The Molecular Setup of the Avian Head Mesoderm and Its Implication for Craniofacial Myogenesis†

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The head mesoderm is the mesodermal tissue on either side of the brain, from forebrain to hindbrain levels, and gives rise to the genuine head muscles. Its relatedness to the more posterior paraxial mesoderm, the somites, which generate the muscles of the trunk, is conversely debated. To gain insight into the molecular setup of the head mesoderm, its similarity or dissimilarity to the somitic mesoderm, and the implications of its setup for the progress of muscle formation, we investigated the expression of markers (1) for mesoderm segmentation and boundary formation, (2) for regional specification and somitogenesis and (3) for the positive and negative control of myogenic differentiation. We show that the head mesoderm is molecularly distinct from somites. It is not segmented; even the boundary to the first somite is ill-defined. Importantly, the head mesoderm lacks the transcription factors driving muscle differentiation while genes suppressing differentiation and promoting cell proliferation are expressed. These factors show anteroposteriorly and dorsoventrally regionalised but overlapping expression. Notably, expression extends into the areas that actively contribute to the heart, overlapping with the expression of cardiac markers. *Developmental Dynamics 235:2845–2860, 2006.* © 2006 Wiley-Liss, Inc.

Key words: chick; embryo; head mesoderm; segmentation; differentiation; skeletal muscles; heart; *Alx4*; *Chordin*; *Cyp26C1*; *Dll1*; *EphA4*; *Gata3*; *Isl1*; *Lunatic Fringe*; *Myf5*; *MyoR*; *Nkx2.5*; *Notch1*; *Paraxis*; *Pax3*; *Pitx2*; *Raldh2*; *Tbx1*; *Twist*.

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INTRODUCTION

The vertebrate head mesoderm is the mesoderm-derived, mesenchymal tissue on either side of the neural tube from forebrain to hindbrain levels (reviewed by Wachtler and Jacob, 1986; Kuratani, 2005). It consists of the paraxial mesoderm and the remnant of the originally axial, pre-chordal mesoderm that is pushed posteriorly through the growing forebrain. The

ABBREVIATIONS aip anterior intestinal portal **a psm** anterior presomitic mesoderm **as** aortic sac **di/m** diencephalon/mesencephalon **ect** surface ectoderm (future epidermis) **end** endoderm **ep** epiblast **fg** foregut **hm** head mesoderm **hn** Hensen's node **hp** head process **ht** heart (at HH10 consisting of parts of the ventricles only) **m** mesencephalon **mes** mesoderm **ncc** neural crest cells **not** notochord **np** neural plate **nt** neural tube **op** otic placode **p** prosencephalon **pc** pericardial cavity (cardiac aspect of the coelom) **ph** pharynx **phf** primary heart field **ps** primitive streak **r1/2** rhombomere 1 and 2 (metenceplalon) **r3** rhombomere 3 **r5/6** rhombomeres 5 and 6 **r7** rhombomere 7 **s1** 1st somite (with ill-defined boundary to head mesoderm) **s2** 2nd somite **scp**subcephalic pocket **sompl** somatopleura of the lateral mesoderm **spc** spinal cord **splpl** splanchnopleura of the lateral mesoderm

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head mesoderm contributes to the base of the skull, but most importantly, delivers the jaw closure muscles (1st arch muscles); muscles to open the jaw, to control the cranial openings, and to facilitate facial expression (2nd arch muscles); and the muscles of the upper pharynx and larynx that assist swallowing and breathing (3rd arch muscles; Noden, 1983; Jacob et al., 1984; Wachtler et al., 1984; Couly et al., 1992; Evans and Noden, 2006; reviewed by Wachtler and Jacob, 1986; Kuratani, 2005). Moreover, the head mesoderm delivers the extraocular muscles that rotate the eyeball and, hence, aid vision (Noden, 1983; Jacob et al., 1984; Wachtler et al., 1984; Couly et al., 1992; Evans and Noden, 2006; reviewed by Wachtler and Jacob, 1986; Kuratani, 2005). Thus, although these head muscles do not contribute to locomotion, they perform vital functions for any individual.

At the level of the uppermost spinal cord, which posteriorly follows the hindbrain and is sometimes referred to as rhombomere 8 (reviewed in Lumsden, 2004), the neighbouring paraxial mesoderm is known as occipital somites (reviewed by Wachtler and Jacob, 1986; Kuratani, 2005). These somites deliver the remaining bones of the skull base, the hypobranchial/tongue muscles, and the muscles of the posterior branchial arches, and, hence, contribute to the head as much as the head mesoderm (Hamilton and Hinsch, 1956; Noden, 1983; Couly et al., 1992; Huang et al., 1997; reviewed by Wachtler and Jacob, 1986; Kuratani, 2005). Yet, the occipital somites are arranged as reiterated, epithelial balls of cells (Hamilton and Hinsch, 1956; Huang et al., 1997). They are serially homologous with somites in the trunk, which constitute the trunk paraxial mesoderm and deliver the vertebral column and entire trunk musculature (reviewed by Buckingham et al., 2003; Dubrulle and Pourquie, 2004). Moreover, occipital somites have secondarily been incorporated into the head during vertebrate evolution, possibly to accommodate for increasing brain sizes (Gans and Northcutt, 1983). Thus, they are essentially trunk derivatives.

