *Scl*, and *ρ-globin* and the RBC antigen; Fig. 4A,B; data not shown). This suggested that although Notch activation leads to almost exclusive SMC contribution, the lack of Notch activity does not affect the induction or differentiation of SMCs. We then investigated whether relative percentages of SMCs and BC/ECs are affected by changes in Notch activity. When a control GFP-expressing construct was electroporated into ventral mesoderm precursors at stage HH3 and analyzed at stage HH5, about half (47%; 1005/2156) of the precursors were seen to be co-positive for *Scl* (Fig. 4C), reflecting the normal segregation of SMC progenitors and BC/EC progenitors. By contrast, only 8.5% (98/1126) of dENotch1-expressing cells showed *Scl* co-positivity (Fig. 4C), which was in agreement with our observation that dENotch1-positve cells at later stages have an exclusive SMC contribution. The cell-autonomous reduction of Notch activity in ventral mesoderm precursors with a dominant-negative Suppressor of Hairless [DnSu(H)] expression construct resulted in a small but statistically significant increase in their contribution to the *Scl*-positve lineage (57%; 1733/3057; Fig. 4C). Because electroporation in chicken embryos generally results in a small percentage of cells within the ventral mesoderm population expressing exogenously introduced genes, we performed similar statistical analysis with embryos treated with DAPT, which presumably results in all cells having strongly reduced Notch activity. Similar to DnSu(H) expression, DAPT treatment from stage HH3 to stage HH10 resulted in a small but statistically significant increase in BC contribution (DMSO control, 43%, n=904; DAPT, 61%, n=1122; Fig. 4D).

Wnt pathway plays an instructive role in SMC lineage specification

To understand what may play an instructive role in SMC lineage specification, we investigated the involvement of the BMP and Wnt pathways in this process. Both pathways are active in the posterior primitive streak, although it is not clear whether their main roles are in dorsoventral patterning by promoting the ventralization of mesoderm, or in lineage specification by promoting either SMC or BC/EC formation. We introduced a constitutively active BMP receptor 1 (CA-ALK6) (Miyagishi et al., 2000; ten Dijke et al., 1994) into the anterior primitive streak, where BMP activity is

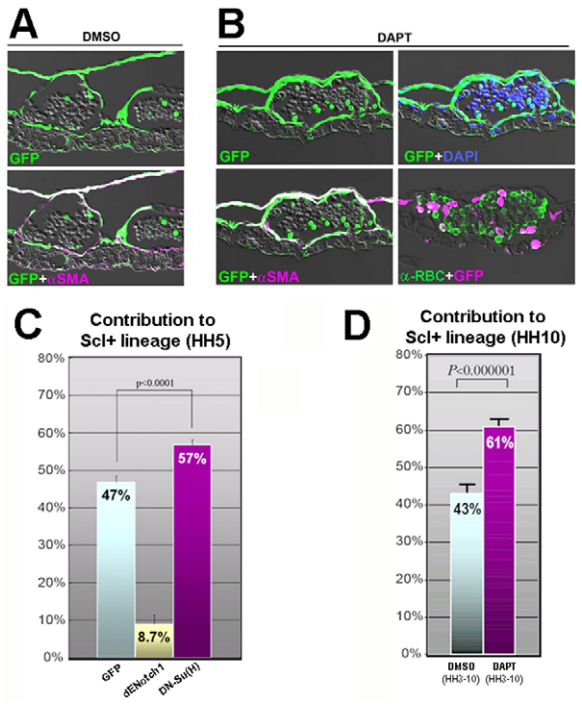

Fig. 4. Reduction of Notch activity results in a small increase in BC/EC contribution. (**A**) Control DMSO treatment from HH3 to HH10. Green indicates that GFP-expressing cells contribute to all three lineages. (**B**) DAPT treatment from HH3 to HH10 increases the contribution of labeled cells to the BC lineage. Although collapsed extraembryonic coelom makes the distinction of SMCs and ECs difficult, the relative contribution to BCs is assessed with the help of DAPI co-staining (top right). These BC lineage located cells are normal blood cells as revealed by αRBC staining (bottom right). (**C**) Quantification of Scl-positive lineage contribution at HH5 for cells expressing either dENotch1 or DN-Su(H). Cell-autonomous Notch pathway activation by dENotch1 results in a strong bias ($P<0.0001$) against the Scl-positive, whereas inhibition by DN-Su(H) causes only a minor increase. (**D**) Quantification of the contribution of GFP-labeled cells in embryos treated with DAPT from HH3-HH10. DAPT causes a small increase in Scl-positive BC contribution (stained with αRBC), in comparison with DMSO control.

