Development 135, 647-657 (2008) doi:10.1242/dev.007989

The contribution of Islet1-expressing splanchnic mesoderm cells to distinct branchiomeric muscles reveals significant heterogeneity in head muscle development

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During embryogenesis, paraxial mesoderm cells contribute skeletal muscle progenitors, whereas cardiac progenitors originate in the lateral splanchnic mesoderm (SpM). Here we focus on a subset of the SpM that contributes to the anterior or secondary heart field (AHF/SHF), and lies adjacent to the cranial paraxial mesoderm (CPM), the precursors for the head musculature. Molecular analyses in chick embryos delineated the boundaries between the CPM, undifferentiated SpM progenitors of the AHF/SHF, and differentiating cardiac cells. We then revealed the regionalization of branchial arch mesoderm: CPM cells contribute to the proximal region of the myogenic core, which gives rise to the mandibular adductor muscle. SpM cells contribute to the myogenic cells in the distal region of the branchial arch that later form the intermandibular muscle. Gene expression analyses of these branchiomeric muscles in chick uncovered a distinct molecular signature for both CPM- and SpM-derived muscles. Islet1 (Isl1) is expressed in the SpM/AHF and branchial arch in both chick and mouse embryos. Lineage studies using *Isl1-Cre* mice revealed the significant contribution of Isl1$^+$ cells to ventral/distal branchiomeric (stylohyoid, mylohyoid and digastric) and laryngeal muscles. By contrast, the Isl1 lineage contributes to mastication muscles (masseter, pterygoid and temporalis) to a lesser extent, with virtually no contribution to intrinsic and extrinsic tongue muscles or extraocular muscles. In addition, in vivo activation of the Wnt/β-catenin pathway in chick embryos resulted in marked inhibition of Isl1, whereas inhibition of this pathway increased Isl1 expression. Our findings demonstrate, for the first time, the contribution of Isl1$^+$ SpM cells to a subset of branchiomeric skeletal muscles.

KEY WORDS: Anterior heart field, Splanchnic mesoderm, Myogenesis, Wnt/β-catenin

INTRODUCTION

In the vertebrate embryo, the segregation of cells into paraxial mesoderm, which forms the basis of skeletal muscle, and lateral mesoderm, which contributes cardiac myocytes, is thought to occur during gastrulation. In vertebrates, myocardial progenitors migrate from the primitive streak anteriolaterally to form bilateral heart fields. During head-fold stages, these progenitors form the cardiac crescent, prior to the formation of the linear heart tube. Studies in chick embryos have demonstrated that subsequently, most of the outflow tract (OFT) is populated by myocardial progenitors from the anterior or secondary heart field (AHF/SHF), which resides in pharyngeal mesoderm dorsal to the heart (Mjaatvedt et al., 2001; Waldo et al., 2001). In mouse embryos, retrospective lineage analysis has demonstrated the presence of two heart fields – the first and the second heart field – based on their timing of entry into the heart and their timing of differentiation (Buckingham et al., 2005). The first heart field primarily contributes to the left ventricle, whereas the second heart field contributes most of the cells of the cardiac OFT and right ventricle (RV), a majority of cells in the atria, and some cells within the left ventricle (Black, 2007; Buckingham et al., 2005; Garry and Olson, 2006; Srivastava, 2006). In chick, the precise boundaries and molecular identities of mesodermal 'fields' are less clear, owing to a lack of genetic and lineage-specific markers for these early progenitors, as well as differences between chick and mouse models (Abu-Issa and Kirby, 2007).

Heart development takes place in close apposition to the developing head. The separation between the heart and the head commences gradually, following heart-looping stages as the heart shifts caudally. The term 'cardio-craniofacial morphogenetic field' reflects the intimate developmental relationship between the head, face and heart, which is also reflected in numerous cardiac and craniofacial birth defects (Hutson and Kirby, 2003).

Cranial paraxial mesoderm (CPM) cells located anterior to the somites, as well as prechordal mesoderm, provide the precursors for approximately 60 distinct skeletal muscles in the head, which are used to facilitate food intake, move the eyeball, provide facial expressions and aid speech in humans (Wachtler and Jacob, 1986). CPM cells stream into the neighboring branchial arches (BAs, also known as pharyngeal arches), the templates of the adult craniofacial structures. Within the BAs, cranial neural crest cells surround the muscle anlagen (Noden, 1983; Trainor et al., 1994); these cells provide multiple signals that regulate cranial muscle patterning and differentiation (Rinon et al., 2007; Tzahor et al., 2003).

It is well accepted that distinct developmental programs control skeletal muscle formation in the trunk and in the head (reviewed by Bothe et al., 2007; Noden and Francis-West, 2006). Moreover, muscle myopathies are known to be differentially linked to a specific trunk or cranial region (Emery, 2002). Similarly, within the head musculature, eye muscles differ from branchiomeric muscles, and there is evidence that branchiomeric muscle development varies among the different BAs (Dong et al., 2006; Kelly et al., 2004).

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Accepted 26 November 2007

A previous study in chick embryos demonstrated that signals from the dorsal neural tube (e.g. Wnt1 and Wnt3a) block cardiogenesis in the adjacent CPM (Tzahor and Lassar, 2001). We further demonstrated in vivo, also in chick embryos, that a subset of CPM cells contributes to both myocardial and endocardial cell populations within the cardiac OFT (Tirosh-Finkel et al., 2006). These two studies revealed that CPM cells contribute to both cardiac and skeletal muscle lineages, and illustrate the plasticity of these cells during embryogenesis. In accordance with these results, recent studies involving various transgenic mouse lines have demonstrated an overlap in the progenitor populations contributing to branchiomeric and cardiac muscle (Dong et al., 2006; Kelly et al., 2001; Verzi et al., 2005) (reviewed by Grifone and Kelly, 2007).

The LIM homeodomain protein Islet1 (Isl1) stands at a nodal point in the self-renewal, differentiation and lineage specification of distinct cardiovascular precursors, and is a major player in the second heart field lineage during embryogenesis (Cai et al., 2003; Laugwitz et al., 2005; Moretti et al., 2006). This transcription factor marks undifferentiated progenitors of the SHF; its expression is downregulated with differentiation (Cai et al., 2003). Genetic removal of *Isl1* in mice showed that *Bmp4* (as well as other Bmp and Fgf family members) is a target of *Isl1* in the AHF (Cai et al., 2003). We demonstrated in chick embryos that Bmp4 induces *Isl1* expression in the CPM, while blocking its expression in neuronal tissues (Tirosh-Finkel et al., 2006). More recently, it has been shown in mice that β-catenin directly targets and activates *Isl1* expression in the AHF (Lin et al., 2007).

In the present study, we characterized the nature of the Isl1$^+$ cardio-craniofacial splanchnic mesoderm, using several lineage-tracing and gene expression techniques in both chick and mouse embryos. At both the cellular and molecular levels, the cardio-craniofacial mesoderm can be divided into two compartments: the CPM and splanchnic mesoderm (SpM), part of which comprises the AHF. Following linear heart tube stages, we found that Isl1$^+$/SpM cells contribute to the distal part of the pharyngeal (branchial) mesoderm, as well as to the cardiac OFT. Molecular and lineage analyses of the head musculature in chick and mouse embryos demonstrated distinct molecular and developmental programs for CPM and Isl1$^+$ SpM-derived branchiomeric muscles. Furthermore, we demonstrate that the Wnt/β-catenin pathway regulates Isl1 (and Nkx2.5) protein expression, presumably by fine-tuning boundary formation within the cardio-craniofacial mesoderm.

MATERIALS AND METHODS

Embryos

Fertilized white eggs from commercial sources were incubated for 1-3 days at 38.5°C in a humidified incubator to HH stage (St.) 3-26 (Hamburger and Hamilton, 1992).

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed using digoxigenin (dig)-labeled antisense riboprobes synthesized from total cDNA. A detailed protocol, as well as specific primers for cDNA probes, are available upon request.

Double-fluorescence in situ hybridization (FISH) on paraffin sections

Paraffin sections were hybridized with two RNA probes, one labeled with dig-UTP and the other with fluorescein-UTP. Post-hybridization, each probe was developed separately using the FITC/Cy3 tyramide amplification system (Perkin Elmer).

Sectioning and histology

For cryosections, embryos were incubated overnight in 20% sucrose in PBS, and then embedded in 7.5% gelatin, 15% sucrose in PBS. Blocks were trimmed and frozen and then sectioned at 20 μm.

Lineage tracing and dye injection

DiI/DiO (D282, C275, Molecular Probes) labeling experiments were performed on St. 8 embryos as described previously (Tirosh-Finkel et al., 2006).