Ever since Oken and Goethe proposed that the skull is made from "head vertebrae," the relationship of the head mesoderm and the occipital somites has roused fascination and controversy (Oken, 1807; Goethe, 1820; reviewed by Kuratani, 2005). The first unresolved problem regards the organisation of the head mesoderm as compared to occipital somites. Genes that control paraxial mesoderm segmentation at trunk levels are expressed in the primitive streak at HH3–4 (Jouve et al., 2002), the time when the head mesoderm is laid down (Garcia-Martinez and Schoenwolf, 1992; Schoenwolf et al., 1992; Psychoyos and Stern, 1996). Moreover, using electronmicroscopy, repetitive swirls of cells were detected in the head mesoderm that suggested the existence of cryptic somites/somitomeres (Meier, 1979; Anderson and Meier, 1981; Meier and Tam, 1982). In addition, in some vertebrate species, the head mesoderm forms so-called head cavities that by some authors are regarded as somite homologues (and hence called head-somites (Balfour, 1878; reviewed in Goodrich, 1958; Kuratani, 2005). Furthermore, in the cephalochordate *Amphioxus*, which for a long time was seen as the closest living chordate relative of vertebrates, somites are found almost up to the anterior end (Goodrich, 1958; Holland, 2000; Delsuc et al., 2006). Thus, it is conceivable that the vertebrate head mesoderm may represent a variation on the theme "somitic paraxial mesoderm." However, no study ever demonstrated that the head somitomeres give rise to head cavities. Moreover, the somitomeres came into disrepute as studies found the cell swirls statistically insignificant (Freund et al., 1996). In addition, the head cavities display a morphological organisation distinct from that of somites (Wachtler and Jacob, 1986). They vary in number in jawed vertebrates (gnathostomes) and are not found in primitive, jaw-less vertebrates (agnathans; Kuratani et al., 1999, reviewed in Holland, 2000; Kuratani, 2005). Thus, head cavities are not a primitive, shared character of vertebrates. Finally, it is unclear whether head somites in Amphioxus represent a derived condition of an animal with severely reduced cranial structures (Goodrich, 1958). Moreover, in the larvae of tunicates, which are now regarded as the closest living relatives of vertebrates, segmented mesoderm is confined to the tail (Delsuc et al., 2006). Thus, a non-segmental organisation of the cranial mesoderm may by a primitive condition of "olfactoria" (tunicates plus vertebrates; Delsuc et al., 2006). Taken together, the problem of head mesoderm organisation versus the organisation of somites is unresolved.

The second problem regards the differentiation of head mesoderm versus somites. Both tissues rely on the MyoD family of muscle-determining factors (MDF) for their myogenic differentiation, and, eventually, they use the same factors to assemble contractile muscle fibres (reviewed in Buckingham et al., 2003). However, Myf5, the first MDF to be expressed in the embryo, employs distinct promoter and enhancer elements in the trunk and in the head (Hadchouel et al., 2000; Summerbell et al., 2000). Moreover, the upstream regulator of somitic myogenesis, Pax3, is not expressed in developing head muscles (Hacker and Guthrie, 1998; Mootoosamy and Dietrich, 2002). Conversely, the related basic helix-loop-helix transcription factors MyoR and Capsulin, the T-box transcription factor Tbx1, and the homeobox transcription factor Pitx2, though also expressed in somites, seem to be required for the development of certain head muscles only (Gage et al., 1999; Kitamura et al., 1999; Lu et al., 2002; Kelly et al., 2004; von Scheven et al., 2006b). Overall, muscle formation from head mesoderm is significantly delayed compared to muscle formation from somites (Noden et al., 1999). Moreover, the head mesoderm can only differentiate into muscle in a head environment (Mootoosamy and Dietrich, 2002). In the head, in addition to the general repressors of muscle differentiation, Bmp4 and Fgf8, Shh and Wnt signalling molecules also suppress muscle formation (Tzahor et al., 2003; von Scheven et al., 2006a); in the trunk, Shh and Wnt signals are required to promote myogenesis (reviewed in Buckingham et al., 2003). Thus, the molecular setup of the head mesoderm that determines its developmental potential and the use of regulatory cascades may be distinct.

To gain insight into the molecular

difference of head and somitic mesoderm that may underpin the different developmental programmes, we comparatively analysed the expression of markers for mesoderm segmentation and somite formation, for anteroposterior positional values, and for the activation or repression of skeletal muscle development. Moreover, since recent studies suggested that the head mesoderm contributes to the outflow tract of the heart (reviewed in Kelly, 2005), we comparatively analysed the expression of cardiac markers. We show that a number of markers distinguish between head and trunk/somitic mesoderm. Moreover, in the head mesoderm, markers for mesoderm segmentation and somitogenesis are either absent or expressed at low levels in a non-segmental fashion. This indicates that head and somitic mesoderm are distinct types of mesoderm, and that the head mesoderm is unsegmented. Most striking was the massed expression of negative regulators of myogenic differentiation in the head mesoderm, while in the occipital somites, predominantly positive regulators were expressed. In the head mesoderm, the expression of the negative myogenic regulators stretched ventrolaterally into the region that expresses cardiac markers and secondarily contributes to the heart. Thus, the head mesoderm is continuous with the splanchnic/cardiac mesoderm and may be withheld from differentiation to make cells available for the heart.

RESULTS

To gain insight into the molecular setup of the head mesoderm in comparison to the somitic/trunk paraxial mesoderm, we investigated the expression of molecular markers in the chick embryo from HH3–4, the time when the head mesoderm is being generated through gastrulation; HH4–5, the time that the generation of somitic mesoderm commences; HH6, the time when the first somite begins to form at the anterior end of the presomitic paraxial mesoderm; HH7, the stage when the first somite (ultimately the second somite in series) is morphologically defined; to HH10, when the first 10 of the 52 chicken somites have been generated and their myogenic differentiation is well underway (Hamburger and Hamilton, 1951; Garcia-Martinez and Schoenwolf, 1992; Schoenwolf et al., 1992; Psychoyos and Stern, 1996; Noden et al., 1999).

We analysed the expression of markers indicative of segmentation and boundary formation (*Notch1, Dll1, Lunatic Fringe, EphA4*; reviewed in Dubrulle and Pourquie, 2004), of anteroposterior specification and somitogenesis (*Paraxis, Raldh2, Cyp26C1*; Burgess et al., 1996; Blentic et al., 2003; Reijntjes et al., 2004), and markers for the progress of myogenic differentiation (*Pitx2, Alx4, MyoR, Tbx1, Twist, Pax3, Myf5*; Hebrok et al., 1994, 1997; Tajbakhsh et al., 1997; Tremblay et al., 1998; Lu et al., 1999; Kioussi et al., 2002; Cheng et al., 2004; Kassar-Duchossoy et al., 2004; Kelly et al., 2004; reviewed in Fuchtbauer, 2002). The expression of the latter was compared with that of *Gata3, Isl1*, and *Nkx2.5*, markers for the cardiogenic regions of the head (Schultheiss et al., 1995; Sheng and Stern, 1999; Yuan and Schoenwolf, 2000; reviewed in Brand, 2003; Kelly, 2005; this study). Expression patterns were determined by whole mount in situ hybridisation, followed by serial cross or sagittal vibratome sectioning. In selected embryos, the position of the node and head process/notochord was marked via staining for *Chordin* (red staining in Figs. 1A–D,M–P,Q, 2; Chapman et al., 2002).

