Development 127, 3865-3876 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
DEV4379

Serrate and Notch specify cell fates in the heart field by suppressing cardiomyogenesis

Melissa S. Rones, Kelly A. McLaughlin, Michael Raffin and Mark Mercola*

Department of Cell Biology, Harvard Medical School, 240 Longwood Avenue, Boston, MA 02115, USA
*Author for correspondence (e-mail: mmercola@hms.harvard.edu)

Accepted 9 June; published on WWW 9 August 2000

SUMMARY

Notch signaling mediates numerous developmental cell fate decisions in organisms ranging from flies to humans, resulting in the generation of multiple cell types from equipotential precursors. In this paper, we present evidence that activation of Notch by its ligand Serrate apportions myogenic and non-myogenic cell fates within the early *Xenopus* heart field. The crescent-shaped field of heart mesoderm is specified initially as cardiomyogenic. While the ventral region of the field forms the myocardial tube, the dorsolateral portions lose myogenic potency and form the dorsal mesocardium and pericardial roof (Raffin, M., Leong, L. M., Rones, M. S., Sparrow, D., Mohun, T. and Mercola, M. (2000) *Dev. Biol.*, 218, 326-340). The local interactions that establish or maintain the distinct myocardial and non-myocardial domains have never been described. Here we show that *Xenopus Notch1* (*Xotch*) and *Serrate1* are expressed in overlapping patterns in the early heart field. Conditional activation or inhibition of the Notch pathway with inducible dominant negative or active forms of the RBP-J/Suppressor of Hairless [Su(H)] transcription factor indicated that activation of Notch feeds back on *Serrate1* gene expression to localize transcripts more dorsolaterally than those of *Notch1*, with overlap in the region of the developing mesocardium. Moreover, Notch pathway activation decreased myocardial gene expression and increased expression of a marker of the mesocardium and pericardial roof, whereas inhibition of Notch signaling had the opposite effect. Activation or inhibition of Notch also regulated contribution of individual cells to the myocardium. Importantly, expression of *Nkx2.5* and *Gata4* remained largely unaffected, indicating that Notch signaling functions downstream of heart field specification. We conclude that Notch signaling through Su(H) suppresses cardiomyogenesis and that this activity is essential for the correct specification of myocardial and non-myocardial cell fates.

Key words: Notch, Serrate, Heart, Myocardium, Mesocardium, *Xenopus*

INTRODUCTION

The vertebrate heart arises from paired primordia of cardiogenic mesoderm specified during gastrulation, in part by signals from the adjacent endoderm (Sater and Jacobson, 1989, 1990a; Nascone and Mercola, 1995; Schultheiss et al., 1995). While several diffusible factors, including members of the family of bone morphogenetic proteins (BMPs), are known to be involved in specification of the heart field (Lough et al., 1996; Schultheiss et al., 1997), little is understood of the subsequent steps required for the determination of the diverse cell types that arise within the field. Recently, chamber- or tissue-specific transcription factors, such as MEF2 family members, the bHLH proteins eHAND and dHAND, and the homeodomain factor Irx4, have been shown by molecular genetic analyses to be involved in the regional control of contractile protein gene expression within the developing myocardium (Lyons, 1996; Mohun and Sparrow, 1997). Yet, despite these advances, the molecular nature of local cell-cell interactions that define the identities of distinct cardiac tissues have not been described.

In *Xenopus*, the heart primordia originate in dorsal mesoderm flanking Spemann's organizer, migrate anteriorly during gastrulation (stage 10-12) to underlie the prospective hindbrain, and then migrate laterally until they fuse at the ventral midline by the neural tube stage. By the early tailbud stage (stage 22), the cardiogenic mesoderm is demarcated by *Nkx2.5* expression and forms a crescent extending upwards from the ventral midline towards the neural tube. During late tailbud stages (stage 28-30), the sheet of cardiogenic mesoderm folds. Shortly thereafter (stage 32-33) a linear myocardial tube lined by endothelial cells has formed and is suspended from the roof of the pericardial cavity by the mesocardium (Fig. 1Ap; Fishman and Chien, 1997; Raffin et al., 2000). Recent fate-mapping studies have shown that mesocardial and pericardial roof cells are derived from the dorsolateral portions of the crescent of cardiogenic mesoderm at stage 22 while the myocardium originates from the intervening ventral region (Raffin et al., 2000). Despite their final fate, the dorsolateral portions of the Nkx2.5/heart field form beating heart tubes with lumens when isolated experimentally, either as explants or by heterotopic

transplantation (Copenhaver, 1924; Ekman, 1925; Jacobson and Duncan, 1968; Sater and Jacobson, 1990b; Raffin et al., 2000). Thus, the entire field is specified initially as cardiomyogenic. However, between stages 22 and 28, signals from prospective myocardium and neurogenic tissue suppress cardiomyogenesis in the dorsolateral portions of the heart field and thereby subdivide the *Nkx2.5*/heart field into ventral myogenic and dorsolateral non-myogenic domains (Raffin et al., 2000). Similarly, fate-mapping experiments in chick and zebrafish have suggested that the region with myocardial potency extends beyond the cells fated to form myocardial tissue (Lee et al., 1994; Cohen-Gould and Mikawa, 1996; Serbedzija et al., 1998).

Notch signaling mediates a wide array of cell fate decisions in both invertebrates and vertebrates (Artavanis-Tsakonas et al., 1995, 1999; Weinmaster, 1997, 1998). Notch receptors are single-pass transmembrane proteins of approximately 300 kilodaltons (kDa) containing a series of highly conserved domains in both their extracellular and intracellular regions. *Notch* orthologues have been isolated from *Xenopus*, chick, zebrafish, mice and humans (Coffman et al., 1990; Ellisen et al., 1991; Weinmaster et al., 1991, 1992; Franco del Amo et al., 1992; Reaume et al., 1992; Bierkamp and Campos-Ortega, 1993; Lardelli et al., 1994; Williams et al., 1995; Myat et al., 1996; Uyttendaele et al., 1996; Westin and Lardelli, 1997). Both the Delta and Serrate/Jagged families of ligands are also single-pass transmembrane proteins with conserved functional domains. Orthologues of these genes have been identified in numerous species and are often expressed coincidentally with various *Notch* family members (Bettenhausen et al., 1995; Chitnis et al., 1995; Henrique et al., 1995; Lindsell et al., 1995; Myat et al., 1996; Shawber et al., 1996a; Dunwoodie et al., 1997; Jen et al., 1997; Haddon et al., 1998). Interaction with either Delta or Serrate induces a cleavage within the cytoplasmic domain of Notch and permits the intracellular domain (ICD) to associate with the transcription factor CBF-1/RBP-J/Suppressor of Hairless [Su(H)]. Translocation of this complex to the nucleus then activates downstream genes that include members of the *Enhancer of split* (*HES* or *ESR* in vertebrates) family that encode basic helix-loop-helix (bHLH) transcription factors. Notch activation is complex and responsiveness is also influenced by additional factors including members of the Fringe family and post-translational modifications of Notch (Logeat et al., 1998; Schroeter et al., 1998; Irvine, 1999).

In *Drosophila*, Notch signaling governs cell fate decisions in all three germ layers (Corbin et al., 1991; Hartenstein et al., 1992). Similarly, Notch signaling in vertebrates influences differentiation of many tissues, including neural ectoderm, somites, cartilage, retina and T-lymphocytes (Austin et al., 1995; Conlon et al., 1995; Chitnis and Kintner, 1996; Robey et al., 1996; de la Pompa et al., 1997; Dorsky et al., 1997; Hrabe de Angelis et al., 1997; Jen et al., 1997, 1999; Wettstein et al., 1997; Wang et al., 1998; Crowe et al., 1999). Although different combinations of ligand, receptor and downstream genes are involved in each of these systems, all involve the initial expression of a Notch receptor and its ligand throughout a field of equivalently specified cells. Resolution of this pattern such that ligand and receptor become expressed preferentially by different cells establishing the basis for the intercellular signaling that eventually subdivides the field into distinct cell types (for review, see Artavanis-Tsakonas et al., 1999). The asymmetry that triggers progression from an equivalence state is poorly understood but, once manifest, is amplified by a feedback loop within each cell such that activation of the Notch receptor results in a decrease in ligand production (see Artavanis-Tsakonas et al., 1999). Studies of the developing nervous system and somites have demonstrated that activation of Notch generally inhibits differentiation and can serve to maintain a precursor population of cells (Austin et al., 1995; Dorsky et al., 1995, 1997; Weinmaster, 1997; Wang et al., 1998). Accordingly, experimental perturbations of Notch signaling can change the fate of cells and/or alter the borders that normally distinguish tissue compartments. Expectedly, mutations in components of the Notch pathway have severe clinical repercussions. For example, translocations of the human *Notch1* locus (*TAN1*) result in leukemia, mutations in human *Notch3* result in complex neurological defects, and mutations in human *Jagged1*/*Serrate1* are associated with Alagille syndrome, an autosomal dominant disorder involving multiple organs, including the heart (Ellisen et al., 1991; Joutel et al., 1996; Li et al., 1997; Oda et al., 1997).

Previous studies have shown expression of *Notch* and *Delta* homologues in the heart region of chick and zebrafish embryos both before and after formation of a linear heart tube (Myat et al., 1996; Westin and Lardelli, 1997). In addition, null mutations in either *Notch1* or *RBPJ* in the mouse lead to complex defects that include pericardial edema and embryonic lethality (Swiatek et al., 1994; Conlon et al., 1995; Oka et al., 1995). However, it is unclear whether these malformations are caused by aberrant heart field patterning or are secondary to other defects, or both. These observations prompted us to determine if Notch signaling plays a direct role in patterning the vertebrate heart. We report that transcripts of *Notch1* (also known as *Xotch*) and *Serrate1* are expressed in the heart field of tailbud stage *Xenopus* embryos during the period when the dorsolateral portions lose cardiomyogenic potency. We also show that activation or inhibition of Notch signaling through Su(H) leads to opposite effects on the size of the myocardial domain and the ability of individual cells to contribute to myocardium. We propose that signaling through the Serrate-Notch-Su(H) pathway is responsible for the normal loss of myogenic potency in the dorsolateral domains of the *Nkx2.5*/heart field and is essential for generating distinct cardiomyogenic and non-myogenic domains from an initial field of cardiomyogenic potency. Interestingly, Notch has been implicated in regulating the number of cardioblasts in the dorsal vessel of *Drosophila* independently of its effect on neural tissue (Corbin et al., 1991; Hartenstein et al., 1992). The similarity between the *Drosophila* data and our model indicate that the cardiogenic function of Notch has been remarkably well conserved throughout evolution.