Implantation of Fz8-CRD-IgG beads

Affi-Gel blue gel beads (150-300 μm; Bio-Rad) were soaked in 200 ng/μl Fz8-CRD-IgG or BSA prior to in vivo implantation into the CPM of St. 8-9 embryos.

Mutant mice and *lacZ* staining

Isl1-Cre and *Rosa26R* strains were crossed to generate embryos at E10.5, 12.5 and 16.5, as previously described (Yang et al., 2006). β-gal staining was performed as previously described (Moretti et al., 2006). Embryos were embedded in paraffin and 8 μm sections were counterstained with Nuclear Fast Red.

Immunofluorescence staining

Sections were blocked with 5% goat serum, 1% BSA in PBS prior to incubation with the primary antibody: Nkx2.5 (Santa-Cruz), β-galactosidase (Sigma), chick MyoD (a gift from Prof. Yablonka-Reuveni, University of Washington School of Medicine, Seattle, WA), Myf5 (a gift from Dr Bruce Paterson, NIH, Bethesda, MD), Isl1, Pax7 and MF20 (DSHB). Secondary antibodies used were Cy3 or Cy2-conjugated-anti-mouse or anti-rabbit IgG (Jackson ImmunoResearch).

Electroporation

Detailed protocols are available upon request.

RESULTS

Molecular characterization of the two heart fields in chick embryos

In order to explore molecular and cellular characteristics of CPM and SpM, and their relative contributions to the developing heart and BAs, we performed in situ hybridization for cardiac markers at cardiac crescent stages in Hamburger Hamilton stage (St.) 8+ chick embryos (Fig. 1), as well as fate-mapping analyses (see Fig. S1 in the supplementary material). Transverse sections of whole-mount in situ hybridization revealed distinct subdomains of cardiac gene expression within the cardiac crescent. *C-actin* and *Gata4* marked differentiating myocardial cells within the ventrolateral aspect of the SpM (Fig. 1A′,D′, respectively; see also Fig. S1 in the supplementary material). *Isl1* and *Nkx2.5* were also expressed within this population, but were uniquely expressed dorsomedially, and extended toward the CPM (Fig. 1B′,C′, respectively). These findings suggest that undifferentiated AHF/SHF/SpM cells (dashed circles in Fig. 1B′,C′), expressing *Nkx2.5* and *Isl1* but not *C-actin* and *Gata4*, reside in the dorsomedial region of the SpM.

To further define the boundaries of distinct mesodermal compartments, we used double-fluorescence in situ hybridization (FISH) (Denkers et al., 2004) on sectioned embryos (Fig. 1E-I). Our results confirmed that *Nkx2.5* and *Isl1* (Fig. 1E-E″), as well as *Fgf10* (Fig. 1F-F″), are co-expressed throughout the SpM. The boundaries of the undifferentiated SpM, which demarcate the AHF/SHF, are delineated by the CPM marker *Cyp26c1* (Fig. 1G-G″) (Bothe and Dietrich, 2006) and by the cardiac differentiation marker *Gata4* (Fig. 1H-H″). *Tbx5*, like *Gata4*, was found to be restricted to the differentiating myocardial cells in the lateral SpM, whereas *Tbx20* was also expressed in the AHF/SpM (Fig. 1I-I″). Taken together, our molecular analyses enabled us to delineate the

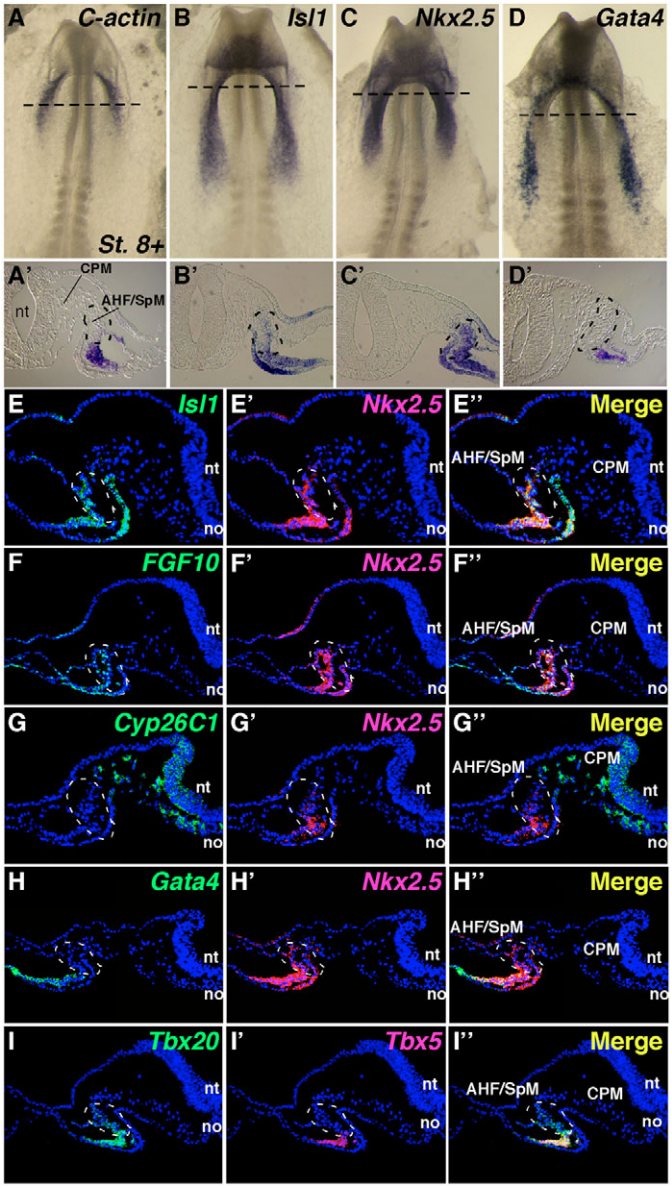

Fig. 1. Histochemical and fluorescence in situ hybridizations identify the molecular boundaries of the AHF/SpM in the craniofacial mesoderm at cardiac crescent stages. The molecular identities of distinct cell populations in the head were determined, using histochemical in situ hybridization (ISH) and a double-fluorescence ISH method in St. 8+ chick embryos. AHF/SpM cells are delineated by dashed ellipses. (**A-D**) Whole-mount ISH for the indicated genes. Dashed lines indicate the plane of the stained transverse sections (**A′-D′**). *Nkx2.5* and *Isl1* are expressed in a broader portion of the SpM (defined as the AHF/SpM), whereas *C-actin* and *Gata4* are expressed in the more-differentiated SpM. (**E-E″**) Fluorescent ISH for *Isl1* (E) and *Nkx2.5* (E′), and their overlay (E″), showing that both genes are expressed in both the differentiated SpM and AHF/SpM. (**F-F″**) *Fgf10* (F), *Nkx2.5* (F′) and overlay (F″). (**G-G″**) The expression of CPM marker *Cyp26c1* (G) and the AHF/SpM marker *Nkx2.5* (G′) defining the boundary between the CPM and the AHF/SpM (G″). (**H-H″**) Expression of the differentiated SpM marker *Gata4* (H) and *Nkx2.5* (H′). The merged image (H″) demonstrates that the AHF can be molecularly identified as comprising *Nkx2.5*$^{+}$ and *Gata4*$^{-}$ cells. (**I-I″**) *Tbx20* expression (I) marking both differentiated SpM and AHF/SpM. *Tbx5* expression (I′) labeled only the differentiated SpM. The merged image (I″) shows that the AHF can be molecularly identified as comprising *Tbx20*$^{+}$/*Tbx5*$^{-}$ cells. nt, neural tube; no, notochord; CPM, cranial paraxial mesoderm; AHF/SpM, anterior heart field/splanchnic mesoderm.

boundaries between the CPM, undifferentiated SpM progenitors of the AHF/SHF, and differentiating cardiac cells in the SpM of St. 8 chick embryos.

Utilizing fluorescent dyes as lineage tracers, we next explored the contribution of differentiated cells within the lateral SpM (DiO, green) and undifferentiated AHF cells within the medial aspect of the SpM (DiI, red) in St. 8 chick embryos (see Fig. S1A in the supplementary material). Control embryos sectioned immediately after labeling were used to validate the accuracy of our method (see Fig. S1A′ in the supplementary material). At St. 10, DiO-labeled cells were found in the looping heart tube (see Fig. S1B″ in the supplementary material), whereas DiI-labeled cells were located within the SpM, underneath the pharynx (see Fig. S1B′ in the supplementary material). At St. 12, both DiO- and DiI-labeled cells were detected within the heart: DiO-labeled cells were detected in the future ventricles, whereas the DiI-labeled AHF/SpM cells were found in the OFT (see Fig. S1C-C″ in the supplementary material).