Markers for Mesoderm Segmentation and Boundary Formation

The trunk paraxial mesoderm including the post-otic, occipital tissues forms reiterated and alike morphological units, i.e., segments (reviewed by Dubrulle and Pourquie, 2004). These segments are organised as epithelial balls of cells, the somites, which form at regular intervals from the anterior end of the not yet segmented paraxial mesoderm (presomitic mesoderm [PSM] or segmental plate). To the posterior end of the PSM, cells are being continuously added from the primitive streak and, later, from the tail bud (i.e., through gastrulation). Mesoderm segmentation is controlled by periodic activation/deactivation of Notch-Delta (Dll) signalling, which is achieved through oscillating expression of the negative regulator of Notch function, the glycosyl transferase Lunatic fringe (Lfng; reviewed by Dubrulle and Pourquie, 2004). This "segmentation clock" is overlaid by a "maturation gradient" consisting of an Fgf8 gradient, high at the posterior end of the PSM, low towards its anterior end. Fgf8 keeps cells in an immature state. When a threshold of low FGF8 levels is reached at the anterior end of the PSM, oscillating gene expression terminates and the expression of Notch-Delta signalling components resolves into stripes in either the anterior or posterior half of a developing segment. This, together with the initiation of EphA4-Ephrin signalling, leads to the establishment of segmental boundaries. To assay for signs of segmentation or boundary formation in the head mesoderm, we investigated the expression of the mRNAs for the *Notch1* receptor, the Notch ligand Delta-like1 (*Dll1*), and the Eph receptor *EphA4.* (Fig.1)

Notch1.

At all stages examined, *Notch1* was expressed in the primitive streak, in line with Henrique et al. (1995). Thus, when head mesodermal cells were generated through gastrulation at HH3–4, they temporarily harboured the mRNA for the *Notch* receptor. From HH5 onwards, expression was found in the condensing somites at the anterior end of the PSM and in the developing neural plate/neural tube. In somites, low-level expression continued in the posterior domain. However, the head mesoderm did not retain *Notch1* expression (data not shown).

Dll1.

Similar to *Notch1*, also *Dll1* was expressed in the primitive streak albeit at higher levels (Fig. 1A–Fi; Henrique et al., 1995). Expression continued in the newly laid down paraxial mesoderm and in cells lining the node and the anteriorly adjacent part of the head process or notochord (Fig. 1A–C, small arrows). However, staining was never found in the head mesoderm. In the newly formed epithelial somites, expression was downregulated and confined to the posterior domain of each somite. This expression was initially weak, intensifying, however, at

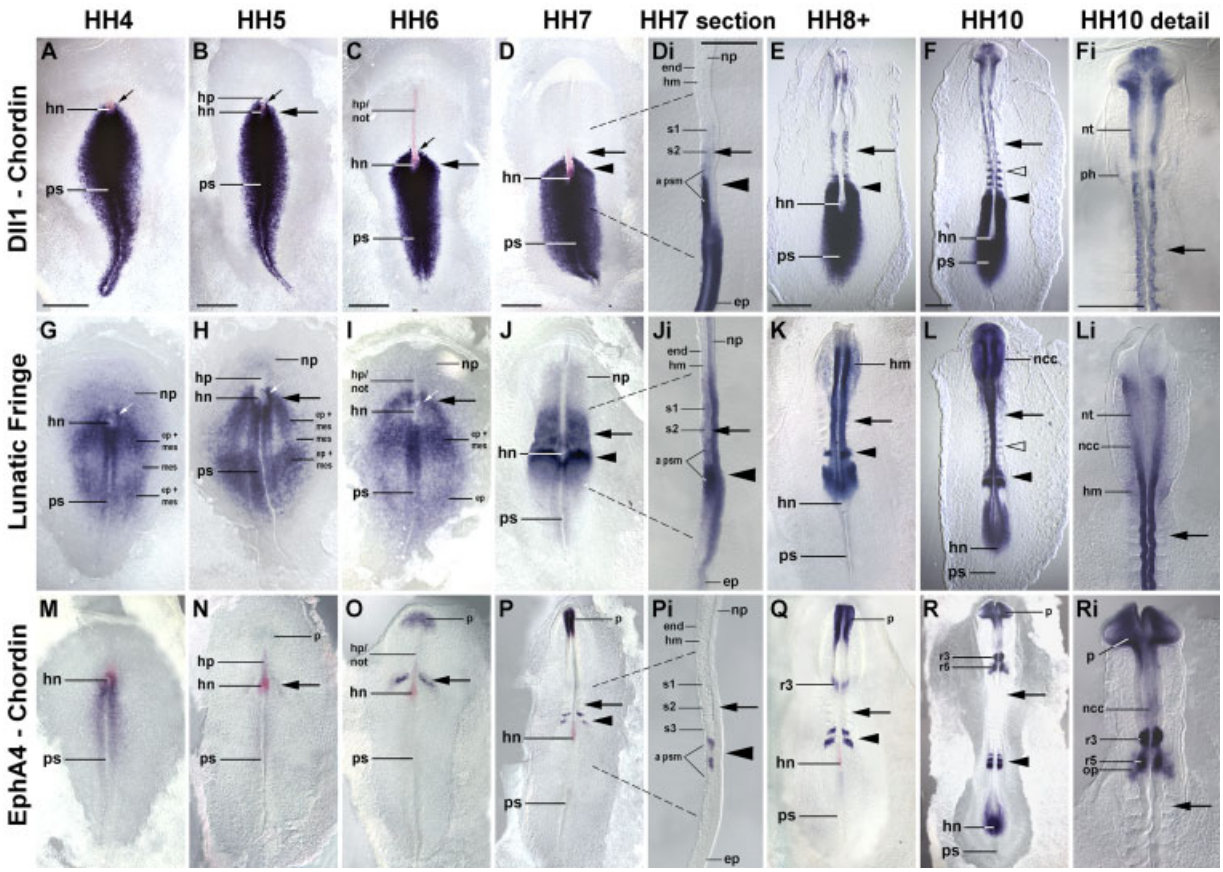
HH4
HH5
HH6
HH7
HH7 section
HH8+
HH10
HH10 detail
Dll1 - Chordin
Lunatic Fringe
EphA4 - Chordin
hn
ps
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Fig. 1.