MATERIALS AND METHODS

Embryos

Embryos were dejellied in 2% cysteine-HCl (pH 7.8), washed and maintained in 0.1× Marc's Modified Ringer's solution (MMR). Embryos were reared at 14-22°C and staged according to Nieuwkoop and Faber (1994).

Microinjection and embryo culture

Capped mRNA for microinjection was synthesized in vitro using the mMessage Machine kit (Ambion). Embryos were injected into one dorsal-vegetal blastomere at the 8-cell stage. cDNAs encoding the inducible constructs, GR-Su(H)VP16, GR-Su(H)^{DBM} and GR-NotchICD were the gift of Robert Davis. GR-Su(H)VP16 was made by fusing the activating domain of VP16 to the carboxyl terminus of *Xenopus* Su(H) (Wettstein et al., 1997) and the ligand binding domain of the human glucocorticoid receptor (GR) to the amino terminus (as in Kolm and Sive, 1995). GR-Su(H)^{DBM} is similar except that it lacks the VP16 activation domain and the Su(H) coding region contains a mutation in the DNA-binding domain (provided by Chris Kintner; Wettstein et al., 1997). Notch-ICD encodes the intracellular domain of Notch, and has been shown previously to activate Notch signaling constitutively. An inducible version was made by fusion of GR to the amino terminus. 250-500 pg of the GR-Su(H) mRNAs, or 500-1000 pg of GR-NotchICD mRNA were injected along with 50-100 pg of nuclear β-galactasidase mRNA as a lineage tracer. Injected embryos were cultured in 0.1× MMR until they reached the desired stages. Induction of GR constructs was by addition of dexamethasone (10 μM) (Sigma). Dexamethasone was changed daily when embryos were cultured longer than 24 hours.

In situ hybridization and probes

Embryos were fixed for 1 hour at room temperature in MEMFA (0.1 M MOPS pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde), rinsed in 1× PBS + 2 mM $MgCl_2$, and incubated in staining solution at 37°C with either X-gal or magenta-gal in order to detect β-galactosidase activity. Embryos were then rinsed in 1× PBS, postfixed in MEMFA for 2 hours and dehydrated in methanol. For in situ hybridization, embryos were rehydrated and processed using digoxigenin-coupled cRNA probes (Harland, 1991). Probes included: cardiac troponin-I (*TnIc*) (Drysdale et al., 1994), cardiac actin (Mohun et al., 1984), *MHCα* (Logan and Mohun, 1993), *Nkx2.5* (Tonissen et al., 1994), *Gata4* (Jiang and Evans, 1996), *Bmp4* (Nishimatsu et al., 1992), *Serrate1* (gift of Chris Kintner), and *Notch1* (also known as *Xotch* and most similar to mouse *Notch1*; Coffman et al., 1990). For double in situ hybridization analysis, the second probe was FITC-coupled. In some cases, following in situ hybridization, embryos were postfixed and dehydrated through a methanol series, transferred to JB4 and embedded according to manufacturer's directions (Polysciences).

RESULTS

We surveyed the spatial and temporal expression pattern of the previously identified *Xenopus* genes *Notch1*, *Serrate1*, *Delta1* and *Delta2*. *Delta1* and *Delta2* mRNAs were not detected in the heart region by in situ hybridization between stages 20 and 30 (Fig. 1C). *Notch1* and *Serrate1* expression were first detected during the early tailbud stages (Fig. 1Ab,c,e,f). Expression of *Notch1* and *Serrate1* between stages 22 and 25 overlap and span an extensive portion of the *Nkx2.5* field (Fig. 1Aa,d). This region comprises the future myocardium as well as extending more dorsolaterally to include the regions that give rise to the dorsal mesocardium and pericardial roof. Expression of both *Serrate1* and *Notch1* is preceded by expression of *Nkx2.5* and precede that of markers of myocardial differentiation, such as *TnIc*, by several hours (Fig. 1Ag,k,o).

As development progresses, the expression patterns of both *Notch1* and *Serrate1* become more refined (Fig. 1Ai,j,m,n,q,r). By stage 30, just prior to heart tube fusion, *Notch1* transcripts are present primarily in the developing myocardium whereas *Serrate1* mRNAs are present primarily in the future dorsal mesocardium but also in the dorsalmost portion of the developing myocardium where they overlap *Notch1* transcripts (Fig. 1Am,n,q,r). The resolution of the dynamic expression patterns of *Notch1* and *Serrate1* occurs during the developmental stages when the dorsolateral portions of the heart field lose myocardial potency (Raffin et al., 2000) and the prospective myocardium begins to express genes indicative of terminal myocardial differentiation. This can be seen most clearly in double in situ hybridization analysis of *TnIc* and *Serrate1* transcripts where *TnIc* marks exclusively the developing myocardium and *Serrate1* has become localized primarily to the dorsal mesocardium and roof of the pericardium (Fig. 1B). The timing and spatial patterns of expression suggest a model in which Notch activity feeds back on *Serrate1*, as in other systems (see Artavanis-Tsakonas et al., 1999). In addition to shifting *Serrate1* expression dorsolaterally, Notch activity might suppress cardiomyocyte differentiation in the dorsolateral domains of the heart field. These possibilities are examined below.

Regulation of endogenous *Serrate1* expression by Notch signaling

To study the consequences of Notch signaling in the developing heart (during the second day of development) without affecting any earlier developmental steps in which Notch functions, we used inducible forms of the transcription factor Su(H). Activated and dominant negative forms of Su(H) were fused to the human glucocorticoid receptor ligand binding domain (Fig. 2; Materials and Methods). GR fusions have been employed to create transcription factors that are maintained in an inactive complex until activated by the addition of the glucocorticoid dexamethasone to the culture media (Kolm and Sive, 1995). By allowing signaling to be manipulated at a time consistent with the endogenous expression of *Notch1* and *Serrate1* in the heart region, this approach circumvented any confounding effects that early constitutive alteration of Notch signaling may cause.

The constructs were tested for both function and inducible regulation by injecting into animal blastomeres and examining the effect on primary neurogenesis, as indicated by β-tubulin expression. We obtained results identical to those of Wettstein et al. (1997), who observed specific alterations in the pattern and number of primary neural precursors. Thus, we concluded that our constructs are inducible and function normally (data not shown).

To address the possibility that a feedback loop refines the expression of *Serrate1* in the heart field, we injected embryos with mRNA encoding either the activated or dominant negative form of Su(H). Embryos were injected into one dorsal-vegetal blastomere at the 8-cell stage with mRNA encoding either GR-Su(H)VP16 [activated Su(H)] or GR-Su(H)^{DBM} [dominant negative Su(H)]. These embryos were cultured in the absence of dexamethasone (uninduced) until stages 18-19, and then maintained in media containing dexamethasone (induced) until fixation at stages 28-31. This time period was chosen to yield functional GR-Su(H)VP16 or GR-Su(H)^{DBM} at a time coincident with endogenous *Notch1* and *Serrate1* expression (as shown in Fig. 1A).

Activation of Notch signaling with GR-Su(H)VP16 decreased the expression of *Serrate1* on the injected side of the