normally inhibited by anti-BMP signals from the Hensen's node. Ectopic CA-ALK6 expression resulted in ectopic induction of both *Scl* (n=4) and *dHand* (n=4; Fig. 5A), suggesting that the main role of the BMP pathway is to ventralize the mesoderm. By contrast, when CA-β-Catenin, which leads to a constitutive activation of the canonical Wnt pathway (Takahashi et al., 2000; Yu et al., 2008), was introduced ectopically, strong induction was observed only with the SMC marker *dHand* (8/8; Fig. 5B). No induction was seen with any of the markers for the BC or EC lineage, including *Scl* (n=3), *Lmo2* (n=3), *Vegfr2* (n=3) and *Ets1* (n=3; Fig. 5B; see also Fig. S6 in the supplementary material), suggesting that the activity of the Wnt pathway plays an instructive role in SMC lineage specification. Supporting this notion, CA-β-Catenin, when introduced in ventral mesoderm territory, resulted in a prominent suppression of *Scl* expression (n=4; Fig. 5B). Unlike ectopic CA-β-Catenin expression, however, ectopic dENotch1 expression in the anterior primitive streak did not lead to ectopic *dHand* expression (data not shown), suggesting that Notch-mediated SMC specification might act in parallel to or downstream of the Wnt pathway. We therefore investigated whether CA-β-Catenin in the anterior primitive streak can induce the ectopic expression of genes involved in Notch signaling. Indeed all three genes tested, *Delta1* (n=2), *L-fringe* (n=3) and *Notch1* (n=4), were induced ectopically by CA-β-Catenin (Fig. 5C). This induction, however, partially requires intact Notch signaling, as the induction of *L-fringe* and *Notch1* was abolished when embryos were treated with DAPT (n=4; see Fig. 5D for *L-fringe*; data not shown for *Notch1*).

The relationship between the BMP and Notch pathways in SMC and BC/EC segregation

As we have mentioned earlier (Fig. 5A), the main role of BMP pathway activity is in the ventralization of mesoderm, leading to the induction of both SMC and BC/EC populations, either directly or indirectly. In support of this, CA-ALK6 electroporated cells in the ventral mesoderm were shown to have the ability to contribute later on to all three lineages (data not shown). In normal gastrulation stage embryos, BMP pathway activity, as revealed by phospho-Smad1/5/8 staining (see Materials and methods), could be detected in all ventral mesoderm cells (see Fig. S7A in the supplementary material). Ectopic CA-ALK6 in the anterior primitive streak resulted in strong ectopic phospho-Smad1/5/8 staining together with staining for co-electroporated GFP (see Fig. S7B in the supplementary material). We next investigated the relationship between BMP-mediated induction of SMC and BC/EC populations, and Notch-mediated segregation of these two populations. In normal stage HH10 embryos, few *Scl*-positive cells could be detected in medial regions of the embryos (corresponding to the medial part of lateral plate mesoderm; Fig. 6A,E; 0%, n=13), whereas strong *dHand* expression can be observed in this region (Fig. 3A). Ectopic CA-ALK6 expression resulted in a large increase in the percentage of embryos with *Scl*-positive cells in medial regions at stage HH10 (Fig. 6B,E; 77%, n=31). This is in agreement with the fact that high BMP activity can promote the induction of BC/ECs, in addition to SMCs, which are already abundantly present in this region in normal embryos. This increase, however, could be effectively blocked by the co-expression of dENotch1 (Fig. 6C,E; CA-ALK6+dENotch1; 27%, n=33) and re-rescued by culturing in the presence of DAPT (Fig. 6D,E; CA-ALK6+dENotch1+DAPT; 90%, n=10). This suggested that BMP activity-induced ventralization of mesoderm is modulated by Notch activity levels, leading to a strong preference for the SMC lineage when Notch activity is high. Supporting this, we observed that CA-ALK6-induced ectopic *Scl* expression was further enhanced in the presence of DAPT treatment (Fig. 6E; CA-ALK6+DAPT; 94%, n=16).