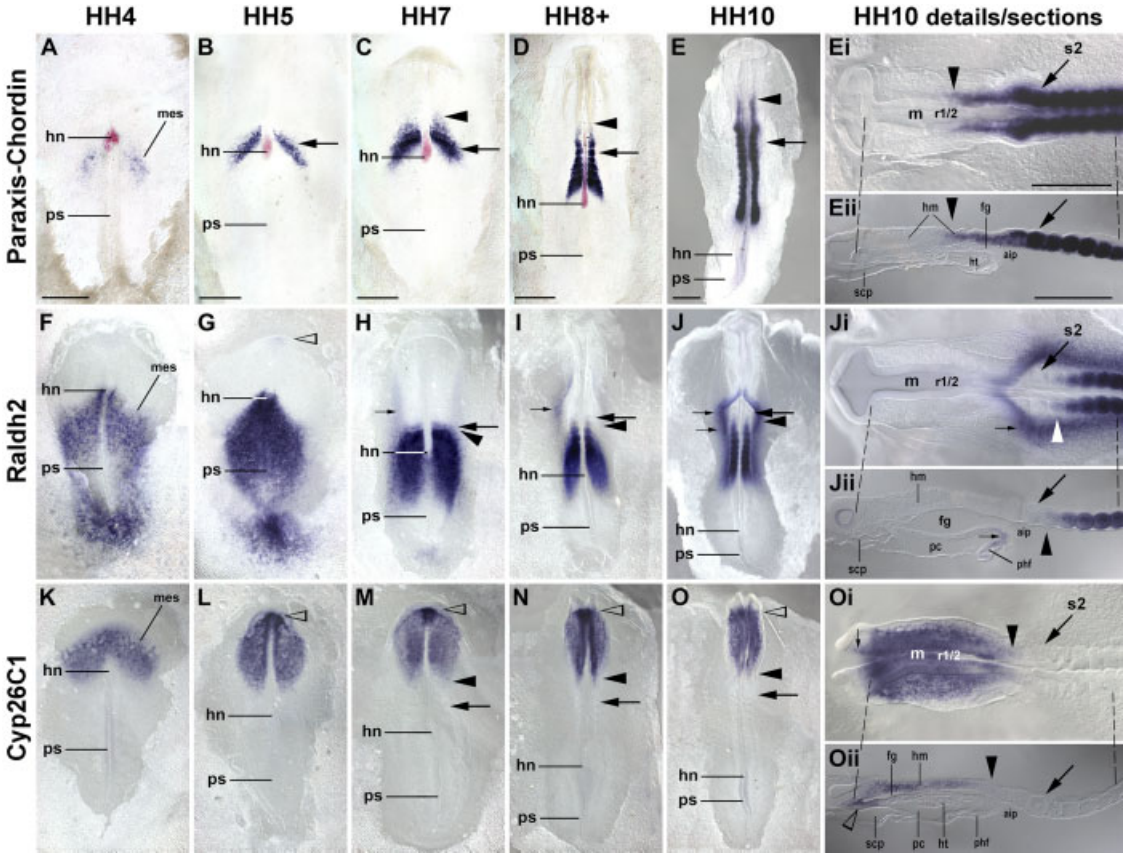
HH4
HH5
HH7
HH8+
HH10
HH10 details/sections
Paraxis-Chordin
Raldh2
Cyp26C1
hn
ps
mes
m
r1/2
s2
hm
fg
ht
aip
scp
pc
phf

Fig. 2.

HH10 (Fig. 1F, open arrowhead). From HH8–HH10, the main cranial expression of *Dll1* resided in the neural tube, with strong expression in the forebrain and more punctuate expression demarcating differentiating neurons (Fig. 1E–Fi), and in the pharynx (Fig. 1Fi, ph). The head mesoderm remained Dll1-negative.

Lfng.

At HH3–4, Lfgn showed a highly dynamic expression in the primitive streak as reported by Jouve et al. (2002). As for *Dll1*, upregulated expression was found in the mesoderm surrounding the node (Fig. 1G, small arrow, and not shown). Posterior to the node, wings of expression continued laterally into the epiblast and mesoderm (Fig. 1G, ep+mes; sections not shown). Between these wings, expression was confined to the lateral aspect of the mesoderm (Fig. 1G, mes). Anterior to the node, the staining encompassed the prospective neural plate (Fig. 1G, np). The head mesoderm was devoid of *Lfgn* staining while the newly formed presomitic mesoderm located lateral and posterior to the node (Fig. 1G, hn) was *Lfng* positive. Expression was similar at HH5–6, with sustained signals within the PSM (Fig. 1H,I, arrows) and in perinodal and peri-chordal cells (Fig. 1H,I, small arrows). From HH7–10 (Fig. 1J–Li), cycling expression continued in the presomitic mesoderm, resolving into a strong stripe of expression at its anterior end (arrowheads). Expression was retained in the anterior half of the developing somites, but was detectable after prolonged staining only (Fig. 1L, open arrowhead). The main expression domain of *Lfgn* in the head resided in the neural plate/neural tube, partially overlapping with the strong expression domain of *Dll1*. However, weak *Lfgn* expression was visible throughout the head mesoderm from HH7–8 onwards (Fig. 1Ji,K), which at HH9–10 became obscured by the comparatively stronger staining in the emigrating neural crest cells (ncc; compare embryos with different signal intensity in Fig. 1L,Li). Nevertheless, the head mesodermal staining was ubiquitously distributed; expression never resolved into stripes associated with segmentation or boundary formation.

EphA4.

