Mesoderm progenitor cells of common origin contribute to the head musculature and the cardiac outflow tract

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During early embryogenesis, heart and skeletal muscle progenitor cells are thought to derive from distinct regions of the mesoderm (i.e. the lateral plate mesoderm and paraxial mesoderm, respectively). In the present study, we have employed both in vitro and in vivo experimental systems in the avian embryo to explore how mesoderm progenitors in the head differentiate into both heart and skeletal muscles. Using fate-mapping studies, gene expression analyses, and manipulation of signaling pathways in the chick embryo, we demonstrate that cells from the cranial paraxial mesoderm contribute to both myocardial and endocardial cell populations within the cardiac outflow tract. We further show that Bmp signaling affects the specification of mesoderm cells in the head: application of Bmp4, both in vitro and in vivo, induces cardiac differentiation in the cranial paraxial mesoderm and blocks the differentiation of skeletal muscle precursors in these cells. Our results demonstrate that cells within the cranial paraxial mesoderm play a vital role in cardiogenesis, as a new source of cardiac progenitors that populate the cardiac outflow tract in vivo. A deeper understanding of mesodermal lineage specification in the vertebrate head is expected to provide insights into the normal, as well as pathological, aspects of heart and craniofacial development.

KEY WORDS: Myogenesis, Secondary heart field, Cranial paraxial mesoderm, Splanchnic mesoderm, Bmp4

INTRODUCTION

A key event in the establishment of the body plan during vertebrate embryogenesis is the regionalization of the mesoderm into paraxial and lateral compartments. Paraxial mesoderm in the trunk forms the somites, which give rise to the skeletal muscle lineage, as well as to cartilage, endothelial and dermis precursors. During the past decade, the tissues and signaling molecules that induce the formation of skeletal muscle from somites have been intensively studied (Bailey et al., 2001; Buckingham, 2001; Pourquie, 2001). Notably, Wnt signaling molecules secreted from the dorsal neural tube, together with Sonic hedgehog (Shh) from the notochord, induce myogenesis in the trunk, whereas bone morphogenetic protein (Bmp) signals from the lateral plate mesoderm have been shown to block myogenesis in the trunk (Borycki et al., 2000; Munsterberg et al., 1995; Pourquie et al., 1996; Reshef et al., 1998; Stern et al., 1995; Tajbakhsh et al., 1998).

In vertebrates, the head musculature is derived from the paraxial mesoderm located anterior to the somites [termed cranial paraxial mesoderm, CPM (Coulby et al., 1992; Noden, 1983)]. Unlike the paraxial mesoderm in the trunk, the CPM lacks any overt sign of segmentation. Together with cranial neural crest cells, CPM cells fill the branchial (pharyngeal) arches, paired thickenings around the pharynx that will eventually give rise to the facial structures (Noden and Trainor, 2005). It appears that different intrinsic and extrinsic regulatory pathways control skeletal muscle formation in the trunk and in the head, as indicated by the genetic loss of myogenic transcription factors in mice (Kelly et al., 2004; Lu et al., 2002; Rudnicki et al., 1993; Tajbakhsh et al., 1997), as well as by the manipulation of tissues and signaling molecules in chick embryos (Hacker and Guthrie, 1998; Mootosamy and Dietrich, 2002; Noden et al., 1999; Tzahor et al., 2003).

Cardiac progenitor cells are derived from the lateral plate mesoderm. This tissue splits into somatic and splanchnic layers; cells within the splanchnic mesoderm form the cardiac crescent (also called the primary heart field) that later forms the myocardium and endocardium of the heart. In both Xenopus and chick embryos, Bmp and fibroblast growth factor (Fgf) act as potent inducers of cardiac differentiation during early stages of heart formation (Lough and Sugi, 2000; Schlange et al., 2000; Schultheiss et al., 1997). By contrast, members of the canonical Wnt signaling pathway can block cardiac differentiation during these stages (Brott and Sokol, 2005; Foley and Mercola, 2005; Marvin et al., 2001; Schneider and Mercola, 2001; Tzahor and Lassar, 2001).

In the developing head, the lateral mesoderm (also termed splanchnic mesoderm, SpM) is located on the ventral side of the embryo, beneath the floor of the pharynx. It has been shown in both chick (Mjaatvedt et al., 2001; Waldo et al., 2001) and mouse (Cai et al., 2003; Dodou et al., 2004; Kelly et al., 2001; Zaffran et al., 2004) embryos that cells in the head originating in various parts (albeit obscure) of the mesoderm contribute to the formation of the anterior pole of the heart following the formation of the linear heart tube. It was originally thought that all cardiac myocytes arise from the primary heart field; however, these studies suggest that the arterial pole of the heart develops by the addition of cells derived from mesoderm progenitors, now termed the second heart field [SHF (Buckingham et al., 2005)]. In contrast to our relatively broad understanding of the development of the primary heart field (Kirby, 2002; Olson and Schneider, 2003), the nature of the secondary/anterior heart field and its exact anatomical location remain elusive (Abu-Issa et al., 2004).