Because *Isl1* has been shown to be a marker of the AHF/SHF in both mouse (Cai et al., 2003; Moretti et al., 2006) and chick (Tirosh-Finkel et al., 2006) models, we performed immunofluorescence analysis for this protein at relevant stages of cardiac development in chick embryos (Fig. 2). Transverse sections of the head and heart regions were taken at three axial levels and stained with Isl1 antibody. In general, Isl1 expression in all three germ layers matched the in situ hybridization patterns [Fig. 1 and Tirosh-Finkel et al. (Tirosh-Finkel et al., 2006)]. At St. 8, Isl1 was detected throughout the SpM, including the dorsomedial aspect (Fig. 2A-C′), whereas at St. 12 and 18 its mesodermal expression was largely excluded from cardiac myocardium. At St. 12, Isl1 was observed in the SpM underneath the ventral pharynx, as well as in the pharyngeal endoderm (Fig. 2D-F′). Notably, at St. 18, Isl1 expression was detected in the distal part of the myogenic core of the first and second BAs (BA1-2) and surrounding the aortic sac (Fig. 2G-I′). The latter findings point to the possible involvement of Isl1 in skeletal muscle progenitors.

Fate mapping of the Isl1 and Nkx2.5-expressing SpM cells in chick embryos

We previously demonstrated in chick embryos that DiI labeling of the CPM at St. 8 (prior to delamination of cranial neural crest cells) resulted in the presence of labeled cells in both BA1 and in the cardiac OFT [Fig. 3A,A′ and Tirosh-Finkel et al. (Tirosh-Finkel et al., 2006)]. Likewise, both the OFT and BA1 were labeled when DiI was injected into *Isl1*- and *Nkx2.5*-expressing medial SpM (Fig. 3A″,A‴). This experiment indicates that *Nkx2.5*/*Isl1*-expressing undifferentiated SpM cells migrate to both BA1 and the cardiac OFT.

We next labeled both CPM (DiI, red) and *Isl1*- and *Nkx2.5*-expressing undifferentiated SpM (DiO, green) at St. 8 (Fig. 3B-G; the section in B′ indicates that labeling was indeed restricted to the CPM and SpM). At St. 11, DiO-labeled SpM cells were detected in the OFT, whereas DiI-labeled CPM cells remained adjacent to the neural tube (Fig. 3C-C″). Sections of the BA region at St. 12 revealed how CPM and SpM cells populated the myogenic core of the developing BA1: cells from CPM were detected within the

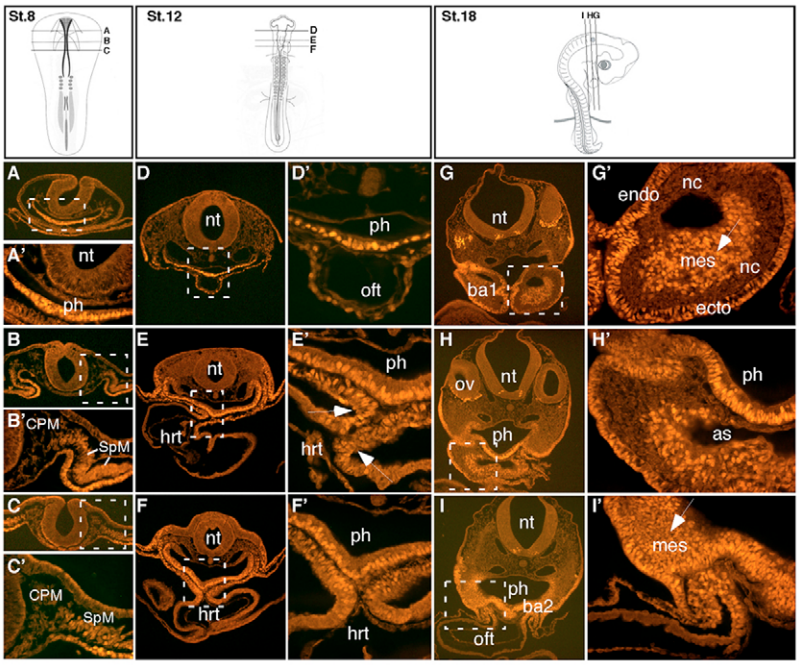

Fig. 2. Expression of Isl1 protein in the AHF and in myogenic progenitors of the branchial arches. Immunofluorescence staining for Isl1 at different stages of chick embryonic development [as illustrated in the top row, modified from The Atlas of Chick Development (Bellairs and Osmond, 1998)]. (**A-C**) Isl1 is expressed in the SpM and the pharyngeal endoderm at St. 8. (**D-F**) At St. 12, Isl1 is restricted to the ventral pharynx and the AHF/SpM at different anterior-posterior levels. Myocardial cells in the linear heart tube show no Isl1 expression. (**G-I**) Isl1 expression (St. 18) is detected in the mesodermal core of BA1-2 but not in the neural crest-derived mesenchyme (nc). Isl1 is also expressed in the aortic sac (as, H). (**A′-I′**) Higher magnifications of the regions indicated by the dashed squares in A-I. Arrows in E′ show Isl1 expression in the border of the SpM. Arrows in G′ and I′ the expression of Isl1 in the mesoderm of BA1 and BA2, respectively. nt, neural tube; ph, pharynx; ecto, ectoderm; endo, endoderm; ba, branchial arch; SpM, splanchnic mesoderm; hrt, heart; oft, outflow tract; ov, otic vesicle; CPM, cranial paraxial mesoderm; mes, mesoderm.

proximal BA, whereas SpM cells filled the distal BA (Fig. 3D′). At this stage, both CPM and SpM cells were observed within the OFT (Fig. 3D″). At St. 16, SpM cells (DiO, green) filled the most-distal region of the myogenic core within BA1, whereas CPM cells (DiI, red) filled the proximal region of the myogenic core (Fig. 3E-G). To investigate the nature of labeled cells from the SpM, DiO was injected into the SpM, and labeled embryos were subsequently stained for Isl1 and Nkx2.5 (Fig. 3F,G, respectively). These markers were co-expressed with the fluorescent dye in distal BA1. Taken together, these results indicate that CPM and undifferentiated Isl1$^+$ and Nkx2.5$^+$ SpM cells contribute to both the cardiac OFT and the mesodermal core of BA1 in a distinct spatial and temporal manner.

To obtain a molecular perspective on the contributions of both CPM and SpM cells to the myogenic core of BA1, we stained transverse sections of BA1 with antibodies against Nkx2.5, Isl1 and Myf5 (see Fig. S2 in the supplementary material). Both Nkx2.5 and Isl1 (see Fig. S2B,C, respectively, in the supplementary material) stained the SpM-derived BA1 (Fig. 3), whereas Myf5 (see Fig. S2E in the supplementary material) was expressed in the CPM-derived proximal region. Collectively, we demonstrate the regionalization of the myogenic core into proximal (CPM-derived, Myf5$^+$) and distal (SpM-derived, Nkx2.5$^+$, Isl1$^+$) subdomains.

We then explored the molecular and anatomical characteristics of CPM- and SpM-derived branchiomeric muscles (Fig. 4). Dye labeling of either proximal or distal BA1 myogenic populations at St. 15, followed by immunostaining for MyoD at St. 26, revealed that the mandibular adductor muscle in birds (equivalent to the masseter in mammals) is derived from the proximal region of the myogenic core, whereas distal BA1 myoblasts form the intermandibular muscle (Fig. 1A-A‴) (see also Marcucio and Noden, 1999; Noden et al., 1999). At St. 20, Myf5 and Isl1 double staining in BA1 matched the proximal/distal regionalization of CPM and SpM cells in the myogenic core (Fig. 4B,B′; compare with Fig. 3E-E″). Notably, at this stage, Pax7 and Myf5 co-expression was detected in the dorsal/proximal region of the myogenic core, but not in the distal, Isl1$^+$ region (Fig. 4B″).