Expression of *EphA4* was first found in the primitive streak (Fig. 1M and not shown), fading away at HH5 (Fig. 1N). At this stage, weak expression was seen in the head process (Fig. 1N, hp). Moreover, expression in the anterior neural plate (prospective prosencephalon, p) and in the condensing first somite (arrow) began. This expression intensified at HH6 (Fig. 1O, p, arrow). At HH7, expression was evident in the developing forebrain and in the anterior PSM, here labelling the anterior territory of the condensing somite and the next somite to form (Fig. 1P,Pi, arrowhead). Expression in the forebrain and in the actively developing somites was also seen at HH8–10 (Fig. 1Q–Ri). The number of *EphA4* stripes in the forming somites varied from 2–4 according to the stage of somite formation at the anterior end of the presomitic mesoderm and the intensity of staining, in line with Kulesa and Fraser (2002). From HH8–10, expression commenced in the emerging rhombomeres r3 and r5, and the otic placode (Fig. 1Q–Ri,r3,r5,op). From forebrain to metencephalic levels, expression was also found in the fusing neural folds, the emerging neural crest cells (Fig. 1Ri, ncc), and in the floor plate, while at rhombomere r3–5 levels, the wall of the neural tube was stained (sections not shown). At HH10, staining intensified in the remnant of the primitive streak and the posterior end of the neural plate (Fig. 1R.Ri). However, no staining was ever seen in the head mesoderm, suggesting that boundaries were not established.

Fig. 1. Expression of markers for mesoderm segmentation and boundary formation. **A–Fi:** *Deltal-like 1* (*Dll1*); **G–Li:** *Lunatic Fringe* (*Lfng*); **M–Ri:** *EphA4*. A–D,M–P are double-stained for *Chordin* (red). Dorsal views except in Di, Ji, Pi (parasagittal sections); anterior to the top in all, dorsal to the right in Di, Ji, Pi. Scale bars in A–Fi = 500 μm for respective column. Abbreviations: see list of abbreviations. Large arrows, the level of the second somite in series; small arrows, staining in perichordal cells; arrowheads, the anterior PSM; open arrowheads, staining in the somites. At HH4, expression of all markers is found in the primitive streak, labelling prospective mesodermal cells, including the future head mesoderm generated by the anterior streak. Once the cells left the streak, *Dll1* and *Lfng* expression is shut down in the head mesoderm but continues in the developing presomitic paraxial mesoderm (PSM) and, subsequently, in the somites. In the anterior PSM, expression resolves into stripes (*Dll1*, posterior somite half; *Lfng*, anterior somite half; F,L, open arrowheads) associated with the formation and maintenance of somite boundaries. *EphA4* expression in the PSM commences at HH5 and is always associated with the formation of somite boundaries. Note that at all stages, *Dll1* and *EphA4* are not expressed in the head mesoderm. *Lfng* is expressed at low levels throughout the head mesoderm from HH7–8, and in neural crest cells from HH9–10 onwards.

Fig. 2. Expression of markers for the head and the somitic mesoderm. **A–Eii:** *Paraxis*. **F–Jii:** *Raldh2*. **K–Oii:** *Cyp26C1*. Dorsal views except in Eii, Jii, Oii (parasagittal sections). Anterior to the top in A–E, F–J, K–O; to the left in Ei–ii, Ji–ii, Oi–ii; dorsal to the top in Eii, Jii, Oii. Scale bars in A–E = 500 μm for respective column; in Ei = 500 μm for (Ei, Ji, Oi); in Eii = 500 μm for (Eii, Jii, Oii). Abbreviations: see list of abbreviations. Large arrows, the level of the second somite in series; small arrows, the lateral mesoderm; arrowheads, the anterior expression boundary for *Paraxis*/*Raldh2* and the posterior expression boundary for *Cyp26C1*; open arrowheads, the prechordal mesendoderm. Note that *Paraxis* and *Raldh2* expression is largely confined to the somitic, expression of *Cyp26C1* to the head mesoderm. However, the anterior expression boundaries for the somitic markers and the posterior expression boundary for *Cyp26C1* are ill-defined. Moreover, the boundaries do not align.

Markers Discriminating Between Head and Somitic Mesoderm

So far, our expression analysis showed that, although head mesodermal cells may experience waves of Notch-Delta signalling when they are generated in the primitive streak, this does not result in the manifestation of boundaries. Contrarily, in the occipital region and further down in the trunk, the paraxial mesoderm readily segments. Thus, the head mesoderm and trunk/somitic mesoderm may harbour a different molecular setup. To further investigate this possibility, we studied the expression of markers that had been suggested to discriminate between head and somitic mesoderm, namely

1. The bHLH transcription factor

Paraxis, which is associated with epithelial somite formation (Burgess et al., 1996);
2. the aldehyde dehydrogenase Raldh2, which is required to generate retinoic acid (RA), in turn required for the posteriorisation of tissues and the establishment of Hox/HOM mediated positional values in the neural tube from hindbrain levels to the tail, in the somites, and in the gut endoderm (Burke, 2000; Blentic et al., 2003; Kmita and Duboule, 2003);
3. Cyp26C1, a member of the P450 superfamily of enzymes that converts RA into an inactive metabolite, thereby protecting the anterior region of the embryo from RA- and Hox/HOM mediated posteriorisation (Burke, 2000; Kmita and Duboule, 2003; Reijntjes et al., 2004).

As before, embryos from HH3–10 were analysed (Fig. 2; HH3,6,9 not shown).

Paraxis.

Paraxis expression commenced HH4, labelling the newly generated presomitic mesoderm in a salt-and–pepper pattern (Fig. 2A, mes; red staining for *Chordin*). Expression intensified at HH5–7 when the anterior PSM condensed and formed the first somite (Fig. 2B,C, arrows). At HH8, the condensing mesenchmye of the anterior PSM plus all somites present at this stage expressed *Paraxis*; this pattern was maintained until HH10 and beyond, in line with Barnes et al. (1997), Šošic et al. (1997) (Fig. 2D–Eii). The head mesoderm lacked *Paraxis* expression. However, the anterior boundary for the somitic *Paraxis* expression was ill-defined, with expression tapering into the head mesoderm, in a strongly stained specimen reaching the boundary of met- and myelencephalon (Fig. 2C–Eii, arrowheads).

Raldh2.