DISCUSSION

The number of cell types present in the extraembryonic mesoderm has not been carefully investigated. Here, we show that in addition to BCs and ECs, SMCs represent another major component of the extraembryonic mesoderm. Although other possible minor cell types cannot be ruled out, these three lineages together can account for almost all of the cells present in the extraembryonic mesoderm prior to the establishment of the circulation (Fig. 7B). Our studies also suggest that dHAND is a molecular marker and a crucial transcriptional regulator of the SMC lineage (Fig. 7A). The extraembryonic SMC lineage is composed of two anatomically distinct layers: the vascular SMC and the somatic SMC. Although the smooth muscle component of the extraembryonic splanchnopleure (the vascular SMC layer) has been well described, we show here that the extraembryonic somatopleural mesoderm is also largely composed of smooth muscle cells. The medial part of

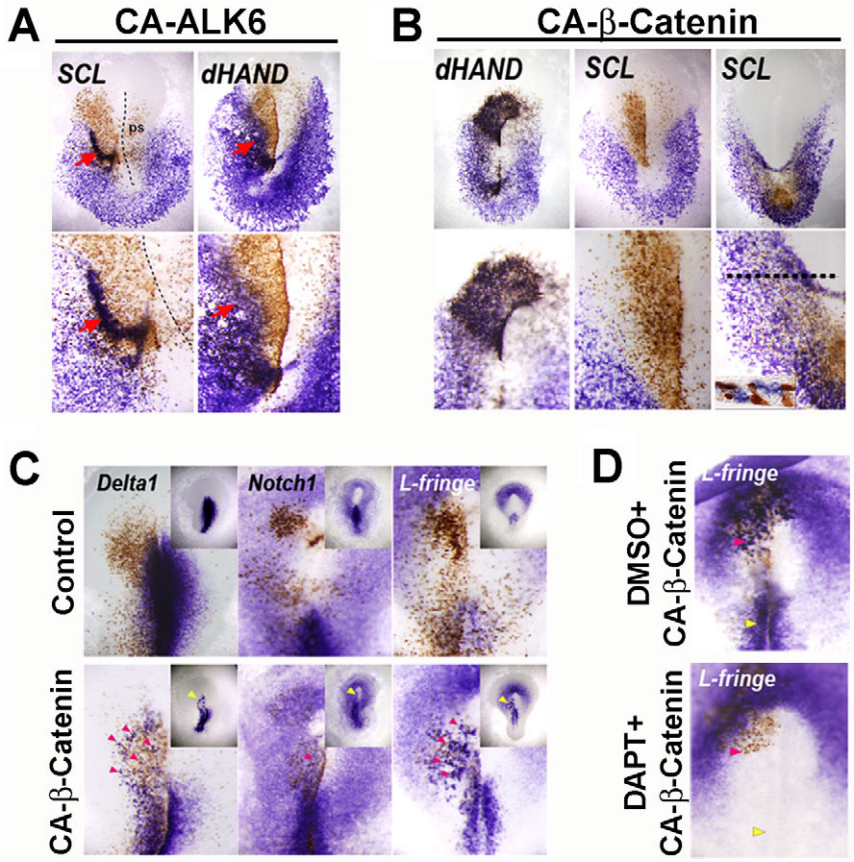

Fig. 5. Roles of BMP and Wnt in SMC and BC/EC induction. Chick embryos were electroporated with expression constructs at HH3 and analyzed at HH4. Brown, cells expressing electroporated genes; blue, in situ staining with indicated genes. (**A**) Ectopic activation of the BMP pathway by CA-ALK6 leads to the ectopic expression of both *Scl* and *dHand* (red arrows). (**B**) Ectopic activation of the Wnt pathway by CA-β-Catenin results in ectopic *dHand*, but not *Scl*, expression. CA-β-Catenin overexpression in the endogenous *Scl*-expressing region results in its inhibition. Dotted line indicates the level of the section shown in the inset. (**C**) Ectopic Wnt pathway activation upregulates Notch pathway genes. (Top panels) Control GFP electroporation; (bottom panels) CA-β-Catenin electroporation. Insets indicate whole-embryo views. Yellow arrowhead in insets indicates magnified region. Red arrowheads indicate ectopic induction. (**D**) CA-β-Catenin induction of Notch pathway genes, *L-fringe*, shown here, is abolished by DAPT treatment (compare red arrowheads in top and bottom panels). Yellow arrowheads indicate the inhibition of endogenous L-fringe in the posterior primitive streak by DAPT.