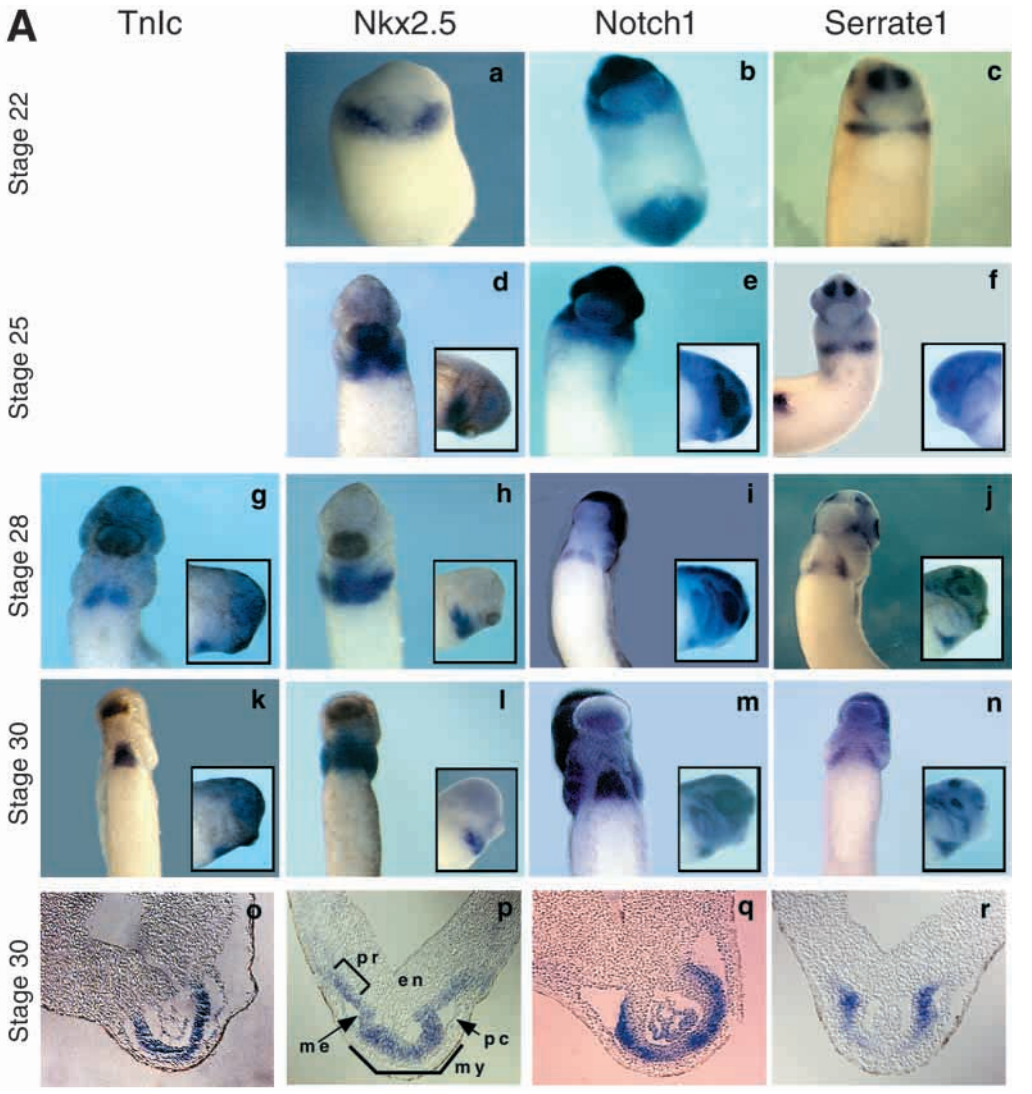

Fig. 1. Expression patterns of *Notch1* and *Serrate1* within the Nkx2.5/heart field. (Aa-n) Expression patterns of *Nkx2.5*, *TnIc*, *Notch1* and *Serrate1* by whole-mount in situ hybridization from stages 22 to 30. Embryos are shown ventrally with anterior oriented to the top. Inset shows anterior lateral views. Note the dynamic expression patterns within the cardiogenic mesoderm: expression of *Notch1* and *Serrate1* largely overlap at stages 22-25 but become distinct at later stages as terminal markers of myocardial differentiation are expressed. (o-r) Stage 30 embryos were embedded following in situ hybridization and sectioned transversely. By this stage, the patterns have become refined such that expression of *Serrate1* extends more dorsolaterally than that of *Notch1*. Overlap is restricted to the region spanning the developing mesocardium. (B) Transverse sections of double in situ hybridization of *TnIc* (in red) and *Serrate1* (in blue). At this stage, the expression of *Serrate1* has been resolved to the dorsolateral margins of the cardiogenic mesoderm and is largely excluded from the myocardium marked by *TnIc*. (C) Expression of *Delta1* and *Delta2* by whole-mount in situ hybridization. Transcripts are present in the developing nervous system and in the branchial arches (indicated by yellow arrow) but absent from the developing heart region. Abbreviations: en, endoderm; me, mesocardium; my, myocardium; pc, pericardium; pr, pericardial roof; ta, truncus arteriosus.

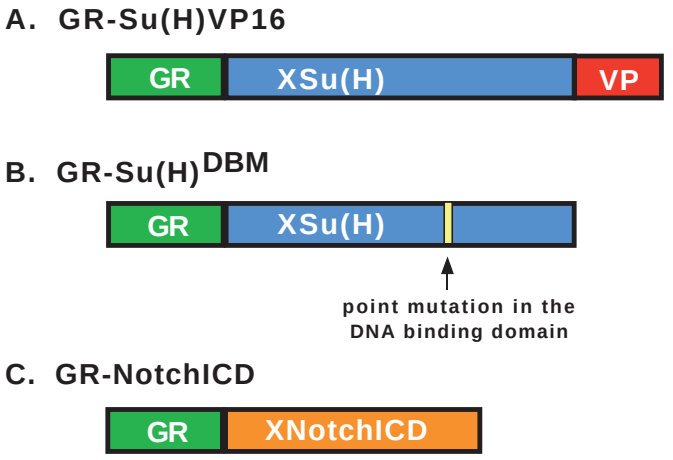

Fig. 2. Schematic of the inducible Su(H) constructs. (A) The conditionally activate GR-Su(H)VP16 construct was made by fusing the human glucocorticoid receptor ligand binding domain (green) in frame to the amino terminus and the VP16 activation domain (red) to the carboxy terminus of wild-type *Xenopus* Su(H) (blue). (B) The dominant negative GR-Su(H)^{DBM} was made by fusing the human glucocorticoid receptor ligand binding domain (green) in frame to a construct harboring point mutations (yellow) in the DNA binding domain of *Xenopus* Su(H) (blue) (Wettstein et al., 1997). (C) GR-NotchICD was made by fusing the GR domain (green) in frame to the intracellular domain of *Xenopus* Notch (Notch-ICD) (orange).

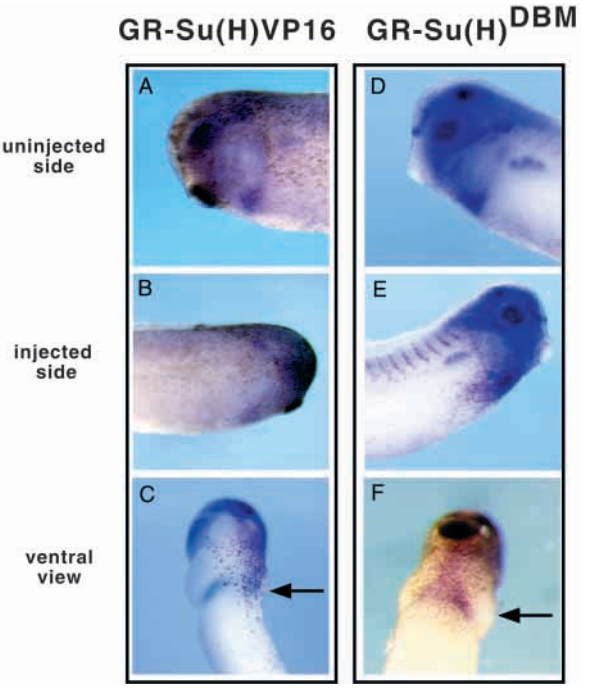

Fig. 3. Notch signaling influences the expression of *Serrate1*. *Serrate1* expression was examined in embryos injected with mRNAs encoding either GR-Su(H)VP16 or GR-Su(H)^{DBM} into one dorsovegetal blastomere at the 8-cell stage as described in the Materials and Methods. mRNA encoding β-galactasidase was co-injected as a lineage tracer. Embryos were induced with dexamethasone at stages 18-19 and cultured until fixation at stages 28-31. (A,B) Expression of *Serrate1* on the uninjected and injected sides, respectively, of an embryo injected with GR-Su(H)VP16 and induced with dexamethasone. (C) Ventral view of a sibling GR-Su(H)VP16 injected embryo. Note the decreased expression of *Serrate1* on the injected side (arrow). (D,E) Expression of *Serrate1* on the uninjected and injected side, respectively, of an embryo injected with GR-Su(H)^{DBM} and induced with dexamethasone. (F) Ventral view of a sibling GR-Su(H)^{DBM}-injected embryo. Note the increased expression of *Serrate1* on the injected side (arrow). *Serrate1* was unaffected in injected embryos cultured in the absence of dexamethasone (data not shown).

embryo compared to that on the uninjected side, which served as an internal control (Fig. 3A-C). A specific decrease was observed in 76.4% of injected embryos (n=144) treated with dexamethasone. In contrast, suppression of Notch signaling by GR-Su(H)^{DBM} increased expression of *Serrate1* in 61.5% of injected embryos (n=78) (Fig. 3D-F). Sibling embryos injected with GR-Su(H)VP16 or GR-Su(H)^{DBM} but not incubated with dexamethasone were affected either minimally or not at all (data not shown). Moreover, no change in gene expression was observed in uninjected embryos cultured in dexamethasone, indicating that the hormone itself has no effect (data not shown). As for GR-Su(H)VP16, injection of mRNA for NotchICD decreased *Serrate1* expression (80.5%, n=87, data not shown). Thus, we conclude that a feedback loop might be responsible for the resolution of *Notch1* and *Serrate1* expression in the heart field.

Activation and inhibition of Notch signaling affect myocardial gene expression differentially

We next asked whether Notch signaling in the heart affects myocardial-specific genes. We examined several genes encoding contractile proteins including *troponin Ic* (*TnIc*), *myosin heavy chain* (*MHCα*) and *cardiac actin* (*c. actin*). These genes are expressed identically throughout the developing myocardium and are considered markers of myocardial differentiation (Mohun et al., 1984; Logan and Mohun, 1993; Drysdale et al., 1994). 60-80% of embryos injected with GR-Su(H)VP16 and treated with dexamethasone after stage 18-19 showed severely reduced or no expression of each of these genes (Fig. 4B,D; summarized in Fig. 6). In contrast, control embryos cultured in the absence of dexamethasone showed symmetrical expression on both the injected and the uninjected sides (Figs 4A,C, 6). A decrease in contractile protein gene expression could be explained by the apoptotic elimination of myocardial cells. However, TUNEL analysis employed to detect fragmented chromatin revealed no change in the pattern of apoptotic cells (data not shown). This argues against apoptotic elimination as does previous work demonstrating that the activation of Notch signaling can protect cells from apoptosis (Deftos et al., 1998; Jehn et al., 1999; Shelly et al., 1999). Additionally, we examined the effect of conditionally activating Notch signaling using GR-NotchICD, as has been done previously (Wettstein et al., 1997). As for GR-Su(H)VP16, GR-NotchICD caused a decrease in the myocardial marker *TnIc* (Fig. 4J; 47.7%, n=130).