In line with earlier reports (Blentic et al., 2003), *Raldh2* expression commenced at HH4 in the anterior primitive streak and the neighbouring, newly generated mesoderm, accompanied by a weaker expression domain in the posterior streak (Fig. 2F,G). At HH5–6, weak expression was found temporarily in the prechordal region (Fig. 2G, open arrowhead). However, the most prominent expression remained in the anterior streak and newly formed mesoderm, including the PSM. The anterior border of expression resided at node levels, suggesting that *Raldh2* staining only partially overlapped with that of *Paraxis* (compare Fig. 2B,G).

At HH7, the anterior boundary of *Raldh2* expression (Fig. 2H, arrowhead) became less distinct as the morphologically defined first somite (arrow) only weakly expressed *Raldh2*, followed by strong expression in the PSM (Fig. 2H). Anteriorly, besides faint expression in the lateral mesoderm (small arrow), no signals were detected. It has to be noted that the "first" somite is the second in series, preceded by a somite with an ill-defined, incompletely epithelialised anterior border (Hamilton and Hinsch, 1956; Huang et al., 1997). Thus, at HH7 the head mesoderm plus the first somite in series were devoid of *Raldh2* expression.

At HH8, expression in the lateral mesoderm intensified, with slightly elevated signals on the left side (Fig. 2I, small arrow). At HH10, this expression lined the inflow tract of the heart, which forms from the posterior end of the primary heart field (reviewed by Brand, 2003; Kelly, 2005), the newly formed somites, and the anterior PSM (Fig. 2J,Jii, small arrows). Paraxial *Raldh2* expression at HH8–10 weakly labelled somites No. 2–3, followed by strong expression in the remaining somites and the anterior PSM (Fig. 2I,J,Ji,Jii; arrow marks somite No. 2). Thus, strong *Raldh2* expression encompassed the lower occipital and all cervical somites, omitting the head mesoderm and anteriormost occipital somites as well as prospective somites further down the trunk.

Cyp26C1.

Cyp26C1 expression commenced at HH4 in the anteriormost mesoderm, just reaching the node (Reijntjes et al., 2004), (Fig. 2K). At HH5, expression was upregulated in the prechordal mesendoderm (Fig. 2L, open arrowhead), but remained unchanged in the paraxial (underneath the neural plate) and lateral/cardiac (lateral to the neural plate) head mesoderm (Fig. 2L). This pattern persisted throughout the stages examined here. At HH10, additional expression of *Cyp26C1* was found in the anterior neural tube (Fig. 2Oi, small arrow). For all expression domains, however, signals ceased at the level of the posterior hindbrain, not encompassing the somitic area (arrowheads). Thus, the anterior expression boundaries of the "somitic markers" are ill-defined and neither align with the border of the first somite nor with each other. Nevertheless, *Cyp26C1* may set up the head mesoderm as distinct, RA-devoid tissue.

Markers for the Repression and Promotion of Myogenic Differentiation

Our analysis showed that markers for mesoderm segmentation, boundary formation, somite epithelialisation, and retinoic acid metabolism were differentially expressed in the head and somitic mesoderm. The most striking feature of the head mesoderm, however, is its delayed myogenic differentiation compared to myogenesis from somites (Noden et al., 1999). To further explore the difference between head and somitic mesoderm and to investigate the molecular basis of the delayed myogenic differentiation in the head, we comparatively analysed the expression of positive and negative regulators of myogenesis, namely

1. the bicoid-type homeodomain transcription factor Pitx2, a promoter of myoblast proliferation and repressor of differentiation in vitro and, besides other processes, a regulator of extraocular muscle and heart development in vivo (Gage et al., 1999; Kitamura et al., 1999; Kioussi et al., 2002; Liu et al., 2002);
2. the paired-type homeodomain containing transcription factor Alx4, required for body wall closure, limb and skull development (Qu et al., 1997). Important for this study, Alx4 is associated with mitotically active somitic muscle precursors, but has to be downregulated prior to myogenic differentiation (Cheng et al., 2004, and unpublished observations);
3. the bHLH transcription factor

and repressor of myogenic differentiation, MyoR, which, together with its paralogue Capsulin, is specifically required for branchiomeric muscle formation (Lu et al., 1999, 2002; Funato et al., 2003; von Scheven et al., 2006b);

4. the T-box containing transcription factor Tbx1, candidate gene for human DiGeorge syndrome and required for the formation of the cardiac outflow tract and branchiomeric muscles (Kelly et al., 2004; Xu et al., 2004). *Tbx1* may directly or indirectly regulate branchiomeric *Myf5* expression (Kelly et al., 2004); however, its main function is a positive control of cell proliferation (Xu et al., 2005);
5. the bHLH transcription factor Twist, a negative regulator of myogenic differentiation (Hebrok et al., 1994, 1997; reviewed in Fuchtbauer, 2002);
6. the paired and homeodomain containing transcription factor Pax3, master regulator of trunk myogenesis upstream of *MyoD* and expressed in all somitic muscle precursors (Tajbakhsh et al., 1997; Tremblay et al., 1998);
7. Myf5, the first of the MDF to be expressed in the embryo, indicating the onset of myogenic differentiation (Saitoh et al., 1993; Kassar-Duchossoy et al., 2004).

Since recent studies established that the head mesoderm contributes to the secondary heart field, which delivers the outflow tract of the heart (reviewed by Brand, 2003; Kelly, 2005), we included in our analysis

8. the homeodomain containing transcription factor Gata3, whose endodermal expression at HH10 demarcates the cardiogenic regions of the head (Sheng and Stern, 1999; this study),
9. the LIM homeodomain containing transcription factor Isl1, which is required for secondary heart field formation, regulating precursor proliferation (Cai et al., 2003),
10. the Nk2-class homeodomain containing transcription factor Nkx2.5, which is essential for heart development (Lyons et al., 1995).

The same stages were analysed as before; here we focus on HH10 embryos as at this stage, somitic myogenesis is well under way (whole heads: Fig. 3; cross-sections: Fig. 4 and 4 continued; the results are summarised in Supplementary Table 1, which can be viewed at www.interscience.wiley.com/jpages/1058-8388/supmat).

Pitx2.