In a previous study in chick embryos, we demonstrated that signals from the dorsal neural tube (e.g. Wnt1 and Wnt3a) block cardiogenesis in the adjacent CPM (Tzahor and Lassar, 2001). The identification of a secondary heart field in vertebrate embryos led us to consider whether the CPM contributes in any way to this recently discovered myocardial lineage. Accordingly, in the present study, we established cellular and molecular models aimed at exploring, both in vitro and in vivo, how head mesoderm cells are specified in...
the avian embryo. Our findings show that Bmp signals affect both cardiac and skeletal muscle cell fates: Bmp4 promotes cardiogenesis and, at the same time, blocks skeletal muscle differentiation in head mesoderm progenitors. Furthermore, our fate-mapping studies reveal, for the first time, that cells within the CPM contribute to the myocardium and endocardium of the cardiac outflow tract (OFT). This cell population may represent an additional source of cardiac progenitors during vertebrate embryogenesis. We therefore propose that the developmental programs of mesoderm progenitors that contribute to the head musculature and the OFT are tightly linked, and are controlled by Bmp signaling levels.

MATERIALS AND METHODS

Explant culture
In order to distinguish between the CPM and splanchic mesoderm (SpM), the tubular heart was first removed from stage 10 chick embryos. CPM explants comprising surface ectoderm (along with some cranial neural crest cells) plus endoderm from the roof of the pharynx, and SpM explants, including the endoderm of the pharynx, were dissected separately. Explants were cultured in a collagen drop covered with medium (10% FCS in cMEM medium, chick embryo extract 2.5%, penicillin/streptomycin 0.5%) in four-well plates. Control or BMP4-conditioned medium, harvested from HEK-293 cells stably expressing human BMP4-HA, were added to the explants. To block Bmp signaling, 500 ng/ml of recombinant Fc-Noggin protein (R&D Systems) was added to the medium. RNA was harvested using a Versagene Cell Kit (Genta Systems). cDNA was synthesized from DNase-treated total RNA, using a M-MLV reverse transcriptase-mediated extension of random primers, followed by a PCR amplification using different sets of primers for cardiac and skeletal muscle markers (primer sequences are available upon request).

In situ hybridization
Whole-mount in situ hybridization was performed using digoxigenin-labeled antisense riboprobes synthesized from the cDNA. A full list of the in situ hybridization probes and a detailed protocol are available upon request. Images were obtained using a Leica MZ16FA stereomicroscope attached to a digital camera (DC300F, Leica Microsystems). For paraffin sectioning, fixed embryos were dehydrated (ethanol/xylene), washed, and transferred to paraffin. The embryos were sectioned at 10-15 μm, using a Leica microtome.

Implantation of cell aggregates
HEK-293 or HEK-293-BMP4 cells were transferred to an agar plate for 24 hours to form aggregates, which were then selected for implantation into stage 9 embryos (Tzahor et al., 2003). The eggs were sealed and incubated for another 24 hours, embryos were fixed with 4% PFA, and in situ hybridization analysis was performed.

In-ovo dye injection
Fate-mapping experiments were performed on stage 8-10 embryos. Micropipettes attached to a micromanipulator were filled with Dil or CM-Dil (D282, C7001, Molecular Probes) at 5 or 2.5 mg/ml, respectively, in ethanol, followed by dilution in tetracyclol (1:2). The dye was pressure-injected into the right or left CPM. Following fixation, bright field and fluorescent images were taken.

Quail-chick chimeras
Quail grafts of the CPM, at the level of rhombomere 1-3 (some of which were labeled with Dil for visualization), were dissected out. A corresponding piece of chick CPM was removed, and the quail graft was implanted at this site. The eggs were incubated for 24 hours. Operated embryos were selected for sectioning and QCPN detection.

Immunohistochemistry
Fixed embryos were embedded in paraffin and sectioned as described above. Sections were blocked with 5% goat serum, 1% BSA in PBS, prior to incubation with the primary antibody [MF20 (1:10), QCPN (DSHB)], followed by Cy3-conjugated anti-mouse IgG (1:100).

RESULTS

Characterizing the nature of cardiogenesis and myogenesis in the head mesoderm, in vivo
We previously observed using explant culture assays that head mesoderm cells undergo cardiogenesis (Tzahor and Lassar, 2001), as well as skeletal muscle, differentiation (Tzahor et al., 2003) in vitro. We now applied a more defined explant culture system to study the differentiation potentials of the cranial paraxial mesoderm (CPM) and splanchic mesoderm (SpM) populations (Fig. 1, see also Materials and methods). At the time of dissection, stage 10 SpM explants expressed the cardiac differentiation marker Nkx2.5, as well as members of the Gata family of zinc finger transcription factors. The expression of *Gata4* and the structural myocardial marker *ventricular myosin heavy chain* (vMHC) was augmented after three days in culture (Fig. 1B). The SpM explants also expressed the recently identified SHF marker *Islet1* [*Isl1* (Cai et al., 2003)], although its levels gradually decreased in culture. Interestingly, *Capsulin*, a bHLH transcription factor involved in head muscle formation in the mouse (Lu et al., 2002), was expressed in SpM explants that were cultured for three days (Fig. 1B). Thus, SpM explants are committed to the cardiac lineage, and can undergo further cardiac differentiation in vitro, a finding consistent with SHF cells.