In contrast to the effects of GR-Su(H)VP16 and GR-NotchICD, targeted expression of GR-Su(H)^{DBM} increased expression of *TnIc*, *MHCα* and *cardiac actin* on the injected side of the embryo (Fig. 4F,H; summarized in Fig. 6). As before, the effects were observed only on the injected side of dexamethasone-treated embryos. Interestingly, while the extent of myocardial gene expression was expanded, it never extended

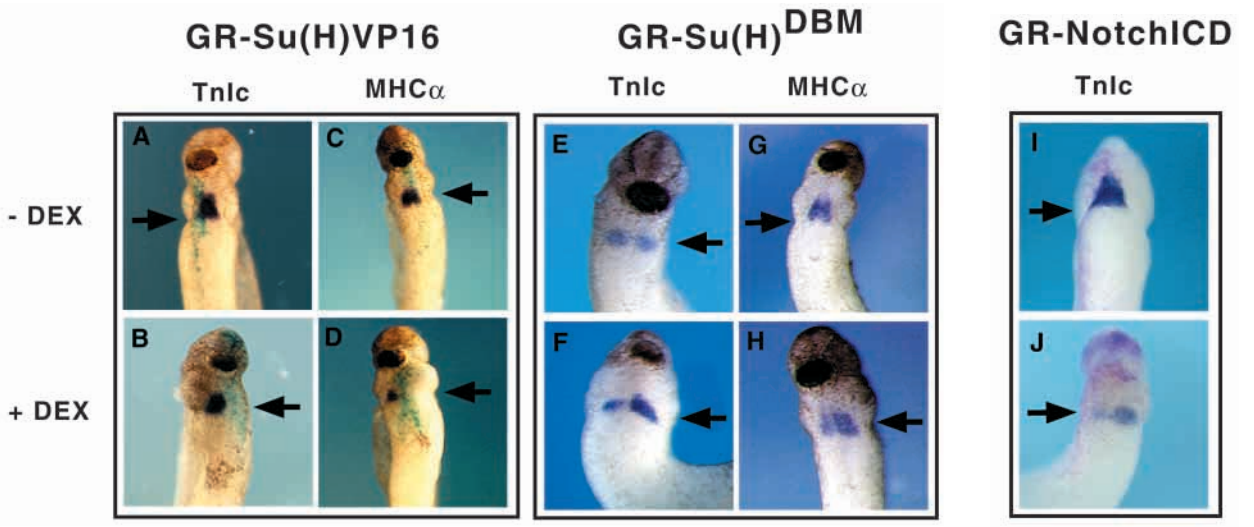

Fig. 4. Inverse effects of GR-Su(H)VP16 and GR-Su(H)^{DBM} on myocardial gene expression. Expression of contractile protein genes *TnIc* and *MHCα* were examined in embryos injected with mRNA encoding either GR-Su(H)VP16 or GR-Su(H)^{DBM}. All embryos are oriented ventrally with anterior at top. Arrows indicate the injected side of the embryo. (A-D) Embryos injected with GR-Su(H)VP16. Control injected embryos cultured in the absense of dexamethasone (uninduced, A,C) had symmetric expression of *TnIc* and *MHCα.* In contrast, injected embryos cultured in the presence of dexamethasone (induced, B,D) showed dramatically decreased expression on the injected sides. (E-H) The opposite effect was seen in GR-Su(H)^{DBM}-injected embryos. Embryos cultured in the absence of dexamethasone had symmetric expression of *TnIc* and *MHCα,* whereas those induced with dexamethasone showed increased expression of the myocardial markers on the injected sides. (I,J) Similar to the effects observed with GR-Su(H)VP16, activation of Notch signaling with GR-NotchICD decreased myocardial gene expression on the injected side in a dexamethasone dependent manner.

outside the domain of *Nkx2.5* expression, even when GR-Su(H)^{DBM} was injected outside of this region. Instead, expression appeared to expand to occupy the dorsolateral regions of the *Nkx2.5* domain, which normally form mesocardium and pericardial roof tissues. This may reflect the requirement of *Nkx2* family members (in particular *Nkx2.3* and *Nkx2.5*) for myocardial gene expression (Fu et al., 1998; Grow and Krieg, 1998). The opposite effects on myocardial gene expression elicited by the activating and inhibiting Su(H) constructs suggest that endogenous Notch signaling suppresses cardiomyogenesis while lack of Notch signaling permits myocardial differentiation.

Notch signaling affects myocardial differentiation downstream of the establishment of the early heart field

Both the timing of endogenous *Notch1* and *Serrate1* expression (Fig. 1A) and the effects of GR-Su(H)VP16, GR-NotchICD

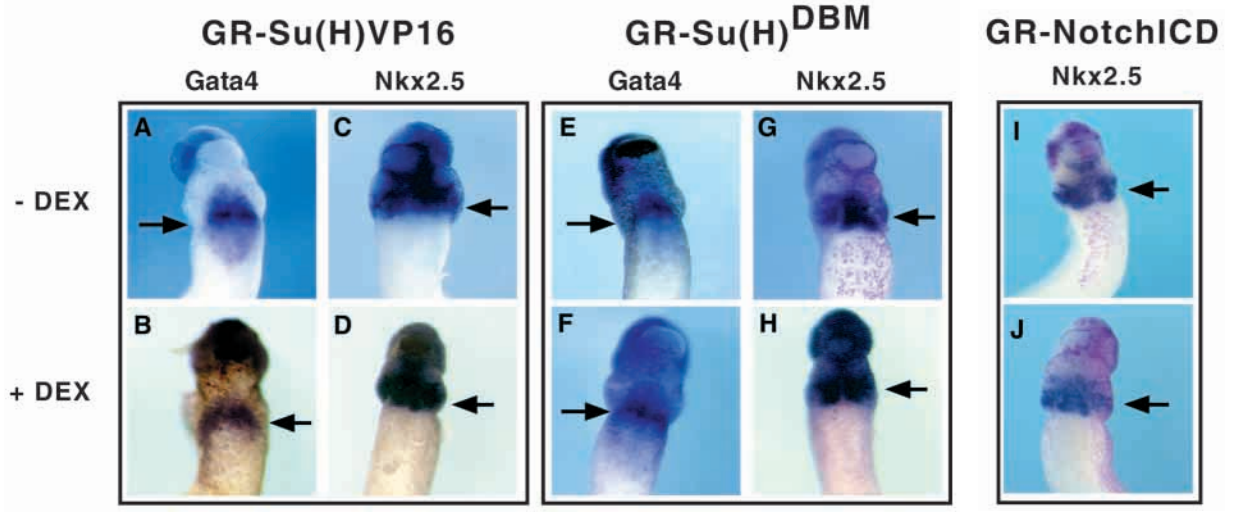

Fig. 5. Heart field markers *Nkx2.5* and *Gata4* are largely unaffected by perturbations in Notch signaling after stage 19-20. Embryos were injected with mRNA encoding either GR-Su(H)VP16 (A-D), GR-Su(H)^{DBM} (E-H), or GR-NotchICD (I,J), treated as in Fig. 3, and assayed for expression of the early heart field markers *Nkx2.5* and *Gata4*. All embryos are shown ventrally with anterior to the top. Arrows indicate the injected side of the embryo. Note the bilaterally symmetric expression of the early heart field markers in all cases.

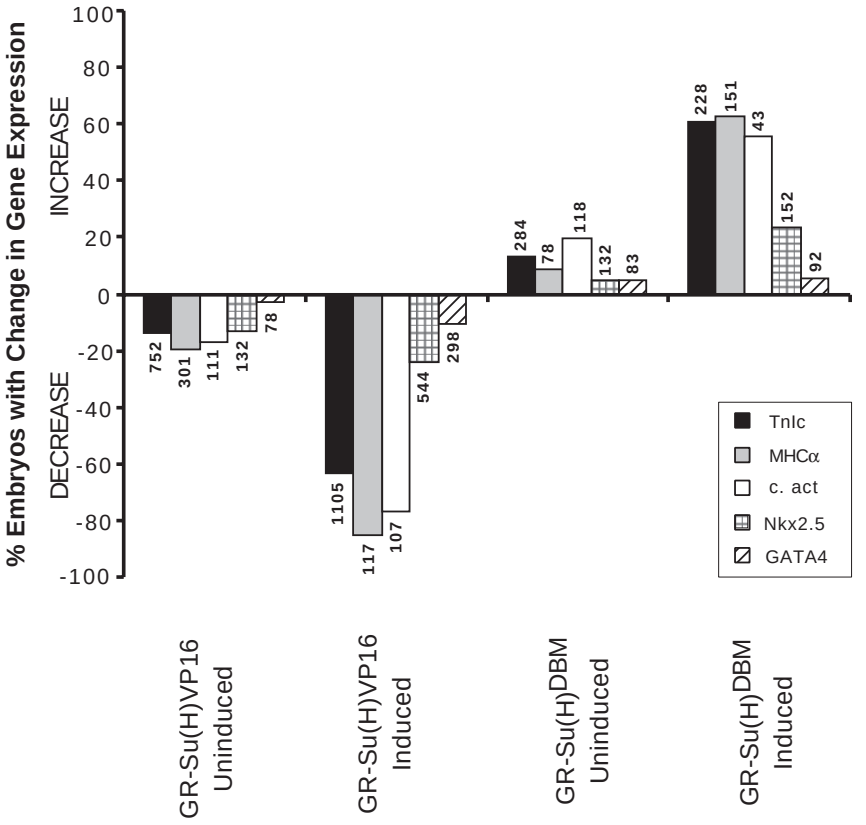

Fig. 6. Summary of the effects of Notch signaling on cardiac gene expression. The percentage of injected embryos showing either an increase or a decrease in gene expression on the injected side is indicated on the Y-axis and markers examined in each case indicated along the X-axis. The total number of embryos analyzed for each gene examined is indicated in parentheses. Activation and suppression of Notch signaling elicited opposite effects on markers of terminal myocardial differentiation but did not greatly alter expression of genes that mark the early heart field (compare *TnIc*, *MHCα*, c. actin with *Nkx2.5* and *Gata4*). Although the Su(H) constructs functioned conditionally, the minimal effects observed in the absence of dexamethasone suggest some residual activity. Standard error from the mean was less than 5% for embryos cultured in the absence of dexamethasone and less than 9% for all embryos cultured in the presence of dexamethasone.

and GR-Su(H)^{DBM} on myocardial gene expression (Fig. 4) are consistent with a role for Notch signaling in cardiogenesis after the early heart field is established. We therefore examined two markers of the early heart field, *Nkx2.5* and *Gata4* (Tonissen et al., 1994; Jiang and Evans, 1996). Unlike the pronounced effects on myocardial genes, dexamethasone activation of GR-Su(H)VP16, GR-Su(H)^{DBM} or GR-NotchICD after stage 18-19 had no or minimal effect on *Nkx2.5* and *Gata4* (Figs 5, 6). Thus, the observed effects on myocardial gene expression do not reflect a generalized perturbation of mesodermal patterning or an alteration in the specification of the heart field, including the amount of cardiogenic mesoderm present in the embryo. Instead, we conclude that endogenous activation of Notch controls cardiac cell fate after the specification of the *Nkx2.5*/heart field.