Pitx2 expression labelled the head mesoderm as early as HH6, in line with St Amand et al. (1998). The strongest signals resided underneath the neural folds, overlapping with the expression of *Isl1*. Moreover, expression faded in a posterior direction, not quite reaching the first somite (not shown). At HH10, Pitx2 expression strongly labelled the head mesoderm from diencephalic to mid-myelencephalic levels (Fig. 3A). The most prominent signals resided again laterally, namely in the region covering the lateral edges of the pharyngeal endoderm (Fig. 4Ai–vi). *Pitx2* expression encompassed the head mesodermal expression domain of *MyoR* (Fig. 4Ai–vi, Ci–vi), and overlapped laterally with the domain of *Isl1*, touching the expression domain of *Nkx2.5* (Fig. 4Ai–iii, Ii–iii, Ji–iii; red arrowheads). In a medial direction, *Pitx2* expression overlapped with the *Alx4* expression domain (Fig. 4Ai–iv,Bi–iv), and at myelencephalic levels, with the expression domains of *Tbx1* and *Twist* (Fig. 4Aiv,Div,Eiv). In addition to the expression in the head mesoderm, *Pitx2* labelled the splanchnic/cardiac mesoderm on the left side of the embryo (Fig. 4Ai–vi, splpl) and the prospective oral membrane (Fig. 4Ai).

Alx4.

At HH5, *Alx4* was expressed on either side of the primitive streak and along the posterior margin of the neural plate (not shown). Expression in the head mesoderm commenced at HH8, i.e., later than *Pitx2*, followed by marked expression in the developing somites (not shown). At HH9–10, expression in the somites declined, while the staining in the head mesoderm intensified. In dorsal views, strong expression in the head mesoderm from di- to metencephalic levels and a distinct patch of expression in the roof of rhombomeres 4–7 was evident (Fig. 3B). Cross-sections confirmed the prominent expression in the anterior head mesoderm, strongest along the walls of the neural tube, overlapping with the mesodermal expression domains for *Tbx1* and *Twist* (Fig. 4Bi–v,Di–v,Ei–v). Expression weakened in lateral direction, overlapping with the expression domains of *Pitx2, MyoR*, and reaching the expression domains of the cardiac markers (Fig. 4Bi–iii, red arrowheads). Weak expression was also found in the posterior head mesoderm (Fig. 4Biii–v) and in the somites (Fig. 4Bvi). Notably, the emerging neural crest cells did not yet express the gene, but will be *Alx4* positive later (Takahashi et al., 1998).

MyoR.

MyoR expression commenced at HH9-10, i.e., later than *Pitx2* and *Alx4* expression (von Scheven et al., 2006b and Fig. 3C). The most prominent expression was found in the lateral aspect of the head mesoderm from the diencephalic to upper myelencephalic levels (Fig. 4Ci–iii), with the strongest signals underneath the forebrain and anterior midbrain (Fig. 4Ci). Expression coincided with *Pitx2* expression and overlapped with the expression domains of *Isl1* and at diencephalic levels, with the expression of *Nkx2.5* (Fig. 4Ci–iii, Ai–iii, Ii–iii, Ji–iii, red arrowheads). Further posterior, a weaker *MyoR* expression domain marked the splanchnic aspect of the lateral mesoderm lining the anterior intestinal portal, which will contribute to the inflow tract of the heart (Fig. 4Cv), coinciding with signals for *Isl1* and *Nkx2.5* (Fig. 4Cv,Iv,Jv).

Tbx1.

From HH7–10, mesodermal *Tbx1* signals were found adjacent to the hindbrain (Fig. 3D and not shown). Cross-sections at HH10− showed that the signals resided close to the notochord and floor plate, overlapping with signals for *Alx4* (Fig. 4Di–v, Ai–v) and in line with the regulation of *Tbx1* expression by Shh (Garg et al., 2001; Yamagishi et al., 2003). At para- and post-otic levels, expression broadened, reaching into the splanchic/cardiac mesoderm (Fig. 4Div–v,Iv–vi,Jv–vi; red arrowheads). Besides the head mesoderm, prominent expression was also found in the ventral pharyngeal endoderm, coinciding with *Gata3,*