We therefore wanted to check whether these two BA1-derived muscles differ molecularly. Strikingly, we found that at St. 26 [embryonic day 5 (E5)], the mandibular adductor complex expressed Myf5, Pax7, myosin heavy chain (MHC) (Fig. 4D-E′) and MyoD (not shown). By contrast, the intermandibular muscle anlagen (derived from the SpM, Fig. 4A‴), expressed Isl1, Myf5 (Fig. 4E′,E″) and MyoD (Fig. 4F′,F″; note that Isl1 and MyoD are not expressed in the same cells). Pax7 expression in the intermandibular muscle anlagen was absent and MHC expression was significantly delayed, compared with the mandibular adductor or the adjacent genioglossal muscle that connects the tongue to the mandible (Fig. 4F‴,G‴) and is derived from myoblasts in the third BA (Marcucio and Noden, 1999). At E7 (St. 31) of chick embryonic development, Pax7 and MHC expression was observed in all muscles at the expense of Isl1, which was diminished (Fig. 4H-H‴). Thus, Isl1 expression in the (SpM-derived) intermandibular anlagen correlates with its delayed differentiation, similar to Isl1$^+$ cardioblasts in the AHF (Fig. 2). These novel findings suggest that there are distinct developmental programs for CPM- and SpM-derived branchiomeric muscles (as summarized in Fig. 4C).

To assess the contribution of Isl1$^+$ cells to the head musculature in mouse embryos, we employed the Cre-loxP system to genetically mark Isl1 progenitors by crossing *Isl1-Cre* mice (Yang et al., 2006) with the transgenic reporter line *R26R*. Staining for β-galactosidase (β-gal) in whole-mount and sectioned *Isl1-Cre;R26R* embryos (E10.5) revealed a contribution of *Isl1*$^+$ progenitors to the myogenic core of BA1 (Fig. 5A,A′). Double staining with anti-β-gal and MyoD antibodies was performed on *Isl1-Cre;R26R* embryos (E12.5) to further demonstrate the contribution of the Isl1$^+$ precursors to branchiomeric muscles, but not to the extraocular, tongue or trunk muscles (data not shown).

In order to carefully assess the myogenic contribution of Isl1$^+$ cells in mice, we analyzed E16.5 *Isl1-Cre;R26R* sectioned embryos (Fig. 5B-F). β-gal staining was strongly detected in distinct branchiomeric muscles, such as stylohyoid muscle (Fig. 5D),

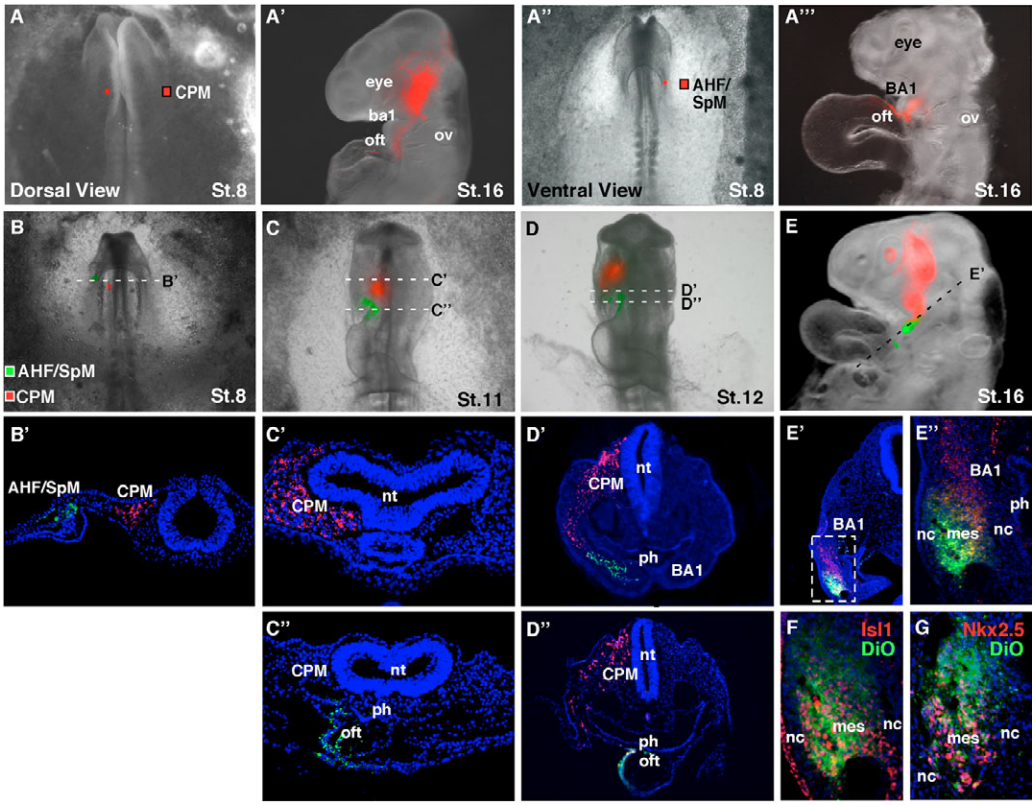

Fig. 3. Regionalization of the myogenic core of BA1 by CPM and SpM cells. (**A**,**A′**) Injection of DiI to the CPM (A) of a St. 8 chick embryo. After 36 hours incubation, the labeled cells streamed toward BA1. Notably, some of the CPM cells were seen in the distal outflow tract (A′). (**A″**,**A‴**) Injection of DiI into the AHF/SpM (A″) of a St. 8 embryo. After 36 hours incubation, labeled cells populated the outflow tract; some of the cells were seen in the distal portion of BA1 (A‴). (**B**,**B′**) Double injection of DiI (CPM) and DiO (AHF/SpM) into a St. 8 embryo (B) as shown in the transverse section (B′) (*n*=22). (**C-C″**) After 18 hours incubation (C), at St. 11, the DiI was found in the CPM (C′) anterior to the location of the DiO in the outflow tract (C″). (**D-D″**) After 24 hours (St. 12, D) the DiI was found at the most-proximal area of the developing BA (D′), as well as in the outflow tract (yellow staining in D″). The DiO was located in the distal part of the forming BA (D′) and in the cardiac outflow tract (D″). (**E-E″**) After 48 hours (St. 16, E) the DiI was found in the most-proximal area of the mesoderm core, whereas the DiO was located in the distal part of the BA1 core (E′). BA1 viewed at higher resolution (E″) demonstrates that the dye is restricted to the mesodermal core, and doesn't appear in the neural crest cells (nc) surrounding the core. (**F**,**G**) DiO was injected into the AHF/SpM of St. 8 embryos that were subsequently immunostained (red) for the AHF markers Isl1 (F) and Nkx2.5 (G). Co-localization of the DiO and the two AHF markers is shown in the distal part of the arch. Note that the DiO only labeled mesoderm cells (Isl1$^+$ and Nkx2.5$^+$), and not neural crest cells. oft, outflow tract; nt, neural tube; BA1, branchial arch 1; mes, mesoderm; hrt, heart; ph, pharynx; ecto, ectoderm; CPM, cranial paraxial mesoderm; AHF/SpM, anterior heart field/splanchnic mesoderm; ov, otic vesicle.

mylohyoid muscle, posterior digastric muscle, extrinsic laryngeal muscles (e.g. inferior constrictor, Fig. 5E), distal facial muscles (e.g. buccinator) and facial subcutaneous muscles (data not shown). Staining was also detected in other tissues, including cranial motoneurons and ganglia, salivary glands, the esophagus and connective tissues (Fig. 5C). We also detected partial β-gal staining in the mastication muscles that control the movement of the mandible, the masseter and pterygoid (Fig. 5F).

Alternatively, we stained serial frontal sections of E16.5 *Isl1-Cre;R26R* embryos with either anti-β-gal (Fig. 5G,H) or MF20 (Fig. 5G′,H′) antibodies. The results revealed a strong β-gal staining in the mylohyoid and anterior digastric muscles, a partial staining in the masseter, pterygoid and temporalis and a lack of staining in the tongue, genioglossal and extraocular muscles (Fig. 5G-H′). The partial staining observed in the masseter, pterygoid and temporalis could result from the fusion of a small number of Isl1$^+$ myonuclei that form the synsitium. Taken together, our genetic lineage-tracing studies clearly demonstrate that *Isl1*$^+$ cells contribute to a subset of branchiomeric muscles.

The Wnt/β-catenin pathway regulates heart field boundaries, differentiation and morphogenesis

Previous studies in chick and frog embryos suggested that members of the canonical Wnt signaling pathway can act as repressors of cardiac differentiation (Brott and Sokol, 2005; Foley and Mercola, 2005; Marvin et al., 2001; Schneider and Mercola, 2001; Tzahor and Lassar, 2001). Recent studies in mouse and zebrafish embryos, as well as in embryonic stem cells, have shed new light on the regulation of cardiogenesis by the Wnt/β-catenin pathway (Ai et al., 2007; Cohen et al., 2007; Kwon et al., 2007; Lin et al., 2007; Qyang et al., 2007; Ueno et al., 2007). Ablation of β-catenin in mice resulted in embryonic lethality, which was attributed to defects in second heart field derivatives: the right ventricular chamber, OFT and pharyngeal mesoderm (reviewed by Tzahor, 2007).