In contrast to the SpM, CPM explants express the skeletal muscle markers *MyoD* and *Myogenin* after three days in culture (Fig. 1B). Notably, *Myf5* expression was consistently detected in both CPM and SpM explants. In addition, the expression of *Gata5*, *Gata6* and *Isl1* was detected in both CPM and SpM explants, suggesting a degree of plasticity in these mesodermal cells in vitro (Fig. 1B).

In order to identify candidate signaling molecules that regulate skeletal muscle and cardiac differentiation programs, we examined the expression of members of the Bmp, Fgf (Fig. 1C) and Wnt (data not shown) signaling pathways, which are known to be major players during early cardiac and skeletal muscle development. Both Fgfs and Fgf10, as well as the receptors for Fgf and Bmp ligands, were expressed in both CPM and SpM explants. Notably, both Bmp2 and Bmp4 were expressed at higher levels within cultured SpM explants than within cultured CPM explants, a finding that strongly correlates with cardiogenesis (Fig. 1C). Taken together, these explant culture results demonstrate that myogenesis could be observed in CPM explants, whereas cardiogenesis could be observed in SpM explants. Moreover, Bmp signaling pathways may play a crucial role in the specification of these lineages.

Candidate regulatory molecules for head mesoderm specification in vivo
To gain in vivo information on the signaling molecules and tissue-specific transcription factors regulating the differentiation of mesoderm precursors into cardiac and skeletal muscle lineages, we initiated candidate gene expression analyses by in situ hybridization in whole-mount and sectioned chick embryos (Fig. 2). Embryos were sectioned at four different levels, the first branchial arch (BA), the OFT/second BA, the heart/third BA and the inflow tract, to demonstrate the expression of both myogenic and cardiogenic markers (Fig. 2A). Because cells from the SHF migrate to the OFT between embryonic stages 10-22 (Waldo et al., 2001), and because myogenesis in the BAs can be observed at stages 14-15 (Hacker and Guthrie, 1998; Noden et al., 1999), we focused our analyses on stage 15-17 embryos (Fig. 2).

*Nkx2.5*, an early cardiogenic marker, was broadly expressed in the developing heart, as well as in surrounding tissues corresponding to the SHF (Fig. 2A), whereas the *cardiac myosin heavy chain* (cMHC)
was expressed only in myocardial cells within the embryonic heart (Fig. 2B). Nkx2.5 was expressed in the mesodermal core at the distal region of the first BA, the ventral mesenchyme of the second BA (which is directly connected to the OFT at stage 16), the ventral pharyngeal endoderm (the floor of the pharynx), and in the OFT (Fig. 2A1,A2). At the level of the third BA, the splanchnic mesoderm was clearly seen as an epithelial layer underneath the endoderm, both of which express Nkx2.5 but not cMHC (Fig. 2A3). In the most posterior area of the embryonic heart, Nkx2.5, but not cMHC, was expressed in the dorsal mesocardium, whereas both genes were observed in the inflow tract (Fig. 2, compare A4 with B4).

Nkx2.5 is known to associate with members of the Gata family. Although we found that Gata4 was expressed throughout the embryonic heart at stage 16 (Fig. 2C,C3) and at lower levels in the OFT (Fig. 2C2), Gata5 and Gata6 were restricted more to the OFT (Fig. 2D2,E2) and, to a lesser extent, to the tubular heart (Fig. 2D,E,D3,E3). Unlike Gata4, the expression of Gata5 and Gata6 (as well as Nkx2.5) at the level of the second and third BAs was detected in the splanchnic mesoderm beneath the pharynx (Fig. 2C3,D3,E3). All three genes could be detected at the inflow pole of the heart (Fig. 2C4,D4,E4). Thus, our gene expression analyses suggest that in chick embryos, Gata5 and Gata6, but not Gata4, may play important roles in the specification of the SHF.

The LIM homeodomain transcription factor Isl1 is a key molecule in the specification of the SHF lineage during mouse embryogenesis (Cai et al., 2003). It was recently shown that this gene might confer ‘stem cell characteristics’ on cardiac myocytes after birth (Laugwitz et al., 2005). The expression of Isl1 at stage 16 was not restricted to the cardiogenic lineage; yet, similar to Nkx2.5, this gene was expressed in the mesenchyme of the second BA, and in the pharyngeal endoderm (Fig. 2F2), in the splanchnic mesoderm at the level of the third BA (Fig. 2F3), and in the dorsal mesocardium (Fig. 2F4). Unlike Gata4, the expression of Gata5 and Gata6, Isl1 expression was not seen in the heart myocardium (Fig. 2F2,F4) in a manner similar to the expression of Isl1 in the mouse (Cai et al., 2003). Thus, Isl1 expression in stage 16 chick embryos represents a pool of undifferentiated cardiogenic cells within the head mesoderm, consistent with previous reports (Cai et al., 2003; Yuan and Schoenwolf, 2000).