Notch signaling prevents contribution of individual cells to the myocardium

The observed effects of experimental manipulation of Su(H) activity on expression of myocardial genes are consistent with a role for Notch in selection of myocardial versus non-myocardial lineages. To examine this model further, we followed the cellular progeny of injected blastomeres to determine whether alterations in Notch signaling correlate with a change in contribution to myocardial tissue. This analysis extends the marker study by examining alterations of fate of individual cells rather than early patterns of gene expression, which might provide an imprecise prognosis of cell identity. Embryos were co-injected with mRNAs encoding either GR-Su(H)VP16 or GR-Su(H)^{DBM} along with β-galactosidase, induced with dexamethasone at stage 18-19, and then fixed and stained with X-gal at stage 40 to identify the progeny of injected cells. The percentage of embryos with β-galactosidase-positive cells in the myocardium was scored (Fig. 7). As expected, frequent contribution to myocardial and non-myocardial tissues was observed in embryos that were not induced with dexamethasone, indicating that the injected mRNA was properly targeted (Fig. 7Aa,b). However, induction with dexamethasone diminished the myocardial contribution of β-galactosidase-positive cells in embryos where Notch signaling was activated by GR-Su(H)VP16 (Fig. 7Ac). In these embryos, β-galactosidase-positive cells persisted in the embryo but were found predominately in non-myocardial tissue. In addition, the myocardium of these embryos appeared smaller and overall morphology was disorganized. In contrast, embryos expressing the dominant negative Su(H) and treated with dexamethasone showed robust contribution of β-galactosidase-positive cells to the myocardium (Fig. 7Ad). These hearts often appeared larger than those of controls, which had been injected but not treated with dexamethasone. Thus, perturbations of Notch signaling affect not only gene expression but also the fate of cells within the heart field.

Does Notch signaling mediate a choice between myocardial and non-myocardial lineages within the heart field?

One implication of the opposite effects of GR-Su(H)VP16 and GR-Su(H)^{DBM} on cardiomyogenesis, consistent with the spatial patterns of *Serrate1* and *Notch1*, is that endogenous Notch signaling mediates a lineage choice between myocardium and the non-myocardial cells that give rise to the mesocardium and pericardial roof. To explore this possibility,

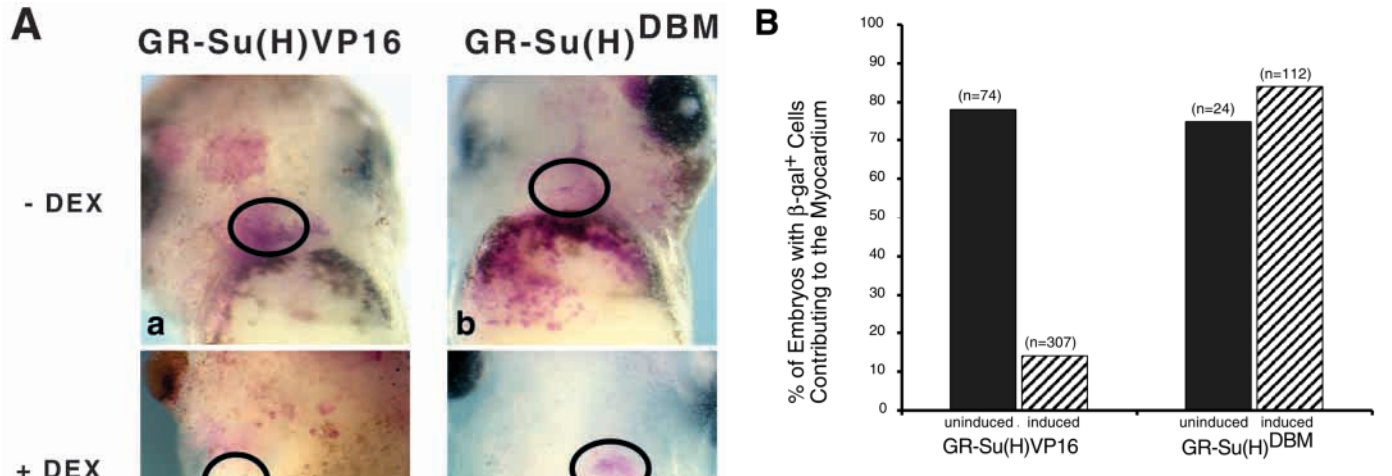

Fig. 7. Activation of Notch inhibits the contribution of individual cells to myocardial tissue. Embryos were injected into one dorsal-vegetal blastomere at the 8-cell stage with mRNA encoding either GR-Su(H)VP16 or GR-Su(H)^{DBM}, treated as described above and fixed at stages 40-42. Embryos were then processed for β-galactosidase activity to identify the fate of the cellular progeny of the injected blastomeres. At this stage, the heart tube has fused and looped, and the ventral ectoderm is transparent allowing for direct visualization of the heart. A black oval surrounds the myocardium. (A) Examples of injected embryos. In the absence of dexamethasone, the progeny of injected blastomeres contribute to the entire heart region as well as to surrounding tissue (a,b). In contrast, β-galactosidase-positive cells are absent in the myocardium of embryos injected with mRNA encoding GR-Su(H)VP16 and treated with dexamethasone, despite contribution to non-myocardial structures (c). β-galactosidase is detected throughout the myocardium of embryos expressing induced GR-Su(H)^{DBM} (d). (B) Percentage of injected embryos with β-galactosidase-positive cells contributing to the myocardium.

we asked if the observed changes in myocardial gene expression are accompanied by commensurate alterations in genes specific for mesocardial or pericardial roof tissue. Two markers for these tissues were examined: *Serrate1*, which is affected by Notch signaling (as shown in Fig. 3), and *Bmp4* (Nishimatsu et al., 1992). In situ hybridization showed that *Bmp4* transcripts are localized specifically in the prospective mesocardium extending into the pericardial roof but not elsewhere in the heart region at stage 28 (Fig. 8Aa-c). In embryos injected with GR-Su(H)VP16 mRNA and cultured in dexamethasone from stage 18-19 until fixation, *Bmp4* expression increased on the injected side of the embryo (Fig. 8B). This increase was noted in 62.7% (n=370) of injected embryos treated with dexamethasone. *Bmp4* expression expanded either ventrally (into tissue which normally forms myocardium) or more laterally. In contrast, GR-Su(H)^{DBM} decreased expression of *Bmp4* on the injected side of the embryo (Fig. 8B). This phenotype was observed in 52.9% (n=121) of injected, dexamethasone-treated embryos. The decrease in gene expression observed in GR-Su(H)^{DBM}-injected embryos was not attributable to apoptotic elimination as determined by TUNEL staining (data not shown). The magnitude of the alteration in *Bmp4* expression, elicited by either GR-Su(H)VP16 or GR-Su(H)^{DBM}, was less than that for either of the myocardial genes examined; however, the percentage of embryos affected was similar. Possible reasons for this observation are discussed below. Nonetheless, the inverse response of myocardial genes and *Bmp4* to both activating and inhibitory Su(H) constructs is consistent with the model that endogenous Notch signaling influences the selection between myocardial and mesocardial/pericardial roof cell fates within the *Nkx2.5*/heart field.

DISCUSSION

Role of Notch in early heart development

An early differentiation event in the cardiogenic mesoderm is the establishment of distinct myocardial and non-myocardial domains. Although the entire crescent of the *Xenopus* Nkx2.5-expressing cardiac mesoderm is specified initially as myogenic, this potency (but not *Nkx2.5* expression) is lost in the dorsolateral portions of the field and these cells eventually adopt mesocardial and pericardial roof fates. The experiments described here show that activation of Notch signaling by GR-Su(H)VP16 and NotchICD caused diminished expression of *Serrate1* (Fig. 3) and the myocardial genes *TnIc*, *MHCα* and *cardiac actin* (Figs 4, 6) and a corresponding increase in expression of *Bmp4*. Inhibition of endogenous Notch signaling by GR-Su(H)^{DBM} resulted in the opposite of these effects. In addition, GR-Su(H)VP16 diminished contribution of individual cells to the myocardium (Fig. 7). Importantly, these changes in gene expression and cell fate were not accompanied by alterations in the expression of *Nkx2.5* and *Gata4*. We conclude that endogenous Notch signaling regulates cell fate downstream of the establishment of the early heart field and that, after stage 20, Notch signaling does not alter expression of these early heart field markers. In addition, our observed regulation of *Serrate1* expression upon injection of Su(H) suggests that the resolution of the largely overlapping patterns

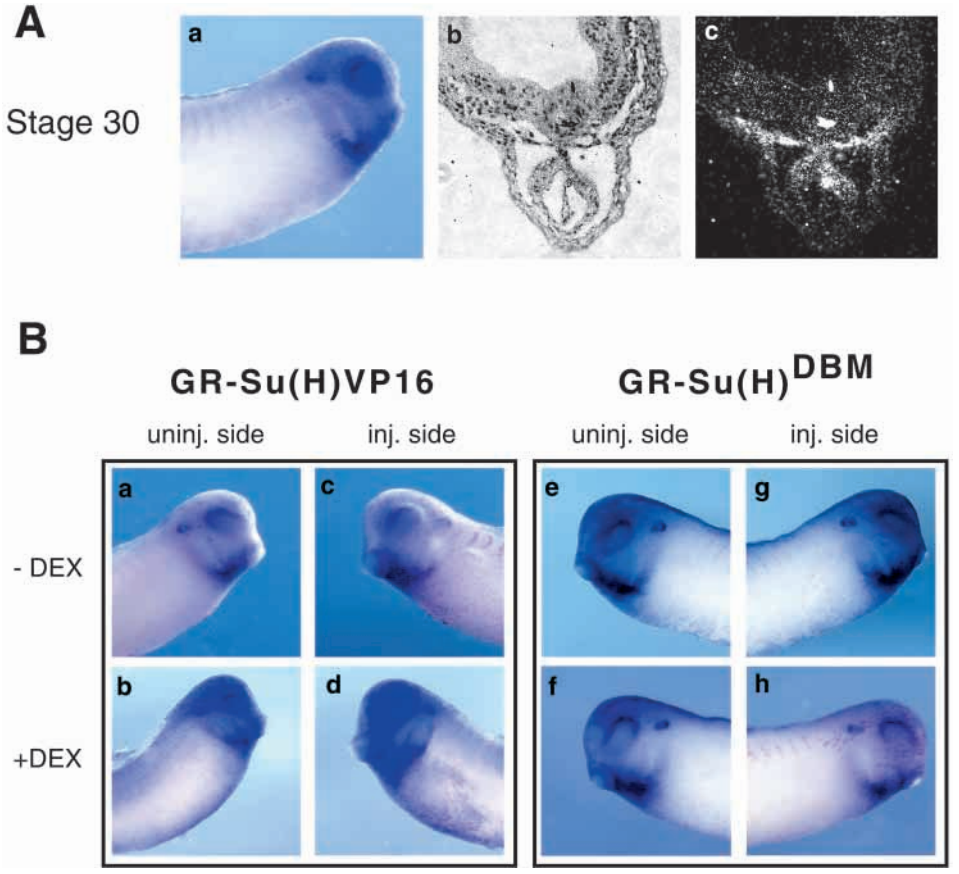

Fig. 8. Notch signaling affects endogenous levels of *Bmp4*, a marker of dorsal mesocardial and pericardial roof cells. (A) *Bmp4* expression marks the pericardial roof and mesocardium at stage 30 shown in whole-mount (a) and transverse section (b,c). (B) *Bmp4* expression in injected embryos. Embryos injected with GR-Su(H)VP16 and induced with dexamethasone displayed increased expression of *Bmp4* on the injected side (b,d). In contrast, injection and induction of GR-Su(H)^{DBM} resulted in a decreased expression on the injected side (f,h). Symmetrical expression was observed in control, sibling embryos that were injected but not induced (a,c,e,g).