The molecular and cellular characterization of distinct mesodermal fields in chick embryos enabled us to explore the role of the Wnt/β-catenin pathway during early and late looping stages. Utilizing an in ovo electroporation system in chick embryos, control *GFP* or *Wnt3a-IRES-GFP* constructs were electroporated at

primitive streak stages to test the effects of the Wnt/β-catenin pathway on cardiac markers (Fig. 6A). Unilateral electroporation of *Wnt3a* into St. 8 chick embryos resulted in the almost complete repression of *Tbx5*, *Nkx2.5* and *Gata4* in differentiated cardiac crescent/SpM cells (Fig. 6B-D, respectively).

We next introduced *Wnt3a-IRES-GFP* into the surface ectoderm of St. 8 embryos (Fig. 6E) and tracked the fate of undifferentiated SpM cells, as marked by the expression of Isl1 and Nkx2.5 proteins. In contrast to control *GFP*-electroporated embryos (Fig. 6F,G), expression of Isl1 (Fig. 6F′) and Nkx2.5 (Fig. 6G′) in *Wnt3a*-electroporated embryos was markedly inhibited on the electroporated side. In addition, normal rightward cardiac looping was reversed in *Wnt3a*-electroporated embryos (compare Fig. 6F′,G′ to the control results in Fig. 6F,G). CPM markers capsulin, *Tbx1* and *Myf5* (not shown) were all repressed on the electroporated side (Fig. 6H-I′), corroborating our earlier findings (Tzahor et al., 2003).

We then tested how inhibition of the Wnt/β-catenin pathway affects cardiogenesis (Fig. 7). In vivo electroporation of the Wnt antagonists *sFrp2* and *sFrp3* into the surface ectoderm (Fig. 7A,B) caused an expansion in the expression domains of both Nkx2.5 and Isl1 (Fig. 7C,D). Furthermore, implantation of beads soaked with a purified Fz8-IgG protein into the CPM of St. 8 embryos (Fig. 7E,F) resulted in the dramatic induction of both Nkx2.5 (Fig. 7G-H′) and Isl1 (Fig. 7I,I′) within BA1 by St. 14. Taken together, these findings indicate that in chick embryos, the Wnt/β-catenin pathway can block

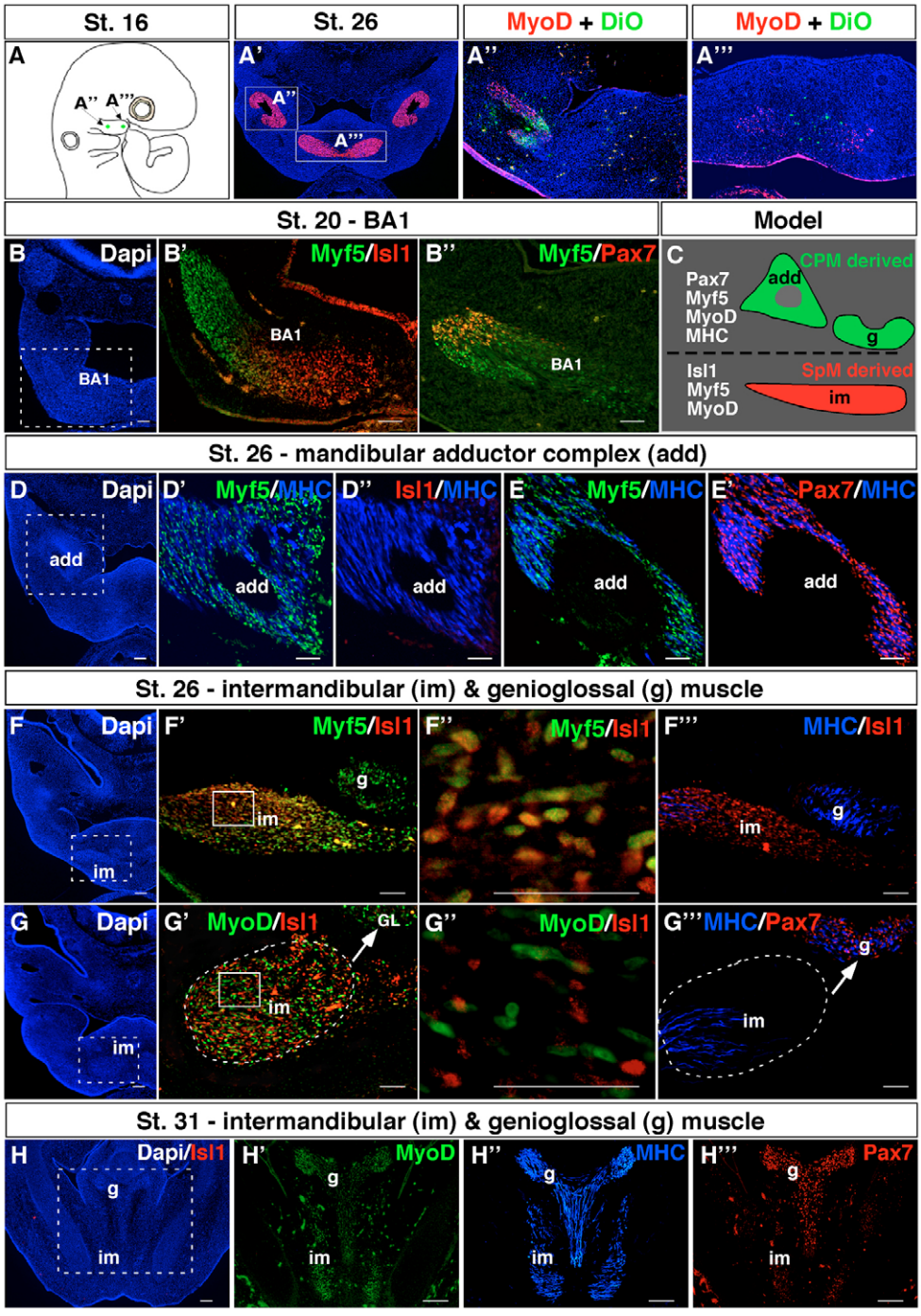

Fig. 4. Distinct programs underlie the development of CPM- and SpM-derived jaw muscles in the chick embryo. (**A-A‴**) Scheme (A) of the dye labeling experiment of proximal and distal BA1 myoblasts (A′). The mandibular adductor complex is illustrated in A″ and the intermandibular muscle is shown in A‴. Co-staining of MyoD and DiO is shown in these muscles (A″,A‴). (**B-B‴**) Transverse section of BA1 in St. 20 embryos stained for Myf5 and Isl1 (B′) and Myf5 with Pax7 (B″). (**C**) Schematic depicting the distinct cellular origins and molecular identities of the branchiomeric muscles. (**D-E′**) A transverse section through the adductor (add) muscle complex at St. 26 stained for the markers indicated. Note that Isl1 shows no apparent expression in this region (D″). (**F-F‴**) A transverse section through the intermandibular (im) muscle (F) stained for Isl1 and Myf5 (F′). Note the co-localization of Isl1 and Myf5 in the enlargement (F″). Staining for MHC and Isl1 shows the lack of MHC in the Isl1$^+$ cells in the intermandibular anlagen (F‴). (**G-G‴**) Similar sections stained for Isl1 and MyoD (G′). Note the mutually exclusive expression of Isl1 and MyoD, as shown in the enlargement (G″). Isl1 was restricted to the intermandibular muscle, and was not expressed in the genioglossal (g) muscle (arrow). A sequential section (G‴) was stained for Pax7 (red) and MHC (blue). Pax7 and Isl1 were not expressed in the same muscles (arrow). (**H-H‴**) Staining for Isl1, MyoD, MHC and Pax7 at the intermandibular region of St. 31, indicating the loss of Isl1 along with the upregulation of MHC and Pax7. Scale bars: 50 μm.