The skeletal muscle marker Capsulin was expressed in the mesodermal core of the first and second BAs (Fig. 2G1,G2), and in the splanchnic mesoderm surrounding the gut (Fig. 2G4). In addition, we observed the expression of Capsulin at the distal end of the OFT (see Fig. S1 in the supplementary material). These findings, together with our data from explant cultures demonstrating the expression of Capsulin in differentiating SpM cells (Fig. 1), lead us to propose that Capsulin, like Isl1, is specifically expressed in paraxial and splanchnic mesoderm cells that contribute to both myogenic and cardiogenic lineages.
Fig. 2. Gene expression analysis of candidate regulatory molecules that specify the head mesoderm. (A-J) Whole-mount in situ hybridization for the indicated genes in stage 16 chick embryos. (A1-J4) Transverse sections at four levels (indicated by the dotted lines in A). (A1-J1) First branchial arch. (A2-J2) Outflow tract/second branchial arch. (A3-J3) Epithelial splanchnic mesoderm/third branchial arch. (A4-J4) Inflow tract. ba1, branchial arch 1; ba2, branchial arch 2; da, dorsal aorta; dm, dorsal mesocardium; end, endoderm; V, trigeminal ganglion; ift, inflow tract; mn, motoneuron; nt, neural tube; oft, outflow tract; ov, otic vesicle; ph, pharynx; spm, splanchnic mesoderm.
Members of the T-box family of proteins were previously shown to play a fundamental role in patterning the developing vertebrate heart (Plageman, Jr and Yutzey, 2005). Tbx1, a T-box transcription factor known to influence SHF formation (Xu et al., 2004) and head muscle formation (Kelly et al., 2004), was strongly expressed in the mesodermal core of the first and second BAs, as well as in the pharyngeal endoderm and the otic vesicle (Fig. 2H1,H2). Tbx1 expression at these axial levels resembled the expression patterns of Isl1, Capsulin and Nkx2.5, although it was not detected in the OFT. Tbx5, in contrast, was not expressed in either the BA or OFT regions (see Fig. S2A in the supplementary material).

Taken together, our gene expression analyses revealed a group of transcription factors (i.e. Gata5, Gata6, Isl1, Tbx1 and Capsulin) that, based on their spatiotemporal expression, are likely to play a role in the development of the SHF lineage in the chick embryo. Although both Tbx1 (Xu et al., 2004) and Isl1 (Cai et al., 2003) were recently shown to be involved in this new myocardial lineage in mice, Gata5, Gata6 and Capsulin represent new candidates for this lineage in chick embryos.

Fgf10 and Fgf8 were also shown to play a crucial role in the SHF lineage in mice (Brown et al., 2004; Kelly et al., 2001). Fgf8 expression was detected in both the endoderm and ectoderm of stage 16 chick embryos, at the boundary between the splanchnic and paraxial mesoderm (Fig. 2J2,J3; see also Fig. S2C in the supplementary material). Furthermore, Mkp3, a MAP kinase regulator that was shown to negatively affect the Fgf8 signaling pathway in the chick limb (Kawakami et al., 2003), is expressed in a similar manner (see Fig. S2B in the supplementary material).

We next looked at the expression of Bmp4 (as well as Bmp2 and Bmp7; see Fig. S1D,E in the supplementary material) in the heart and BA region (Fig. 2I). Bmp4 was expressed in the ectoderm of the BAs, the pharyngeal endoderm, the splanchnic mesoderm, the aortic sac and the dorsal mesocardium (Fig. 2I1-I4, Fig. 6). Notably, the expression of Bmp4 overlapped with that of Isl1 in the splanchnic mesoderm and in the dorsal mesocardium (Fig. 2, compare F3 and F4 with I3 and I4), suggesting that Bmp4 may be involved in the regulation of Isl1 expression.

**Bmp4 induces cardiac gene expression, and blocks skeletal muscle differentiation in the head, both in vitro and in vivo**

The expression of Bmp family members both in SpM explants and in vivo (Figs 1, 2) suggests that this signaling pathway may play a major role in the determination of cardiac and skeletal muscle cell fates. To test this possibility, BMP4-conditioned medium was added to both CPM and SpM explants that had been cultured for two or three days (Fig. 3). BMP4 induced the expression of the cardiac genes Nkx2.5, Gata4, Gata5, Gata6, vMHC and Capsulin in the CPM explants. In parallel, Bmp4 blocked expression of the skeletal muscle markers MyoD, Myf5 and Myogenin that appeared in these same explants after three days in culture. Myf5 expression was also abolished in SpM explants following BMP4 administration. Time-
course RT-PCR and real-time PCR analyses of CPM cells in culture (data not shown) revealed the expression of cardiogenic markers during the first day, followed by a downregulation of these markers and an upregulation of myogenic differentiation at day three. Although SpM explants expressed cardiac differentiation markers in the absence of ectopic BMP4 (Fig. 1; these explants also express endogenous Bmp2 and Bmp4), ectopic application of BMP4 increased the expression of Gata4 and Gata5 after two days in culture (Fig. 3A). Importantly, in all cases we noted that administration of BMP4 (or Bmp2, data not shown) induced beating of the SpM-derived cardiomyocytes, suggesting that in vitro, BMP levels control the differentiation and function of myocytes derived from the splanchnic mesoderm.