of *Notch1* and *Serrate1* mRNA expression at stage 22-25 to the minimally overlapping pattern seen at stage 29-30 (Fig. 1), occurs via feedback inhibition of *Serrate1* by Notch activation. Finally, an important conclusion is that endogenous Serrate-Notch-Su(H) signaling inhibits cardiomyogenesis. This is evident by comparison of the opposite changes in myocardial gene expression (Figs 4, 6) as well as the diversion of cardiomyogenic cells to non-myocardial fates by activation of GR-Su(H)VP16 (Fig. 7). These results, taken together with the spatial and temporal patterns of *Notch1* and *Serrate1* expression (Fig. 1), suggest that endogenous Serrate1/Notch1/Su(H) signaling is involved in the suppression of cardiomyogenesis that normally occurs in the dorsolateral portions of the *Nkx2.5*/heart field between stages 22 and 28.

Dependence on Su(H)

Our results suggest that Su(H) is both necessary and sufficient to mediate the suppression of cardiomyogenic differentiation. However, Su(H)-independent signaling from Notch has been demonstrated (Shawber et al., 1996b; Matsuno et al., 1997; Nofziger et al., 1999; Wilson-Rawls et al., 1999). Whether or not such a pathway also functions during cardiogenesis has not yet been addressed. In particular, the expression of *Notch1* and *Serrate1* after the differentiation of myocardial and non-myocardial domains leaves open the possibility that the Su(H)-independent pathway might be involved during later steps of heart development.

Model for Notch action

Two models are consistent with our finding that Notch controls the ability of cardiogenic mesoderm to differentiate (Fig. 9). In a binary cell fate choice model, the presence or absence of Notch signaling causes an *Nkx2.5*-positive cell to adopt either a myocardial or a mesocardial/pericardial roof fate. Alternatively, a differentiation delay model would predict that Notch signaling maintains or prolongs cardiac cells in an undifferentiated state. This is similar to studies of the *Xenopus* retina, where cells transfected with a gene encoding the constitutively active, intracellular domain of *Xenopus* Notch1 do not immediately shift fate but remain transiently undifferentiated (Dorsky et al., 1997). The maintenance or prolongation of a stable precursor population by Notch signaling may be a general feature of the developing nervous system (Austin et al., 1995; Dorsky et al., 1995, 1997; Wang et al., 1998). In the avian neural retina, successive rounds of ligand expression and lateral inhibition are proposed to lead to a controlled exodus of cells from the precursor pool to take on primary, secondary and tertiary differentiated fates (Austin et al., 1995). By analogy, endogenous Notch signaling may block responsiveness to a myocardial differentiation signal in the dorsolateral portions of the *Nkx2.5*/heart field after stage 22-25, when these cells have overlapping *Serrate1* and *Notch1* mRNA expression and lose myocardial potency. However, precise knowledge of the spatial and temporal patterns of endogenous Notch activity as well as more precise temporal control over experimental perturbations of Notch activity will be needed to distinguish between the differentiation delay and simple lineage choice models.

Does Notch activity promote differentiation of mesocardial and pericardial roof tissue?

Expression of *Bmp4* was examined to explore whether the effects on myocardial differentiation caused by GR-Su(H)VP16 and GR-Su(H)^{DBM} were accompanied by a compensatory change in mesocardial and pericardial roof tissue. This possibility is predicted by the lineage choice model, but is also possible in the differentiation delay model

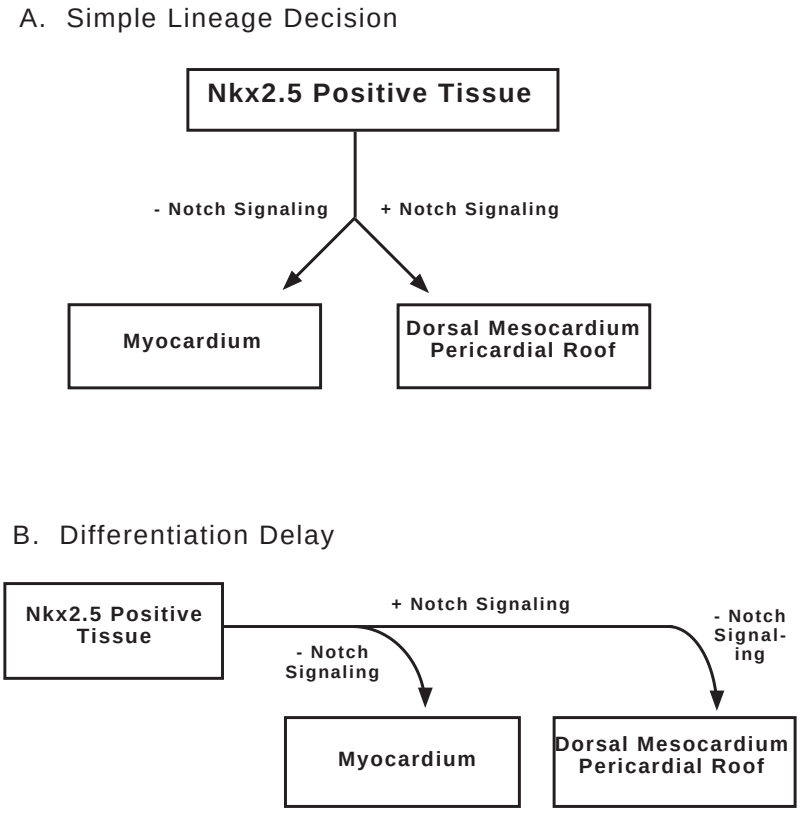

Fig. 9. Two models for the role of Notch signaling during cardiogenesis. In a binary lineage decision model (A), differentiation of an *Nkx2.5*-positive cell towards a myocardial or a mesocardial/pericardial roof fate would depend directly on Notch signaling. In contrast, Notch signaling might act to maintain or prolong a multipotent precursor state (B). In such a model, myocardial differentiation would be permitted in ventral cells in the absence of endogenous Notch signaling, while persistent Notch activity dorsolaterally would block differentiation. Subsequent cessation of Notch signaling dorsolaterally would then allow mesocardial and pericardial roof differentiation. In this model, Notch regulates responsiveness and an endogenous timer and/or extrinsic local cues are needed to specify cell fate.

if the activity of the GR-Su(H) constructs were not sustained. This latter scenario is possible since the injected mRNAs decay over time. In support of the idea that Notch regulates a balance between differentiation as myocardium versus mesocardium/pericardial tissues, we found that *Bmp4* and myocardial genes were inversely regulated by the Su(H) constructs (compare Figs 4 and 8). Although the magnitude of altered *Bmp4* expression (which marks mesocardium and pericardial roof tissue) was less than that seen for the myocardial markers, the percentage of embryos affected was similar. Several factors could account for the moderate magnitude of change in *Bmp4* expression. First, the ability to form myocardium may be restricted to only a fraction of *Bmp4*-positive cells, either because they lack competence or are too distant from any pro-myocardial factors that might exist in or near the prospective myocardium. Second, expression of *Bmp4* itself may not be an ideal marker of mesocardial and pericardial roof specification. We are unaware of additional markers (other than *Serrate1*, which appears regulated by Notch signaling as part of the feedback loop that resolves the overlapping *Notch1*/*Serrate1* expression). The identification of additional markers will be helpful to corroborate our tentative conclusion that suppression of cardiogenesis by Notch activity coincides with an increase in mesocardial and pericardial roof fates.

Multiple roles for Notch signaling in the developing heart

We chose to perturb Notch signaling at the level of the downstream mediator Su(H) in part to overcome the complexity generated by multiple Notch receptors and ligands. Although *Delta1* and *Delta2* transcripts were not detected in the early heart field, the broad specificity of GR-Su(H)VP16 and GR-Su(H)^{DBM} leaves open the possibility that Notch receptors and/or ligands, in addition to Serrate1 and Notch1, influence the myocardial/non-myocardial lineage decision. Moreover, it is likely that Notch signaling plays additional roles during cardiogenesis, either prior to the specification of *Nkx2.5* or after the initial determination of myocardial and non-myocardial tissues. Such a hypothesis is similar to the conclusions drawn from studies on somitogenesis and neurogenesis, in which Notch activity has been shown to act at multiple stages to contribute to complex tissue patterning (Fuerstenberg and Giniger, 1998; Chitnis, 1999; Sestan et al., 1999). A role for endogenous Notch signaling in the heart prior to establishment of the *Nkx2.5*/heart field could contribute to the slight effect that activation of the Su(H) constructs had on expression of *Nkx2.5* and *Gata4* (Fig. 6). Expression of Notch receptor and ligand mRNA and protein in the developing heart of the mouse, chick and zebrafish also point to possible later functions, in particular in the developing myocardial tube, outflow tract and endocardium (Franco del Amo et al., 1992; Reaume et al., 1992; Bierkamp and Campos-Ortega, 1993; Williams et al., 1995; Myat et al., 1996; Westin and Lardelli, 1997). This would be consistent with cardiac defects that characterize the preponderance of patients with Alagille syndrome caused by autosomal dominant mutations in the *Jagged1* gene (Krantz et al., 1997; Li et al., 1997; Oda et al., 1997).