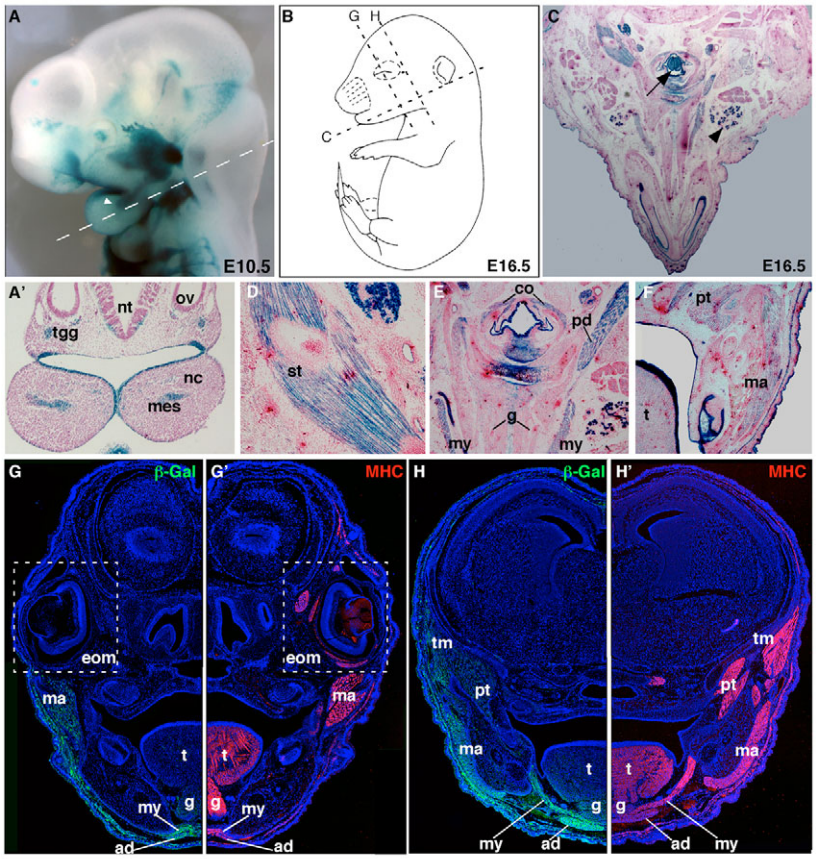

Fig. 5. Contribution of Isl1+ progenitors and their progeny to a subset of branchiomeric muscles in the mouse. (**A**,**A′**) Expression of the *lacZ* reporter in the mesodermal core (white arrowhead) of BA1 in E10.5 *Isl1-Cre/R26R* whole-mount (A) or sectioned (A′) embryo. Dashed line in A indicates plane of section. (**B**) Illustration of the various sections of the E16.5 *Isl1-Cre/R26R* embryos, modified from the Atlas of Mouse Development (Kaufman, 1992). (**C-F**) X-Gal staining for the *lacZ* reporter in transverse sections of an E16.5 *Isl1-Cre/R26R* embryo. The Isl1 lineage gives rise to multiple tissues including branchiomeric muscles (C,D,E), the salivary glands (arrowhead), and the oropharynx (arrow). (**G-H′**) Immunostaining for β-gal (G,H) and MF20 (MHC; G′,H′) on serial frontal paraffin sections of an E16.5 *Isl1-Cre/R26R* embryo (shown in B). For clarity, a half-section of β-gal was combined with the adjacent half of the MF20 staining. Note the co-expression of β-gal and MF20 in a subset of branchiomeric muscles, but not in tongue or extraocular muscles (eom, marked by squares in G,G′). The masseter (ma) has both superficial and deep masses and the pterygoid (pt) has medial and lateral masses (H′). nt, neural tube; ov, otic vesicle; nc, neural crest; mes, mesoderm; t, tongue; my, mylohyoid; ad, anterior digastric; g, genioglossal; co, constrictor; st, stylohyoid; pd, posterior digastric; tm, temporalis.

cardiac and skeletal muscle differentiation in vivo; moreover, antagonists of this pathway induced Isl1 and Nkx2.5 expression in the SpM of both the AHF and BA mesoderm.

DISCUSSION

In the present study, we used both avian and mouse embryonic models to perform cell lineage and molecular analyses of CPM and SpM, in order to systematically track both cardiac and skeletal muscle lineages. Our results uncovered the significant contribution of SpM cells to the BA mesoderm and later to the jaw musculature, underscoring their cardio-craniofacial potential. Using *Isl1-Cre* mice, we revealed that *Isl1*$^{+}$ cells contribute to a subset of branchiomeric muscles. Likewise, in the chick, we demonstrated that Isl1 is expressed in a subset of SpM-derived BA1 muscles. In addition, we examined the Wnt/β-catenin pathway, and showed that it can regulate the specification, differentiation and morphogenesis of cells derived from the Isl1/Nkx2.5/SpM field.

Molecular characterization of the heart fields in chick and mouse embryos

Despite accumulating evidence concerning the subdivision of cardiac progenitor populations into distinct heart fields in the mouse, the exact anatomical locations of these fields in other vertebrates remains unclear (Abu-Issa and Kirby, 2007). In the chick, we defined the AHF field as being located in the dorsomedial region of the SpM in St. 8 embryos in agreement with the current model of two heart fields in the mouse (Buckingham et al., 2005; Srivastava, 2006), which is based on both molecular (*Isl1*, *Nkx2.5*, *Fgf10*, *Tbx20*) and anatomical considerations (Fig. 8A). In contrast to the mouse, in which *Isl1* and *Fgf10* expression were shown to be restricted to the dorsomedial domain of the cardiac crescent (Cai et al., 2003; Kelly et al., 2001), in the chick, both genes are expressed throughout the cardiac crescent. However, downregulation of these markers within differentiating cardiac cells occurs during linear heart tube stages, as the heart begins to beat.

What is the relationship between the contribution of the CPM (Tirosh-Finkel et al., 2006) and AHF/SHF/SpM to the cardiac OFT? Although CPM cells migrate distally to the BAs, where some of these cells infiltrate the AHF/SHF, each cell population seems to contribute to the cardiac OFT in a distinct manner. The entrance of AHF/SHF/SpM cells into the cardiac OFT precedes that of the CPM cells, which also seem to follow a different migratory path. We propose that the contributions of the CPM and AHF/SHF/SpM to the anterior pole of the heart are distinct, both spatially and temporally.

Distinct mesodermal origins for branchiomeric muscles

Findings in mice already suggested that SpM cells contribute to both the pharyngeal mesoderm and the cardiac OFT (Dong et al., 2006; Kelly et al., 2001; Verzi et al., 2005). However, the specific contribution of Isl1^{+} SpM cells to the distal part of the myogenic core in BA1, and later to distinct branchiomeric muscles, remained unclear. Our findings in the chick demonstrate that branchiomeric skeletal muscles derive from both CPM and Isl1^{+}/SpM (Fig. 8C); furthermore, CPM- and SpM-derived BA1 muscles are molecularly distinct.

Previous studies in chick embryos are consistent with the separation of myoblasts in BA1 into proximal (mandibular adductor) and distal (intermandibular) jaw muscles (Marcucio and Noden, 1999; Noden et al., 1999). Interestingly, Pax7 is expressed in CPM- but not SpM-derived myoblasts, whereas Isl1 is expressed in the SpM-derived branchiomeric muscles. Because