In order to block the endogenous BMP signals, we applied the Bmp antagonist Noggin to our explant culture system (Fig. 3B). In SpM explants treated with Noggin, the expression level of vMHC (but not other cardiac markers) was reduced, whereas the levels of the skeletal muscle markers MyoD and Myogenin were slightly elevated (Fig. 3B). Similar results were obtained using virally expressed Noggin (data not shown). CPM explants treated with Noggin were not significantly affected, in line with the significant level of expression of endogenous Noggin in these cells (Fig. 1C). These results, along with the expression of Myf5 and Capsulin in SpM explants, suggest that SpM cells may contain a few myogenic progenitors that can undergo myogenesis if BMP levels are sufficiently low. Alternatively, SpM cells that are committed to the cardiogenic lineage, but not fully differentiated, could transdifferentiate into myogenic progenitors if BMP levels remain low. Together, our explant culture data demonstrate that, in vitro, BMP4 promotes a cardiac cell fate in both CPM and SpM explants, and blocks skeletal muscle formation in CPM explants.

Because data from our in vitro assays demonstrated that application of BMP4 to CPM cells promotes cardiogenesis, we next tested whether ectopic Bmp4 could induce cardiogenesis in vivo (Fig. 4). Pellets of cells overexpressing BMP4 were implanted into the right side of the CPM of stage 9-10 chick embryos (Fig. 4A,B). BMP4 strongly induced Nkx2.5 gene expression on the right side of the head mesoderm and ectoderm, as evidenced in whole-mount (Fig. 4C1) and sectioned embryos (Fig. 4C2,C3). We further tested the impact of BMP4 on Gata4 gene expression (Fig. 4D). Although we did not detect significant induction of Gata4 or Gata6 expression (data not shown), Gata5 expression was induced by BMP4 in the BA region (Fig. 4D1,D2,D3).

The expression patterns of Bmp4 and Isl1 genes (Fig. 2) indicated that BMP4 might regulate Isl1 expression. BMP4 cells that were implanted in the right side of the CPM (Fig. 4E) blocked the neuronal expression of Isl1 in the cranial nerve ganglia V (Fig. 4E1,E2). By contrast, BMP4 induced the expression of this gene in the BA mesenchyme, as well as in the ectoderm and endoderm (Fig. 4E3). We therefore suggest that BMP4 acts upstream of Isl1 in vivo.

Capsulin is expressed in both paraxial and splanchnic mesoderm, suggesting its possible role in both cardiac and skeletal muscle lineage specification in the head (Figs 1, 2). BMP4 induced Capsulin expression in the mesenchyme of the BA (Fig. 4F1,F2,F3), similar to its effect on Isl1 and consistent with the effects of BMP4 in vitro (Fig. 3A). Considering that BMP signals are known to play an inhibitory role in skeletal muscle differentiation, these results suggest that Capsulin may act as a repressor of head muscle differentiation. Furthermore, BMP4 efficiently blocked Myf5 expression (Fig. 4G1,G2,G3), and reduced Tbx1 expression (Fig. 4H1,H2,H3) in the BA region. The expression of Myf5 at the site of lateral rectus progenitors (Mootooosamy and Dietrich, 2002) was also inhibited in response to BMP4 application (Fig. 4G1,G2,G3). Thus, both in vitro and in vivo experimental systems demonstrate that BMP4 plays a crucial role in determining the fate of the head mesoderm: ectopic application of BMP4 protein induced the expression of cardiac marker genes, and blocked myogenic differentiation.

Cranial paraxial mesoderm cells contribute to both the myocardial and endocardial layers of the cardiac outflow tract

The fact that Bmp signaling induces cardiogenesis in cranial paraxial mesoderm in vitro and in vivo, prompted us to investigate the contribution of these cells to the developing heart at the looping stages. To test, in vivo, our hypothesis that CPM cells are recruited to the developing heart, we employed fate-mapping analyses in the chick embryo (Figs 5, 6). DiI was injected into the left or right sides of the CPM of stage 9-11 embryos in ovo at the axial level of rhombomers 1-4 (Fig. 5A,E). The location of the incorporated DiI was subsequently followed at stages 17-22. Most of the CPM cells migrated into the first BA (Fig. 5B,F) in accordance with previous studies (Hacker and Guthrie, 1998). Strikingly, in most embryos, DiI-labeled cells from the CPM were detected in both the OFT and the aortic sac (Fig. 5C,D,G,H). Although DiI-labeled CPM cells from the left side of the embryos could be detected in the inner curvature of the OFT (Fig. 5D), DiI injected into the right side was detected in the outer curvature of the OFT (Fig. 5H; summarized in Table S1 in the supplementary material). This new finding shows that cells from the cranial paraxial mesoderm migrate to the OFT in vivo.

Previous fate-mapping studies in the chick by Kirby and colleagues demonstrated that neural crest cells originating from the hindbrain corresponding to rhombomers 6-8 are required for normal heart development [thus termed cardiac neural crest cells (Kirby et al., 1983)]. In order to confirm that the DiI-labeled cells in the OFT were derived from the CPM and not from cranial neural crest cells, we injected the DiI at an earlier stage of development (stage 8), prior to the onset of cranial neural crest migration (Fig. 5I-L). In these embryos, DiI-labeled cells were detected in the OFT (Fig. 5K,L), indicating that at the level of the first BA, and, to a lesser extent, the second BA (see Fig. S3 in the supplementary material), CPM cells migrate to the OFT. Furthermore, we observed DiI-labeled cells from the CPM in the OFT in cranial neural crest-ablated embryos (data not shown).