Notch, competence and the acquisition of cardiac fates

Our studies of Notch extend the current model for the subdivision of the heart field. We propose that signaling through the Serrate-Notch-Su(H) pathway mediates the suppression of the cardiomyogenic program in the dorsolateral portion of the *Nkx2.5*/heart field. Although Notch acts to inhibit differentiation, as shown in a number of in vivo and in vitro systems, and we have shown here that inhibition of Notch correlates with an increase in cardiomyogenesis, the impetus to differentiate may be more complex than lack or abrogation of a Notch signal. By analogy with its role in neurogenesis, Notch may regulate competence of cells within the cardiogenic mesoderm to adopt myocardial, mesocardial or pericardial roof fate. The absence of Notch signaling in the ventral portion of the heart field, inferred from our results, may confer

competence to differentiate as myocardium. Also, Notch activity (at least transiently) may be important for differentiation of the mesocardium and pericardial roof. However, cell fate acquisition may also require specific local cues. Therefore, it will be of interest to examine whether locally expressed proteins, such as BMP4, function in concert with Notch to regulate cell fate within the cardiogenic mesoderm.

The authors thank Josh Rutenberg for helpful discussions and help with in situ hybridization during the early stages of this work; and Valerie Schneider for radioactive BMP4 in situ hybridization data. We also acknowledge other members of the Mercola lab, and Drs. Spyros Artavanis-Tsakonas, Karen Symes and Barbara Osborne for insightful comments on the manuscript. Additionally, we thank Drs Chris Kintner, Paul Krieg, Tim Mohun and, in particular, Robert Davis for generous contribution of reagents and Chris Simpson for expert histology. The authors gratefully acknowledge funding from the NIH (RO1 HL59502 to M. M. and F32 HL09740-03 to K. M.).

REFERENCES

Artavanis-Tsakonas, S., Matsuno, K. and Fortini, M. E. (1995). Notch signaling. *Science* **268**, 225-232.

Artavanis-Tsakonas, S., Rand, M. D. and Lake, R. J. (1999). Notch signaling: cell fate control and signal transduction in development. *Science* **284**, 770-776.

Austin, C. P., Feldman, D. E., Ida, J. A. and Cepko, C. L. (1995). Vertebrate retinal ganglion cells are selected from competent progenitors by the action of Notch. *Development* **121**, 3637-3650.

Bettenhausen, B., de Angelis, M. H., Simon, D., Guenet, J. L. and Gossler, A. (1995). Transient and restricted expression during mouse embryogenesis of Dll1, a murine gene closely related to Drosophila Delta. *Development* **121**, 2407-2418.

Bierkamp, C. and Campos-Ortega, J. A. (1993). A zebrafish homologue of the Drosophila neurogenic gene Notch and its pattern of transcription during early embryogenesis. *Mech. Dev.* **43**, 87-100.

Chitnis, A., Henrique, D., Lewis, J., Ish-Horowicz, D. and Kintner, C. (1995). Primary neurogenesis in Xenopus embryos regulated by a homologue of the Drosophila neurogenic gene Delta. *Nature* **375**, 761-766.

Chitnis, A. and Kintner, C. (1996). Sensitivity of proneural genes to lateral inhibition affects the pattern of primary neurons in Xenopus embryos. *Development* **122**, 2295-2301.

Chitnis, A. B. (1999). Control of neurogenesis – lessons from frogs, fish, and flies. *Curr. Opin. Neurobiol.* **9**, 18-25.

Coffman, C., Harris, W. and Kintner, C. (1990). Xotch, the Xenopus homolog of Drosophila Notch. *Science* **249**, 1438-1441.

Cohen-Gould, L. and Mikawa, T. (1996). The fate diversity of mesodermal cells within the heart field during chicken early embryogenesis. *Dev. Biol.* **177**, 265-273.

Conlon, R. A., Reaume, A. G. and Rossant, J. (1995). Notch1 is required for the coordinate segmentation of somites. *Development* **121**, 1533-1545.

Copenhaver, W. M. (1924). Experiments on the development of the heart of Amblystoma punctatum. *J. Exp. Zool.* **43**, 321-371.

Corbin, V., Michelson, A. M., Abmayr, S. M., Neel, V., Alcamo, E., Maniatis, T. and Young, M. W. (1991). A role for the Drosophila neurogenic genes in mesoderm differentiation. *Cell* **67**, 311-323.

Crowe, R., Zikherman, J. and Niswander, L. (1999). Delta-1 negatively regulates the transition from prehypertrophic to hypertrophic chondrocytes during cartilage formation. *Development* **126**, 987-998.

de la Pompa, J. L., Wakeham, A., Correia, K. M., Samper, E., Brown, S., Aguilera, R. J., Nakano, T., Honjo, T., Mak, T. W., Rossant, J. and Conlon, R. A. (1997). Conservation of the Notch signalling pathway in mammalian neurogenesis. *Development* **124**, 1139-1148.

Deftos, M. L., He, Y. W., Ojala, E. W. and Bevan, M. J. (1998). Correlating notch signaling with thymocyte maturation. *Immunity* **9**, 777-786.

Dorsky, R., Chang, W. S., Rapaport, D. H. and Harris, W. A. (1997). Regulation of neuronal diversity in the Xenopus retina by Delta signalling. *Nature* **385**, 67-69.

Dorsky, R. I., Rapaport, D. H. and Harris, W. A. (1995). Xotch inhibits cell differentiation in the Xenopus retina. *Neuron* **14**, 487-496.

Drysdale, T. A., Patterson, K., Crawford, M. J. and Krieg, P. A. (1994). Cardiac troponin I is a heart-specific marker in the Xenopus embryo: expression during abnormal heart morphogenesis. *Dev. Biol.* **165**, 432-441.

Dunwoodie, S. L., Henrique, D., Harrison, S. M. and Beddington, R. S. P. (1997). Mouse Dll3: a novel divergent delta gene which may complement the function of other delta homologues during early pattern formation in the mouse embryo. *Development* **124**, 3065-3076.

Ekman, G. (1925). Experimentelle Beitrge zur Herzentwicklung der Amphibien. *Wilhelm Roux Arch. EntwMech. Org.* **106**, 320-352.

Ellisen, L. W., Bird, J., West, D. C., Soreng, A. L., Reynolds, T. C., Smith, S. D., and Sklar, J. (1991). The human homolog of the Drosophila Notch gene is broken by chromosomal translocations in T lymphoblastic neoplasms. *Cell* **66**, 649-661.

Fishman, M. C. and Chien, K. R. (1997). Fashioning the vertebrate heart: earliest embryonic decisions. *Development* **124**, 2099-2117.

Franco del Amo, F., Smith, D. E., Swiatek, P. J., Gendron-Maguire, M., Greenspan, R. J., McMahon, A. P. and Gridley, T. (1992). Expression pattern of motch, a mouse homolog of Drosophila Notch, suggests an important role in early post-implantation mouse development. *Development* **115**, 737-744.

Fu, Y., Yan, W., Mohun, T. J. and Evans, S. M. (1998). Vertebrate tinman homologues XNkx2.3 and XNkx2.5 are required for heart formation in a functionally redundant manner. *Development* **125**, 4439-4449.

Fuerstenberg, S. and Giniger, E. (1998). Multiple roles for Notch in Drosophila myogenesis. *Dev. Biol.* **201**, 66-77.

Grow, M. W. and Krieg, P. A. (1998). Tinman function is essential for vertebrate heart development: elimination of cardiac differentiation by dominant inhibitory mutants of the tinman-related genes, Nkx2.3 and Nkx2.5. *Dev. Biol.* **204**, 187-196.

Haddon, C., Jiang, Y.-J., Smithers, L. and Lewis, J. (1998). Delta-notch signaling and the patterning of sensory cell differentiation in the zebrafish ear: evidence from the mind bomb mutant. *Development* **125**, 4637-4644.

Harland, R. M. (1991). In situ hybridization: an improved whole mount method for Xenopus embryos. *Methods Cell Biol.* **36**, 685-695.

Hartenstein, A. Y., Rugendorff, A., Tepass, U. and Hartenstein, V. (1992). The function of the neurogenic genes during epithelial development in the Drosophila embryo. *Development* **116**, 1203-1220.

Henrique, D., Adam, J., Myat, A., Chitnis, A., Lewis, J. and Ish-Horowicz, D. (1995). Expression of a Delta homologue in prospective neurons in the chick. *Nature* **375**, 787-790.

Hrabe de Angelis, M., McIntyre, J. and Gossler, A. (1997). Maintenance of somite borders in mice requires the Delta homologue Dll1. *Nature* **386**, 717-721.

Irvine, K. D. (1999). Fringe, notch, and making developmental boundaries. *Curr. Opin. Genet. Dev.* **9**, 434-441.

Jacobson, A. G. and Duncan, J. T. (1968). Heart induction in salamanders. *J. Exp. Zool.* **167**, 79-103.

Jehn, B. M., Bielke, W., Pear, W. S. and Osborne, B. A. (1999). Protective effects of notch-1 on TCR-induced apoptosis. *J. Immunol.* **162**, 635-638.

Jen, W. C., Gawantka, V., Pollet, N., Niehrs, C. and Kintner, C. (1999). Periodic repression of Notch pathway genes governs the segmentation of Xenopus embryos. *Genes Dev.* **13**, 1486-1499.

Jen, W. C., Wettstein, D., Turner, D., Chitnis, A. and Kintner, C. (1997). The Notch ligand, X-Delta, mediates segmentation of the paraxial mesoderm in Xenopus embryos. *Development* **124**, 1169-1178.

Jiang, Y. and Evans, T. (1996). The Xenopus GATA 4,5,6 genes are associated with cardiac specification and can regulate cardiac-specific transcription during embryogenesis. *Dev. Biol.* **174**, 258-270.

Joutel, A., Corpechot, C., Ducros, A., Vahedi, K., Chabiat, H., Mouton, P., Alamowitch, S., Domenga, V., Cecillion, M., Marechasl, E., Maciazek, J., Vayssiere, C., Cruaud, C., Cabamos, E.-A., Rochoux, M. M., Weissenbach, J., Bach, J. F., Bousser, M. G. and Tournier-Lasserve, E. (1996). Notch3 mutations in CADASIL, a hereditary adult onset condition causing stroke and dementia. *Nature* **383**, 707-710.