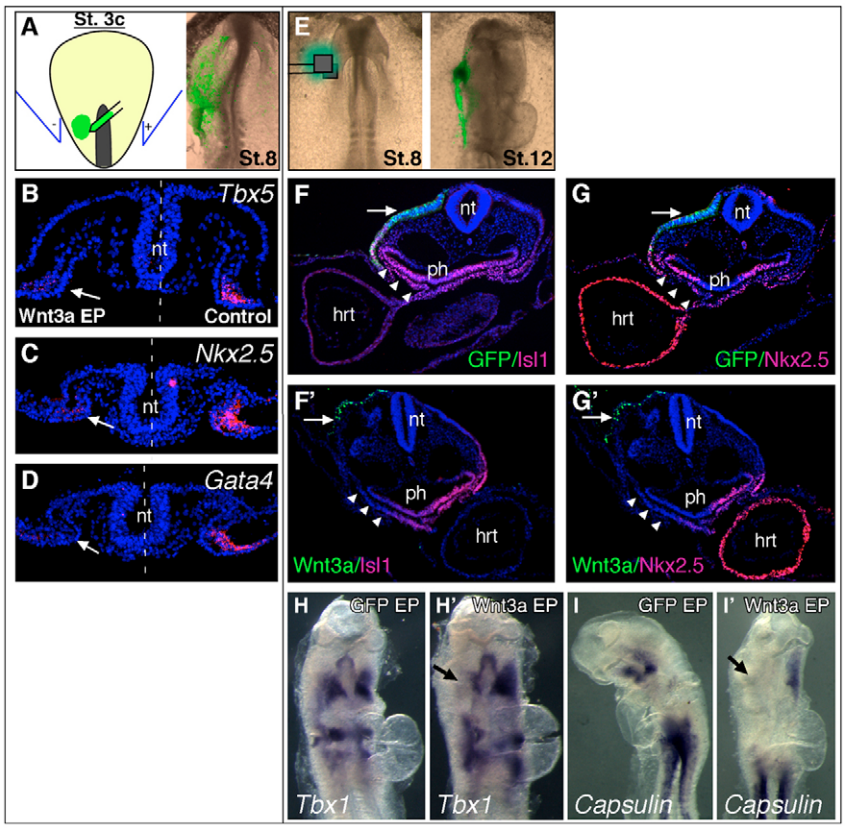

Fig. 6. The Wnt/β-catenin signaling pathway negatively regulates specification and differentiation of cells from the SpM and CPM in chick embryos. (**A**) Schematic representation of the experimental procedure showing the electroporation of the construct into a St. 3c embryo, which was analyzed 16 hours later, at St. 8. (**B-D**) Fluorescent ISH of the St. 8 electroporated embryos for the markers indicated. As a result of Wnt3a activity, the expression levels of *Tbx5* (B), *Nkx2.5*(C) and *Gata4* (D) transcripts were reduced on the electroporated side (arrows; *n*=14). (**E**) Representation of the experimental procedure showing the electroporation of the *pCIG-Wnt3a-IRES-GFP* construct into a St. 8 embryo, and its analysis at St. 12 (note a reverse cardiac looping). (**F**,**G**) St. 12 embryos stained for Isl1 (F) and Nkx2.5 (G) following electroporation with *pCAGG-GFP* (arrows). The normal expression of both proteins was not affected (arrowheads). (**F′**,**G′**) St. 12 embryos, electroporated with *pCIG-Wnt3a-IRES-GFP* marked by the GFP expression in the surface ectoderm. Both Isl1 (F′) and Nkx2.5 (G′) levels in the SpM of the AHF (arrowheads) were reduced on the electroporated side (*n*=17). (**H-I′**) Whole-mount ISH for *Tbx1* and capsulin in St. 13 embryos electroporated with either *pCAGG-GFP* or *pCIG-Wnt3a-IRES-GFP*. Both *Tbx1* (H′) and capsulin (I′) levels in the BA were reduced (arrows) on the *Wnt3a*-electroporated side (*n*=9). nt, neural tube; hrt, heart; ph, pharynx.

MHC expression is delayed in SpM-derived Isl1-expressing myoblasts, we suggest that it acts as a repressor of myogenic differentiation in a manner similar to its expression in undifferentiated second heart field cells before they enter the heart. It is tempting to speculate that Isl1 might also play a role in the regulation of quiescence and self-renewal of satellite cells in branchiomeric muscles, analogous to the role of Pax7 in trunk skeletal muscles

Our lineage studies in both chick and mouse models provide a clear example of lineage heterogeneity within craniofacial muscles. In both species, Isl1^{+} SpM cells contribute to a set of branchiomeric muscles at the base of the mandible facilitating its opening: the intermandibular muscle in the chick, and the mylohyoid, stylohyoid, digastric and other (distal) facial muscles in the mouse. By contrast, there is a relatively minor contribution of Isl1^{+} cells to the mastication muscles in the mouse (masseter, pterygoid and

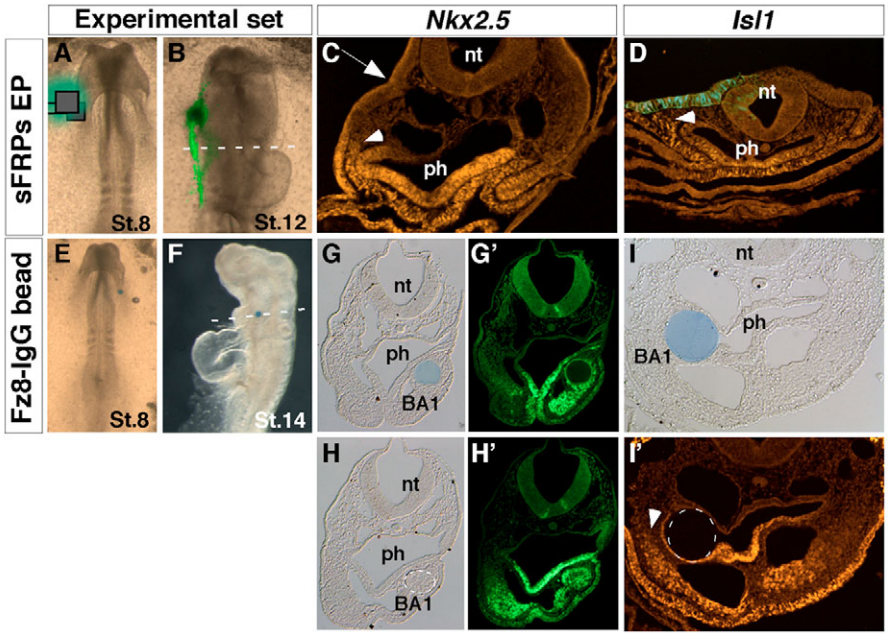

Fig. 7. Antagonists of the Wnt/β-catenin pathway promote Isl1 and Nkx2.5 expression in AHF/SpM and branchial arches. (**A**,**B**) Representation of the electroporation of *pCIG-sFrp2-IRES-GFP* together with *pCIG-sFrp3-IRES-GFP* constructs between St. 8 and St. 12. Dashed line indicates the plane of the transverse sections. (**C**) Immunostaining for Nkx2.5 at St. 12 indicating that Nkx2.5 protein level increased (arrowhead) on the electroporated side (arrow). (**D**) Immunostaining for Isl1 at St. 12. GFP expression in the surface ectoderm marks cells expressing the sFrp2 and sFrp3 proteins. Isl1 level in the SpM (arrowhead) was increased on the electroporated side (*n*=7). (**E**,**F**) Fz8-IgG-soaked bead insertion (E, St. 8) and the same embryo 23 hours later (F, St. 14). (**G-I**) Transverse sections showing the location of the implanted bead (blue circle in G,I and dashed circle in H). (**G′-I′**) Immunostaining of sections G-I. As a result of the Fz8-IgG activity, both Nkx2.5 (G′,H′) and Isl1 (I′) levels in BA1 increased around the bead (*n*=4). EP, electroporation; nt, neural tube; BA1, branchial arch 1; hrt, heart; ph, pharynx.

temporalis) or to the mandibular adductor complex in the chick. Furthermore, in both species, the intrinsic and extrinsic muscles of the tongue (e.g. genioglossal) and extraocular muscles are not derived from the Isl1+ SpM lineage. The slightly broader contribution of the Isl1+ lineage to branchiomeric muscle, observed in the mouse, could result from differences in the lineage allocations between the two species. Similarly, the contribution of Isl1+ cells of the second heart field is broader in the mouse than that in the chick. Clearly, methodological and experimental differences affect lineage comparisons between chick and mouse models. In our *R26R* muscle lineage analyses in mice, it is important to appreciate that the fusion of a few β-gal+ myoblasts is likely to result in staining of the entire myofiber (e.g. masseter, pterygoid and temporalis, Fig. 5).

Tbx1 (Kelly et al., 2004), as well as capsulin and MyoR (Lu et al., 2002), have been shown to act as upstream regulators of branchiomeric muscle development. In capsulin/*MyoR* double mutants (Lu et al., 2002), the masseter, pterygoids and temporalis were missing, whereas the distal BA1 muscles (e.g. anterior digastric and mylohyoid, both derived from Isl1+ cells, Fig. 5) were not affected. Our findings in both chick and mouse experimental models, which reveal that jaw muscles are composed of at least two distinct myogenic lineages (CPM-derived and Isl1+ SpM-derived muscles), provide a plausible developmental explanation for this unique muscle phenotype.

It was recently demonstrated in mice that Isl1/Nkx2.5/Flk1-positive cells within the SpM are multipotent cardiovascular progenitors that give rise to cardiac myocytes, smooth muscle and endothelial lineages within the heart (Moretti et al., 2006; Wu et al., 2006). We show that Isl1+ cells represent multipotential progenitors of both cardiovascular and skeletal muscle lineages.