extraembryonic somatopleure is known to contribute to the amnionic membrane, the rhythmic contraction of which is attributed to its smooth muscle cells (Adamstone, 1948; Oppenheim, 1966; Pierce, 1933; Romanoff, 1960; Wu et al., 2001). The lateral part of the extraembryonic somatopleure gives rise to the chorionic membrane, with the majority of its mesoderm component later on fusing with the mesoderm component of the allantois to form the chorioallantoic membrane. It is therefore unclear what the functions of SMCs might be there. With the SMC markers used in our studies, we could not distinguish either between the vascular SMC and the somatic SMC, or between the medial and lateral parts of the somatic SMC.

In chickens, two Notch genes (*Notch1* and *Notch2*) have been reported so far. This is supported by our genomic and transcriptomic analyses. We show in this study that Notch1 is the main receptor mediating Notch signaling during early ventral mesoderm

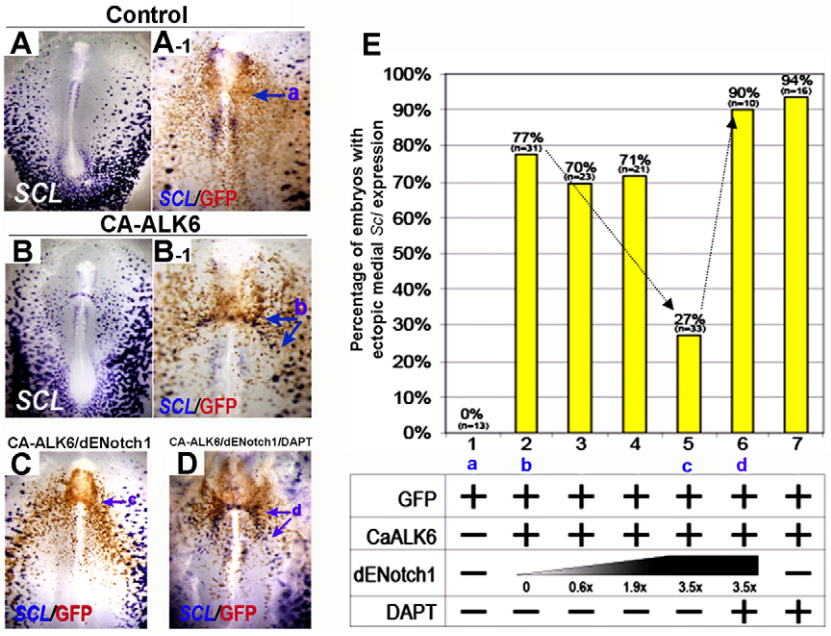

Fig. 6. BMP-induced ectopic medial *Scl* expression is regulated by Notch activity. (**A**) Control GFP expression does not change *Scl* expression, which progressively weakens more medially. The presence of faint staining in part of the dorsal aorta is normal. (Left) Whole embryo view before anti-GFP staining, but after in situ analysis with *Scl*; (right) magnified view of the electroporated region after GFP staining of the embryo shown in the left panel. Arrow indicates the electroporated region with no *Scl* induction. Letter next to the arrow corresponds to the statistical analysis in E. (**B**) CA-ALK6 induces strong ectopic *Scl* expression medially (arrows in right panel), which is also prominent in the whole embryo view shown in the left panel. (**C**) CA-ALK6-induced ectopic *Scl* expression is abolished by dENotch1 co-expression. (**D**) DAPT treatment rescues induction by CA-ALK6. (**E**) Quantification of *Scl* ectopic induction by CA-ALK6. The dose effect of dENotch1 is indicated by the number reflecting the dENotch1 construct concentration (see also Materials and methods).

differentiation. Other Notch pathway components, including *Delta1*, *Hariy2*, *Herp2*, *L-fringe* and *Nrarp*, are also expressed in the posterior primitive streak. Most of these genes, however, are downregulated during active migration and later differentiation, suggesting that the pathway is active only during ventral mesoderm formation and early migration. Nevertheless, it has been well documented that the Notch pathway also plays a role in endothelial cell differentiation, arterial/venous vessel specification and SMC/EC interaction. These aspects appear to take place after the initial lineage specification of SMC, EC and BC, and are not addressed in this study. Indeed, later on, after the lineage specification but before the initiation of circulation, we detected an upregulation of *Notch2* and *Delta4* in yolk sac tissues in our transcriptomic analysis, suggesting that the Notch pathway, albeit utilizing different members, is being reactivated for later functions.