Kolm, P. J. and Sive, H. L. (1995). Efficient hormone-inducible protein function in Xenopus laevis. *Dev. Biol.* **171**, 267-272.

Krantz, I. D., Piccoli, D. A. and Spinner, N. B. (1997). Alagille syndrome. *J. Med. Genetics* **34**, 152-157.

Lardelli, M., Dahlstrand, J. and Lendahl, U. (1994). The novel Notch homologue mouse Notch 3 lacks specific epidermal growth factor-repeats and is expressed in proliferating neuroepithelium. *Mech. Dev.* **46**, 123-136.

Lee, R. K., Stanier, D. Y. R., Weinstein, B. M. and Fishman, M. C. (1994).

Cardiovascular development in the zebrafish: endocardial progenitors are sequestered within the heart field. *Development* **120**, 3361-3366.

Li, L., Krantz, I. D., Deng, Y., Genin, A., Banta, A. B., Collins, C. C., Qi, M., Trask, B. J., Kuo, W. L., Cochran, J., Costa, T., Pierpont, M., Rand, E. B., Piccoli, D. A., Hood, L. and Spinner, N. B. (1997). Alagille syndrome is caused by mutations in human Jagged1, which encodes a ligand for Notch1. *Nature Genetics* **16**, 243-251.

Lindsell, C. E., Shawber, C. J., Boulter, J. and Weinmaster, G. (1995). Jagged: A mammalian ligand that activates Notch1. *Cell* **80**, 909-917.

Logan, M. and Mohun, T. (1993). Induction of cardiac muscle differentiation in isolated animal pole explants of Xenopus laevis embryos. *Development* **118**, 865-875.

Logeat, F., Bessia, C., Brou, C., LeBail, O., Jarriault, S., Seidah, N. G. and Israel, A. (1998). The Notch1 receptor is cleaved constituitively by a furin-like convertase. *Proc. Nat. Acad. Sci. USA* **95**, 8108-8112.

Lough, J., Barron, M., Brogley, M., Sugi, Y., Bolender, D. L. and Zhu, X. (1996). Combined BMP-2 and FGF-4, but neither factor alone, induces cardiogenesis in non-precardiac embryonic mesoderm. *Dev. Biol.* **178**, 198-202.

Lyons, G. E. (1996). Vertebrate heart development. *Curr. Opin. Genet. Dev.* **6**, 454-460.

Matsuno, K., Go, M. J., Sun, X., Eastman, D. S. and Artavanis-Tsakonas, S. (1997). Suppressor of Hairless independent events in Notch signaling imply novel pathway elements. *Development* **124**, 4265-4273.

Mohun, T. and Sparrow, D. (1997). Early steps in vertebrate cardiogenesis. *Curr. Opin. Genet. Dev.* **7**, 628-633.

Mohun, T. J., Brennan, S., Dathan, N., Fairman, S. and Gurdon, J. B. (1984). Cell type-specific activation of actin genes in the early amphibian embryo. *Nature* **311**, 716-721.

Myat, A., Henrique, D., Ish-Horowicz, D. and Lewis, J. (1996). A chick homologue of serrate and its relationship with notch and delta homologues during central neurogenesis. *Dev. Biol.* **174**, 233-247.

Nascone, N. and Mercola, M. (1995). An inductive role for the endoderm in *Xenopus* cardiogenesis. *Development* **121**, 515-523.

Nieuwkoop, P. D. and Faber, J. (1994). *Normal Table of Xenopus laevis.* Amsterdam: North-Holland Publishing Company.

Nishimatsu, S., Suzuki, A., Shoda, A., Murakami, K. and Ueno, N. (1992). Genes for bone morphogenetic proteins are differentially transcribed in early amphibian embryos. *Biochem. Biophys. Res. Com.* **186**, 1487-1495.

Nofziger, D., Miyamoto, A., Lyons, K. M. and Weinmaster, G. (1999). Notch signaling imposes two distinct blocks in the differentiation of C2C12 myoblasts. *Development* **126**, 1689-1702.

Oda, T., Elkaloun, A. G., Pike, B. L., Okajima, K., Krantz, I. D., Genin, A., Piccoli, D. A., Meltzer, P. S., Spinner, N. B., Collins, F. S. and Chandrasekharappa, S. C. (1997). Mutations in the human Jagged1 gene are responsible for Alagille syndrome. *Nature Genetics* **16**, 235-242.

Oka, C., Nakano, T., Wakeham, A., de la Pompa, J. L., Mori, C., Sakai, T., Okazaki, S., Kawaichi, M., Shiota, K., Mak, T. W. and Honjo, T. (1995). Disruption of the mouse RBP-Jk results in early embryonic death. *Development* **121**, 3291-3301.

Raffin, M., Leong, L. M., Rones, M. S., Sparrow, D., Mohun, T. and Mercola, M. (2000). Subdivision of the cardiac Nkx2.5 expression domain into myogenic and non-myogenic compartments. *Dev. Biol.* **218**, 326-340.

Reaume, A. G., Conlon, R. A., Zirngibl, R., Yamaguchi, T. P. and Rossant, J. (1992). Expression analysis of a Notch homologue in the mouse embryo. *Dev. Biol.* **154**, 377-387.

Robey, E., Chang, D., Itano, A., Cado, D., Alexander, H., Lans, D., Weinmaster, G. and Salmon, P. (1996). An activated form of Notch influences the choice between CD4 and CD8 T cell lineages. *Cell* **87**, 483-492.

Sater, A. K. and Jacobson, A. G. (1990a). The role of the dorsal lip in the induction of heart mesoderm in Xenopus laevis. *Development* **108**, 461-470.

Sater, A. K. and Jacobson, A. G. (1989). The specification of heart mesoderm occurs during gastrulation in Xenopus laevis. *Development* **105**, 821-830.

Sater, A. K. and Jacobson, A. G. (1990b). The restriction of the heart morphogenetic field in Xenopus laevis. *Dev. Biol.* **140**, 328-336.

Schroeter, E. H., Kisslinger, J. A. and Kopan, R. (1998). Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. *Nature* **393**, 382-386.

Schultheiss, T. M., Burch, J. B. and Lassar, A. B. (1997). A role for bone morphogenetic proteins in the induction of cardiac myogenesis. *Genes Dev.* **11**, 451-462.

Schultheiss, T. M., Xydas, S. and Lassar, A. B. (1995). Induction of avian cardiac myogenesis by anterior endoderm. *Development* **121**, 4203-14.

Serbedzija, G. N., Chen, J.-N. and Fishman, M. C. (1998). Regulation in the heart field of zebrafish. *Development* **125**, 1095-1101.

Sestan, N., Artavanis-Tsakonas, S. and Rakic, P. (1999). Contact-dependent inhibition of cortical neurite growth mediated by notch signaling. *Science* **286**, 741-746.

Shawber, C., Boulter, S., Lindsell, C. E. and Weinmaster, G. (1996a). Jagged2: a Serrate-like gene expressed during rat embryogenesis. *Dev. Biol.* **180**, 370-376.

Shawber, C., Nofziger, D., Hsieh, J. J.-D., Lindsell, C., Bogler, O., Hayward, D., and Weinmaster, G. (1996b). Notch signaling inhibits muscle cell differentiation through a CBF1 independent pathway. *Development* **122**, 3765-3773.

Shelly, L. L., Fuchs, C. and Miele, L. (1999). Notch-1 inhibits apoptosis in murine erythroleukemia cells and is necessary for differentiation induced by hybrid polar compounds. *J. Cell Biochem.* **73**, 164-175.

Swiatek, P. J., Lindsell, C. E., Franco del Amo, F., Weinmaster, G. and Gridley, T. (1994). Notch1 is essential for postimplantation development in mice. *Genes Dev.* **8**, 707-719.

Tonissen, K. F., Drysdale, T. A., Lints, T. J., Harvey, R. P. and Krieg, P. A. (1994). XNkx-2.5, a Xenopus gene related to Nkx-2.5 and tinman: evidence for a conserved role in cardiac development. *Dev. Biol.* **162**, 325-328.

Uyttendaele, H., Marazzi, G., Wu, G., Yan, Q., Sassoon, D. and Kitajewski, J. (1996). Notch4/int-3, a mammalian proto-oncogene, is an endothelial cell-specific mammalian Notch gene. *Development* **122**, 2251-2259.

Wang, S., Sdrulla, A. D., diSibio, G., Bush, G., Nofziger, D., Hicks, C., Weinmaster, G. and Barres, B. A. (1998). Notch receptor activation inhibits oligodendrocyte differentiation. *Neuron* **21**, 63-75.

Weinmaster, G. (1997). The ins and outs of notch signaling. *Molec. Cell. Neurosci.* **9**, 91-102.

Weinmaster, G. (1998). Notch signaling: direct or what? *Curr. Opin. Genet. Dev.* **8**, 436-442.

Weinmaster, G., Roberts, V. J. and Lemke, G. (1991). A homolog of Drosophila Notch expressed during mammalian development. *Development* **113**, 199-205.

Weinmaster, G., Roberts, V. J. and Lemke, G. (1992). Notch2: a second mammalian Notch gene. *Development* **116**, 931-941.

Westin, J. and Lardelli, M. (1997). Three novel Notch genes in zebrafish: implications for vertebrate Notch gene evolution and function. *Dev. Genes Evol.* **207**, 51-63.

Wettstein, D. A., Turner, D. L. and Kintner, C. (1997). The Xenopus homolog of Drosophila Suppressor of Hairless mediates Notch signaling during primary neurogenesis. *Development* **124**, 693-702.

Williams, R., Lendahl, U. and Lardelli, M. (1995). Complementary and combinatorial patterns of Notch gene family expression during early mouse development. *Mech. Dev.* **53**, 357-368.

Wilson-Rawls, J., Molkentin, J. D., Black, B. L. and Olson, E. N. (1999). Activated Notch inhibits myogenic activity of the MADS-box transcription factor myocyte enhancer factor 2c. *Molec. Cell. Biol.* **19**, 2853-2862.