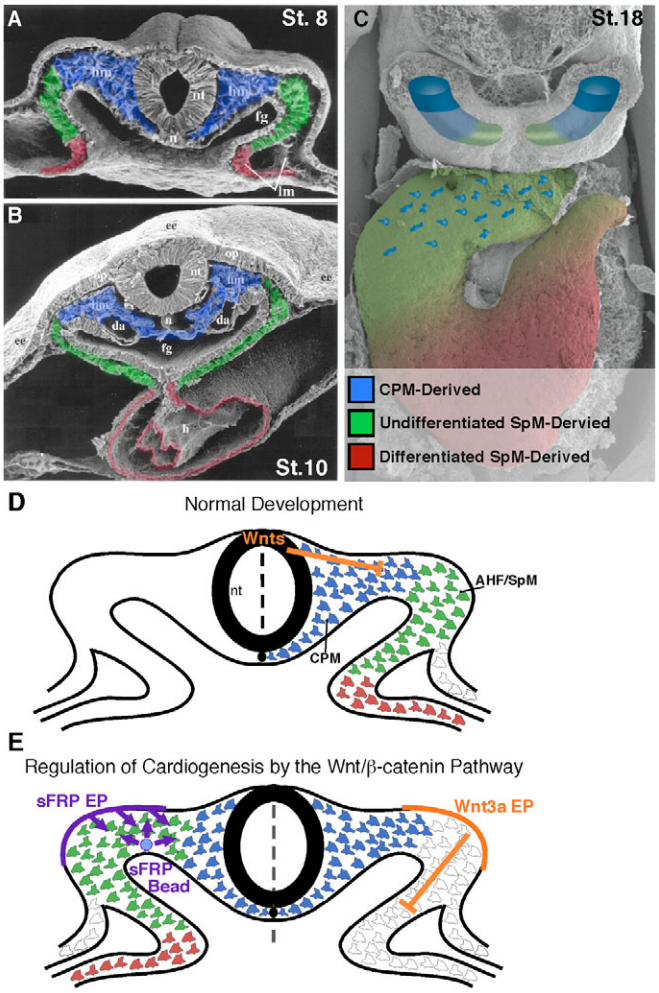

Fig. 8. Model for the molecular regulation of the boundaries within the cardio-craniofacial mesoderm in chick embryos. (**A**) At St. 8, the cardio-craniofacial mesoderm can be divided into CPM (blue), AHF/SpM (green) and differentiated SpM (red). (**B**) At St. 10, the division of the cardio-craniofacial mesoderm becomes more obvious. The AHF/SpM (green) is located underneath the pharynx, just dorsal to the heart. (**C**) At St. 18, both CPM and SpM contribute to BA1 and to the cardiac outflow tract. The distal part of BA1 mesoderm is SpM-derived (green) and is Isl1+, whereas the proximal region is CPM-derived (blue) and Myf5+. In the cardiac outflow tract, the contribution of the AHF/SpM is greater than that of the CPM, the contribution of which is seen in the more-distal part of the heart. (**D**) Heart formation is regulated by a combination of positive and negative signals from surrounding tissues. Whereas a signal(s) from the anterior endoderm works to promote heart formation in concert with BMP signals from the anterior lateral mesoderm, Wnt signals from the axial tissues (orange) repress heart formation in the CPM. (**E**) This study demonstrates that Wnt signaling can repress Isl1 and Nkx2.5 in the AHF/SpM, whereas treatment with Wnt antagonists (sFRP, purple) promotes Isl1 and Nkx2.5 expression. EP, electroporation; fg, foregut; nt, neural tube; da, dorsal aorta; n, notochord; h, heart; lm, lateral mesoderm; CPM, cranial paraxial mesoderm; AHF/SpM, anterior heart field/splanchnic mesoderm. Electron micrographs shown in A and B were reproduced with permission from Prof. Schoenwolf (University of Utah School of Medicine, Salt Lake City, UT).

Wnt/β-catenin signaling and its effect on cardiac and skeletal muscle development

We previously demonstrated in the chick that signals emanating from the neural tube (that can be mimicked by Wnt1 and Wnt3a) block cardiogenesis in the CPM (Tzahor and Lassar, 2001). These findings, and those of two other studies (Marvin et al., 2001; Schneider and Mercola, 2001), suggest that Wnt/β-catenin signaling inhibits cardiogenesis during early embryogenesis. A subsequent study (Foley and Mercola, 2005) demonstrated that Wnt signaling must be inhibited within the endoderm to induce secretion of an as yet unidentified cardiogenic-inducing factor. In fact, numerous studies support the notion that inhibition of the Wnt/β-catenin pathway is required for proper heart development and repair (Barandon et al., 2003; Brott and Sokol, 2005; Lickert et al., 2002; Singh et al., 2007), whereas other studies, mostly in cultured ES cells, suggest that the opposite is true (Nakamura et al., 2003).

Recent studies in mouse and zebrafish embryos, as well as in embryonic stem cells, demonstrate that the Wnt/β-catenin pathway plays distinct, even opposing, roles during various stages and within distinct tissues during cardiac development (reviewed by Tzahor, 2007). The new loss-of-function studies of canonical Wnt signaling in the mouse (Ai et al., 2007; Cohen et al., 2007; Kwon et al., 2007; Lin et al., 2007; Qyang et al., 2007; Ueno et al., 2007) provide compelling evidence that this pathway is required within cardiac progenitors and differentiating cardiac cells for the development of the second heart field (including AHF cells) and its derivatives: the right ventricular chamber, OFT and pharyngeal mesoderm. These studies further demonstrate that Wnt signaling stimulates the proliferation of cardiac progenitors during mouse cardiogenesis.

Using electroporation of Wnt ligands or Wnt inhibitors in chick embryos, we observed either the inhibition of cardiac and skeletal muscle differentiation markers, or the expansion of Isl1 and Nkx2.5, respectively. Similarly, bead implantation of a Wnt inhibitor into the CPM resulted in increased expression of Nkx2.5 and Isl1 within the

SpM-derived myogenic mesoderm of BA1. These results suggest that canonical Wnt signaling can inhibit cardiac and cranial muscle differentiation, which is consistent with findings in mice demonstrating that continuous Wnt signaling prolongs the progenitor state and interferes with differentiation during cardiogenesis.

The cardio-craniofacial mesoderm

Taken together, our past and present studies clearly demonstrate that the cardio-craniofacial mesoderm is tightly regulated by both positive and negative cues from surrounding tissues (Rinon et al., 2007; Tirosh-Finkel et al., 2006; Tzahor et al., 2003; Tzahor and Lassar, 2001). Our findings highlight the heterogeneity of developmental programming among cranial muscles, and confirm that craniofacial myogenesis is developmentally linked to cardiac development (this study) (Tirosh-Finkel et al., 2006; Tzahor and Lassar, 2001) (reviewed by Grifone and Kelly, 2007), suggesting that these tissues share a common evolutionary origin.

The striking parallel between a subset of branchiomeric muscles and the transcriptional networks involved in heart development (this study) (Dong et al., 2006; Kelly et al., 2001; Verzi et al., 2005) is actually seen across vast phylogenetic distances. Nematodes do not possess a heart, yet their pharyngeal muscle contracts like a heart and exhibits electrical activity similar to that of mammalian cardiomyocytes. Moreover, it has been shown that the development of the pharyngeal muscle in nematodes, and of cardiac muscle in vertebrates and insects, is regulated by the homeobox gene *Nkx2.5* (Haun et al., 1998). Thus, unlike skeletal muscles in the trunk, head muscles are likely to have evolved from an ancestral developmental program that gave rise to a contractile tube used for feeding and circulation. Insights into the genetic circuits that drive the evolution and development of heart and craniofacial muscles might shed light on general principles of organogenesis, as well as on the molecular basis of cardiovascular and craniofacial myopathies in humans.

This work was supported by research grants to E.T. from the Estelle Funk Foundation for Biomedical Research, Ruth and Allen Ziegler, the Pasteur-Weizmann Foundation, the Helen and Martin Kimmel Institute for Stem Cell Research, the Y. Leon Benoziyo Institute for Molecular Medicine, a German Israeli Foundation (GIF) Young Investigator Award, the Minerva Foundation with funding from the Federal German Ministry for Education and Research, and the Association Française Contre les Myopathies (AFM). E.N. is a recipient of a travel fellowship from *Development*, and A.M. was supported by the Landa Center for Equal Opportunity Through Education. S.M.E. was supported by research grants NIHRO1, HL074066 and an AHA (fellowship to Z.H.). We thank Margaret Buckingham for her critical review of the manuscript; Michael Zagazki for the EM micrographs; Ori Brenner, Drew Noden and Giovanni Levi for histological and anatomical insights; Laura Burrus and Andrew McMahon for Wnt-related DNA constructs for the electroporation; and our laboratory teams for their insights and support.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/135/4/647/DC1

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