In order to confirm that DiI was injected into the CPM rather than into the splanchnic mesoderm, some of the injected embryos were immediately sectioned transversely to visualize the location of the fluorescence signal. As expected, the injected DiI was located in the CPM (Fig. 5N). Notably, DiI-labeled CPM cells were found in both the myocardial and endocardial layers of the OFT (Fig. 5O,P). Collectively, these DiI labeling experiments demonstrate that cells from the CPM that migrate to the first and second BAs contribute to both the endocardial and myocardial lineages of the cardiac OFT.

To gain insights into the molecular make-up of the CPM cells en route to the OFT, we compared our results from the fate mapping and molecular analyses of sectioned chick embryos (Fig. 6). CM-DiI was injected into the CPM of stage 10 embryos. Stage 16 embryos were fixed and sectioned transversely (Fig. 6A) at the level of the first BA (Fig. 6C), the aortic sac/second BA (Fig. 6C1) and the OFT (Fig. 6C2). The signal resulting from the labeled CPM cells was compared with identical sections of embryos that were stained for Nkx2.5, Gata5, Myf5, Bmp4 and the myocyte marker MF20. As expected, labeled CPM cells were localized in the myogenic core of
Fig. 4. BMP4 induces cardiac gene expression and blocks skeletal muscle differentiation in vivo. (A) Dorsal view of a stage 9 embryo, implanted with cells expressing HA-BMP4 (dashed circle). (B) Western blot analysis of HA-tagged BMP4 in the conditioned medium of the transfected cells. (C-H) In situ hybridization analyses for the indicated genes. Whole-mount and transverse sections of the embryos are shown (left panel, control; right panel, Bmp4 treated). Induction (white arrowhead) or reduction (open arrowhead) of the expression of cardiac and skeletal muscle markers following BMP4 application is indicated. Ectopically-applied Bmp4 induced expression of the cardiac markers Nkx2.5 (n=7/7), Gata5 (n=7/14) and Capsulin (n=5/6). By contrast, BMP4 cells blocked the expression of the skeletal markers Myf5 (n=4/4) and Tbx1 (n=3/4). A dual effect was observed for Isl1: although Bmp4 abolished the expression of Isl1 in the trigeminal ganglion (V), an elevated expression of this gene was found in the mesenchyme and ectoderm of the first BA (n=7/7). As, aortic sac; ba1, ba2, first, second branchial arches; Ir, lateral rectus; nt, neural tube.
Fig. 5. Cranial paraxial mesoderm cells contribute to the myocardium and endocardium of the cardiac outflow tract in vivo. (A-P) Tracking of CPM cells by Dil labelling. Dil injections into the left (A) or right (E,M) CPM of stage 10 (or stage 8, I) chick embryos. Lateral (B,F,J) or transverse (C,G,K) views of embryos at stage 17-18 are shown as an overlay of bright field and fluorescence images. (D,H,L) Higher magnification of dissected hearts. Cells labeled on the left side of the CPM contribute to the inner curvature of the cardiac OFT (D), whereas cells from the right side contribute to the outer curvature (H,L). (O,P) The CPM cells contribute to both the myocardium and endocardium: cross section (O) and transverse section (P) of the OFT. (M,N) Embryo dissected shortly after the Dil injection (M) to verify the location of the dye in the paraxial mesoderm (N). ba1, first branchial arch; ba2, second branchial arch; cc, conus cordis; da, dorsal aorta; end, endocardium; ic, inner curvature; myo, myocardium; nc, notochord; nt, neural tube; oc, outer curvature; oft, outflow tract; ov, otic vesicle; ph, pharynx; rv, right ventricle; ta, truncus arteriosus.

Fig. 6. Cellular and molecular analyses of cranial paraxial mesoderm en route to the heart. (A) Diagram of a stage 16 embryo (ventral view), following removal of the heart. Dotted lines represent the three section levels displayed in the lower panels. (B) Diagram of quail-chick chimera assay. (C1-I2) Transverse sections through the quail-chick chimera, followed by immunostaining with the quail-specific antibody (QCPN, X10 bright field and fluorescence images) (D-D2) Immunostaining for Myosin heavy chain using MF20 antibody. (F-I2) Sections through embryos subjected to whole-mount in situ hybridization with the indicated markers. aaa1, aortic arch artery 1; aaa2, aortic arch artery 2; as, aortic sac; ba1, ba2, first, second branchial arches; end, endocardium; myo, myocardium; nt, neural tube; ov, otic vesicle; ph, pharynx.
the first BA, as indicated by Myf5 in situ hybridization (Fig. 6H). Of the cardiogenic genes mentioned above, only Nkx2.5 could be detected at the level of the first BA (Fig. 6F,G, Fig. 2).

During heart looping stages in chick embryos, the OFT shifts caudally (Waldo et al., 2001), such that at stage 16-18 the aortic sac, which connects the aortic arch arteries to the OFT, is connected caudally (Waldo et al., 2001), such that at stage 16-18 the aortic sac, detected at the level of the first BA (Fig. 6F,G, Fig. 2).