Our data indicate that lineage specification among the ventral mesoderm population involves first the separation of SMC and BC/EC progenitors mediated by the Notch pathway (Fig. 7A). We have previously reported that the FGF pathway plays a crucial role in the separation of BCs and ECs from the BC/EC lineage (Nakazawa et al., 2006). The FGF pathway does not appear to play a prominent role in the early separation of the SMC and BC/EC lineages, as neither constitutively active FGFR nor inhibition of the FGF pathway exhibits any discernable effect on *Scl* or *dHand* expression (see Fig. S6 in the supplementary material; data not shown). We propose that three main cell types in the extraembryonic mesoderm are generated by a two-step binary choice among multipotential ventral mesoderm progenitors: first between SMCs and BC/ECs, and then between BCs and ECs (Fig. 7A,B). A stochastic difference in Notch activities can be reinforced by the mutual inhibition of dHAND and Scl, resulting in the separation of the SMC and BC/EC lineages. A similar model for the early separation of cells contributing to the BC/EC lineage and cells contributing to the extraembryonic coelomic linings was proposed by Sabin (Sabin, 1920), although the SMC nature of coelomic lining cells was not clearly mentioned in that report. The general anatomical organization, proposed in this work, of the extraembryonic mesoderm in the chick embryo, is also supported by ultrastructural studies (Kessel and Fabian, 1985; Murphy and Carlson, 1978).

Molecularly, the antagonistic action of Scl and dHAND (two bHLH transcription factors) seen in our experimental system is supported by several recent findings in other systems. The role of Scl in promoting BC/EC differentiation and in inhibiting vascular SMC differentiation has been reported in a mouse embryoid body differentiation assay (Ema et al., 2003). The mutually antagonistic action of dHAND and Scl in muscle and endothelial lineages, respectively, was reported in zebrafish heart development (Schoenebeck et al., 2007). Furthermore, the mutually antagonistic specification of muscle and BC/EC lineages, with the Notch pathway involved in promoting the muscle lineage, has been reported in several recent in vitro and in vivo studies (Ben-Yair and Kalcheim, 2008; Chen et al., 2008; Cheng et al., 2008; Cohen et al., 2008; Tang et al., 2008; Varadkar et al., 2008; Wang et al., 2007).

The precise function of the Notch pathway in the process of muscle and BC/EC lineage separation, however, remains to be elucidated. Our data suggest that, during chick ventral mesoderm differentiation, the Notch pathway acts together with the BMP and Wnt pathways, and that it plays a ‘permissive’, rather than an ‘instructive’, role in mediating the separation of SMCs and BC/ECs. The Notch pathway does not control the induction of but rather the balance between these two populations. We provide evidence that