The lack of expression of Bmp4 at the first BA level (Fig. 6F) and its expression in the aortic sac/second BA (Fig. 6F1,F1) is correlated with the change in cell fate from myogenic to cardiogenic progenitors. At the level of the OFT, CM-DiI-labeled cells could be detected in the endocardium and myocardium (Fig. 6C2,E2; myocardial cells are MF20 positive), which also express Nkx2.5 and Gata5 (Fig. 6F2,G2).

We next used the quail-chick grafting technique to verify our dye labeling results (Fig. 6B; see also Table S2 in the supplementary material). CPM grafts (with the surface ectoderm) of stage 8 quail embryos were transplanted into the CPM of stage-matched chick embryos (Fig. 6B). Sections of the operated embryos revealed the existence of quail-derived CPM cells (recognized by a QCPN-specific antibody) within the mesenchyme of the first BA (Fig. 6D,D’), the aortic sac/second BA (Fig. 6D1,D1’) and within the endocardium and myocardium of the cardiac OFT (Fig. 6D2,D2’). This experiment further indicates that CPM cells rather than cranial neural crest contribute to the OFT during looping stages. Taken together, our dye marking technique, quail chick grafting procedure and retroviral infection (see Fig. S4 in the supplementary material) confirm the observation that cranial paraxial mesoderm cells contribute to the myocardial and endocardial components of the cardiac OFT.

DISCUSSION

In the present study, we employed both in vitro and in vivo experimental systems in the avian embryo to explore how mesoderm progenitors in the head, differentiate into both heart and craniofacial muscles. Our detailed gene expression analyses of candidate tissue-specific transcription factors and signaling molecules both in vitro and in vivo (Figs 1, 2) revealed a group of transcription factors (Gata5, Gata6, Isl1 and Capsulin) that, based on their spatiotemporal expression, are likely to play a role in the development of the secondary/anterior heart field (SHF) in the chick. These analyses further revealed a considerable degree of plasticity between cardiac and skeletal muscle precursors in the head mesoderm. We also provide evidence that Bmp4 plays a key role in specifying the cell fates of both myogenic and cardiogenic progenitors in the cranial paraxial mesoderm (Figs 3, 4). Data from both fate-mapping experiments and molecular analyses demonstrate that cranial paraxial mesoderm cells represent a source of cardiogenic progenitors that populate the cardiac outflow tract in vivo (Figs 5, 6).

**Bmp signaling induces cardiogenesis and represses head myogenesis during heart looping stages**

Bmp signaling molecules are required for early heart formation in vertebrate embryos (Jiao et al., 2003; Liu et al., 2004; Schlange et al., 2000; Schultheiss et al., 1997; Shi et al., 2000). Similarly, Dpp, the Drosophila ortholog of Bmp, is required for the formation of the dorsal vessel, the equivalent of the heart in flies (Frash, 1995). It was further suggested that Bmp2, which is expressed in the SHF/OFT myocardium, affects the proliferation of these cells in vitro (Waldo et al., 2001).

Here, we demonstrate that the expression of Bmp family members during heart looping and the early stages of myogenesis is correlated with cardiac gene expression (Figs 1, 2). Ectopic application of BMP4 during these stages induced robust expression of Nkx2.5, Gata5, Isl1 and Capsulin in the CPM in vitro (Fig. 3) and in vivo (Fig. 4), whereas inhibition of the BMP signals by Noggin partially blocked cardiogenesis, and induced myogenesis in SpM explants (Fig. 3). Concomitant with the induction of cardiogenesis in the CPM, BMP4 blocked skeletal myogenesis in this tissue, both in vitro and in vivo (Figs 3, 4), consistent with previous studies (Pourquie et al., 1996; Reshef et al., 1998; Tzahor et al., 2003). Ectopic application of BMP2 and BMP4 to SpM cells induced a rhythmic beating of cardiomyocytes in vitro, suggesting a possible role for Bmp signaling in the terminal differentiation of cardiac progenitors.

Loss-of-function studies of Isl1 in mice had previously shown that Bmp4 (as well as other Bmp and Fgf family members) is a target of Isl1 in the SHF (Cai et al., 2003). We now demonstrate that Bmp4 induces Isl1 expression in the SHF, while blocking its expression in neuronal tissue. Thus, there appears to be a positive feedback loop between Isl1 and Bmp4.

**The involvement of cranial paraxial mesoderm in cardiogenesis**

Our fate-mapping experiments using vital dyes and our quail chick transplantation experiments, demonstrate that paraxial mesoderm cells that migrate primarily to the first BA contribute to both myocardial and endocardial layers of the OFT (Figs 5, 6; see also Figs S3, S4 in the supplementary material). DiI-labeled cells from the left side of the CPM were found in the inner curvature of the OFT, whereas those from the right side were detected at the outer curvature of the OFT (Fig. 5). These findings suggest that the differences in origin and in the migratory paths taken by CPM cells as they move toward the OFT could affect the process of rightward heart looping, a critical step during heart morphogenesis in vertebrate embryos. Taken together, our data identify a novel mechanism by which cranial paraxial mesoderm cells adopt a myocardial cell fate. Although we confirmed that DiI-labeled cells within the OFT were derived from the mesoderm and not from cranial neural crest cells (Figs 5, 6), the influence of the cranial neural crest on the migration of CPM cells into the OFT was not addressed in this study.