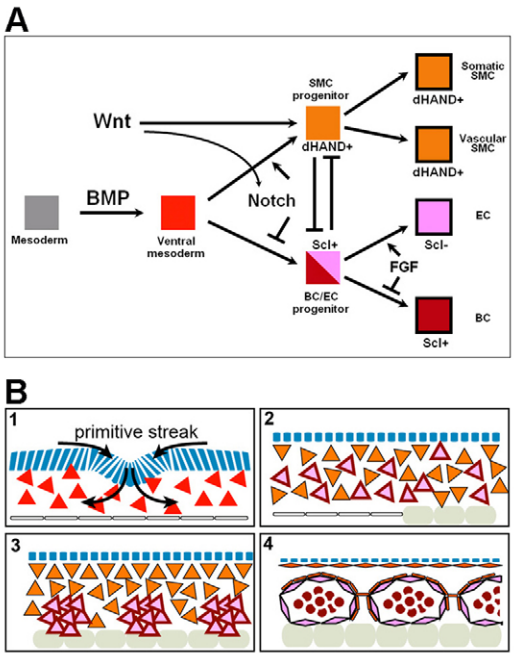

Fig. 7. A model for the separation of SMC, EC and BC lineages, and the role of Notch activity in separating SMC and BC/EC lineages in relationship with those of the BMP and Wnt pathways. (**A**) In this model, the main role of the BMP pathway is to ventralize mesoderm. Wnt pathway activation leads to SMC induction. The balance between SMCs and BC/ECs is mediated by the Notch pathway. After multipotential ventral mesoderm progenitors are segregated into dHAND-positive SMC progenitors and Scl-positive BC/EC progenitors, mutual inhibition of dHAND and Scl leads to fate reinforcement. BC/EC progenitors are further segregated into BCs and ECs, mediated by the FGF pathway. (**B**) A developmental view of how SMC, BC and EC lineages form between HH3 and HH10 in the chick embryo (colors as in A). Soon after the ingression of ventral mesoderm progenitors through the posterior part of the primitive streak (B1), cells are separated into Notch-activity-high and Notch-activity-low types (B2). This separation coordinates with the BMP- and Wnt-mediated induction of BC/EC and SMC progenitors to ensure the proper balance of these two lineages. BC/EC progenitors coalesce to form blood islands (B3). Blood island cells further differentiate into BCs and ECs, and SMC progenitors form both somatic and vascular SMCs (B4).

the induction of these lineages is controlled by the activities of both the BMP pathway, as a general ventral mesoderm inducer, and the canonical Wnt pathway, as a strong SMC lineage inducer. Ectopic activation of the BMP pathway can induce both SMC and BC/EC lineages, with the balance of SMCs and BC/ECs being regulated by Notch activity. It is not clear whether the induction of SMCs by the BMP pathway is a direct or indirect process, or whether it requires an active Wnt pathway. In our analysis, we observed a stronger and wider ectopic *dHand* induction by CA-β-Catenin than by CA-ALK6 around the anterior primitive streak where BMP antagonists are highly expressed, which suggests that the induction of SMCs by the Wnt pathway does not require active BMP signaling. A recent in vitro study suggested that Notch activity promotes the degradation of Scl by facilitating its ubiquitination, and that this process requires the transcriptional regulation of Notch pathway activity through Suppressor of Hairless (Nie et al., 2008). Although we do not have direct evidence in support of a similar phenomenon in our system, it could in principle act as a possible mechanism for the Notch

activity-mediated segregation of SMCs and BC/ECs. Furthermore, Nrarp, in addition to serving as a Notch-activity readout and a feedback regulator of the Notch pathway, has also been shown to positively regulate the canonical Wnt pathway by blocking the ubiquitination and increasing the stability of Lef1 in zebrafish (Ishitani et al., 2005). This might also serve as a possible mechanism for the Notch and Wnt pathway-mediated SMC specification observed in our system.

These observations, however, leave unanswered the question, what controls the induction of the BC/EC lineage? Two possible scenarios could explain the obvious lack of a specific BC/EC-inducing signal. One possibility is that, during normal development, once ventral mesoderm is specified by active BMP signaling, BC/EC differentiation takes place as a default choice. SMC differentiation is promoted by canonical Wnt signaling and the balance of SMCs and BC/ECs is mediated by Notch signaling. The other possibility is that graded levels of BMP pathway activity might have a qualitative difference in whether to induce more SMCs or more BC/ECs. This possibility is supported by the fact that within lateral plate and extraembryonic mesoderm, progressively more *dHand*-positive SMCs and less *Scl*-positive BC/ECs are present dorsally (medially located), where BMP signaling becomes progressively weaker. However, in our analysis, we did not observe a correlation between phospho-Smad1/5/8 signal levels in ventral mesoderm and either *dHand*- or *Scl*-positive signals, nor was the phospho-Smad1/5/8 level changed by either dENotch1 or DN-Su(H) expression.

Taken together, we show that chick extraembryonic SMC and BC/EC lineages are segregated early, and are marked by two bHLH factors, dHAND and Scl, respectively. The segregation involves the Notch pathway. The Notch pathway acts together with the BMP and Wnt pathways in coordinating mesoderm ventralization, ventral mesoderm lineage induction and lineage segregation events. Amniotes exhibit high degrees of conservation in mesoderm patterning and specification. These molecular and cellular events leading to the specification of extraembryonic lineages therefore might not be unique to avian embryos. It will be interesting to investigate whether mammalian extraembryonic mesoderm differentiation involves similar mechanisms.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/136/4/595/DC1

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