It was previously shown that SHF cells in the chick embryo contribute to the myocardium of the OFT (Waldo et al., 2001); later, these mesodermal cells provide smooth muscle cells that form the base of the great arteries (Waldo et al., 2005). Cardiac neural crest cells provide yet another source for smooth muscle cells of the blood vasculature (Hutson and Kirby, 2003). Studies in zebrafish embryos have shown that cardiac neural crest cells contribute to the developing myocardium (Li et al., 2003; Sato and Yost, 2003). Results from quail-chick transplantation assays further suggest that progenitor cells originating in the cephalic mesoderm can give rise to angioblasts that populate the endocardium of the OFT (Noden, 1991). Our data demonstrate that CPM cells can be found in both myocardial and endocardial cell populations. Collectively, these studies suggest that endocardial and myocardial precursors within the OFT derive from multiple origins within the embryo, rather than a common progenitor population.
What, then, is the relationship between the cardiac progenitor populations that constitute the SHF (Buckingham et al., 2005) and those originating in the cranial paraxial mesoderm? In the chick, the SHF includes the epithelial splanchnic mesoderm, beneath the floor of the pharynx, that lies caudal to the OFT (Waldo et al., 2001), whereas the anterior heart field is restricted to the cephalic mesodermal cells surrounding the aortic sac (Mjaatvedt et al., 2001). In mice, the anterior heart field includes the right ventricle and the OFT, as well as the mesodermal core of the pharyngeal arches (Kelly et al., 2001). Our results, which demonstrate that CPM cells at the levels of the first and second BAs (which eventually form the mesodermal core of the pharyngeal arches) contribute to the OFT, are consistent with these descriptions of the anterior heart field. Our results do not rule out a contribution of cardiac progenitors from the splanchnic mesoderm into the OFT, as has been previously shown (Waldo et al., 2005; Waldo et al., 2001). In view of the dynamic nature of heart morphogenesis, our data is consistent with the hypothesis that cardiac progenitor populations from multiple sources (or heart fields) are recruited into the heart at different stages of development, and from various locations (Abu-Issa et al., 2004).

A model for the specification of the cranial paraxial mesoderm in response to Bmp signals

Our results are summarized in a schematic model (Fig. 7), which demonstrates that in chick embryos, Dil-labeled cells from the CPM can migrate along the myogenic core of the first BA, through the aortic sac, and into the cardiac OFT. Initially, these cells represent myoblast precursors, as evidenced by the expression of various skeletal muscle markers (e.g. Myf5, Capsulin and Tbx1; highlighted in the upper box). CPM cells that surround the aortic sac presumably adopt myocardial characteristics (Nkx2.5, Gata5, Isl1 and Capsulin; middle box). CPM-derived cells within the OFT are co-localized with cardiogenic cells expressing Nkx2.5, Gata4 and cMHC (lower box). Our cellular and molecular analyses of the cranial paraxial mesoderm suggest that these cells are gradually specified along the dorsoventral axis, in correlation with Bmp signaling levels.

The expression of Bmp family members – in particular, Bmp4 – in the head region is consistent with its positive role in cardiogenesis: ectopic application of Bmp4 induces cardiac differentiation in CPM cells, and reduces the differentiation of skeletal muscle precursors. Thus, we propose that Bmp4 stands at the apex of a consortium of signaling pathways that promote cardiogenesis and suppress myogenesis in progenitor cell populations originating in the head mesoderm (Fig. 7).

Although CPM cells can undergo myogenic, skeletogenic or angiogenic differentiation (Coulby et al., 1993; Noden, 1983; Noden, 1991), the present study demonstrates that these cells further adopt unique cardiogenic properties. Based on our current results, as well as on those of previous studies, we propose that paraxial mesoderm cells in the head possess the ability to differentiate into a broader range of lineages than does the trunk paraxial mesoderm (Tzahor et al., 2003; Tzahor and Lassar, 2001). Although the initial differentiation and patterning of the somites into both myogenic and skeletogenic lineages take place at sites adjacent to the axial tissues, the differentiation of the CPM cells is repressed by signals (e.g. Wnt1 and Wnt3a) from the neural tube (Tzahor et al., 2003; Tzahor and Lassar, 2001).

During early embryogenesis, heart and skeletal muscle progenitor cells were originally thought to derive from distinct regions of the mesoderm (i.e. the lateral plate mesoderm and paraxial mesoderm, respectively). We propose that the developmental programs of progenitor populations that contribute to both the head musculature and the anterior pole of the heart are tightly linked, consistent with the notion of a single cardiocraniofacial morphogenetic field (Hutson and Kirby, 2003). Accordingly, insults to any component of this field may lead to both cardiac and craniofacial abnormalities. Whether there is a common progenitor cell in the cranial paraxial mesoderm that differentiates into both cardiac and skeletal muscle lineages, or whether this cell population is heterogeneous in nature awaits detailed molecular and cellular analyses. In addition, the impact and function of these cells within the OFT will constitute a major focus of our future research efforts.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/10/1943/DC1

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