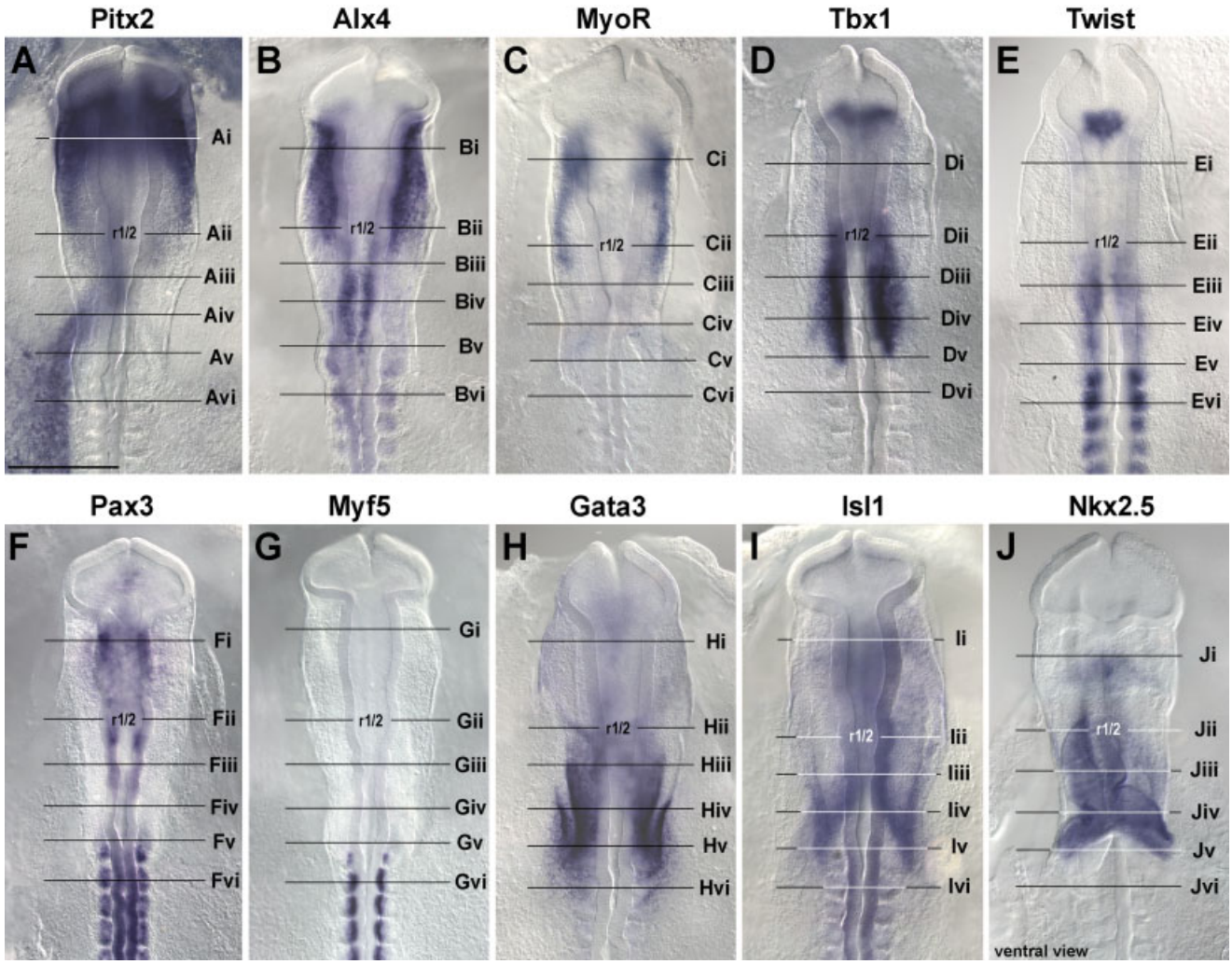

Fig. 3. Expression of repressors and promoters of skeletal muscle differentiation and of cardiac development. HH10 heads with (**A–I**) dorsal views and (**J**) ventral view, anterior to the top, stained for the expression of (A) *Pitx2*, (B) *Alx4*, (C) *MyoR*, (D) *Tbx1*, (E) *Twist*, (F) *Pax3,* (G) *Myf5,* (H) *Gata3*, (I) *Isl1*, (J) *Nkx2.5*. Scale bar in A = 500 μm for all panels. Abbreviations: see list. Note that the head mesoderm is loaded with the known (*Pitx2, MyoR, Twist*) or suspected (*Alx4, Tbx1*) negative regulators of skeletal muscle differentiation and positive regulators of cell proliferation, with *Pitx2, Alx4, MyoR* predominantly labelling the head mesoderm at di-metencephalic levels and *Tbx1* and *Twist* predominantly labelling the tissue at myelencephalic levels. Expression partially overlaps with the expression of cardiac markers (*Gata3, Isl1, Nkx2.5*), while the positive regulators of skeletal myogenesis, *Pax3* and *Myf5*, are absent.

Isl1, and *Nkx2.5* expression (Fig. 4Di–iv, Hi–iv, Ii–iv, Ji–iv); the strongest *Tbx1* signal was found in the territory adjacent to the fusion plate of the bilateral heart rudiments. At HH10+, these *Tbx1* expression domains were accompanied by strong signals in the ectoderm neighbouring the otic placode, and weak expression in the lateral mesoderm flanking the occipital somites (not shown).

Twist.

Twist expression commenced at HH9–10, i.e., later than that of *Tbx1*, strongly labelling the prechordal plate (Fig. 3E and not shown), more weakly labeling the head mesoderm adjacent to di- and mesencephalon (Figs. 3E, 4Eii), more strongly labeling the rhombencephalic head mesoderm (Figs. 3E, 4Eii–v), and then strongly labeling the somites (Figs. 3E, 4Evi). At all axial levels, the most robust expression of *Twist* resided close to the floor plate of the neural tube and the notochord, in the somites labelling the prospective sclerotome. Hence, expression overlapped with that of *Tbx1* (Fig. 4Di–v,Ei–v) and was in line with the established regulation of *Twist* expression through Shh (O'Rourke et al., 2002; Hornik et al., 2004).

Pax3.

Pax3 expression at stages HH5–6 lined the primitive streak and the margins of the developing neural plate, overlapping with *Alx4* expression (not shown). From HH7 onwards, expression was also found in the neural folds at prospective spinal cord levels and in the lateral aspect of developing and newly formed somites underneath (not shown). At HH9–10, the entire neural tube plus the emerging neural crest cells expressed *Pax3* (Figs. 3F,4Fi–vi). Also, all somites expressed *Pax3*, with the expression in mature somites becoming confined to the dorsally located dermomyotome

(Fig. 4Fvi). Interestingly, the somitic expression tapered into the head mesoderm similar to the expression of *Paraxis* (Fig. 4F,Fv), yet the remainder of the head mesoderm was negative of *Pax3*, in line with studies on older embryos (Hacker and Guthrie, 1998; Mootoosamy and Dietrich, 2002).

Myf5.

Expression of *Myf5* was confined to the medial wall of the somites, the site of epaxial myogenesis that delivers the deep muscles of the back (reviewed by Buckingham et al., 2003; Figs. 3G, 4Gvi). At all axial levels, the head mesoderm showed no *Myf5* expression (Fig. 3E, 4Ei–Ev; Noden et al., 1999).

Gata3.

Gata3 expression commenced at HH4, up to HH7, demarcating the prospective surface ectoderm adjacent to the neural folds (Sheng and Stern, 1999, and data not shown). Upon neurolation, this staining persisted in the ventral ectoderm underneath the forebrain that faced the subcephalic pocket (Figs. 3H,4Hi). At HH10, additional ectodermal staining was found in the para and post-otic region overlying the paraxial and medial-most lateral mesoderm (Fig. 4Hiv–Vi). Further into the occipital region, this staining declined while expression in the intermediate mesoderm began (not shown). At HH8, expression began in the foregut endoderm (Sheng and Stern, 1999; and not shown), which developed into the most intense expression domain at HH9–10 (Figs. 3H,4Hi–v). However, at these stages, expression was confined to the lateral and ventral territories only. Thus, at HH9–10 expression lined the cardiogenic mesoderm (secondary heart field; reviewed by Brand, 2003; Kelly, 2005), here overlapping with signals for *Tbx1, Isl1,* and *Nkx2.5* (Fig. 4Di–v,Hi–v,Ii–v,Ji–v).

Isl1.

Isl1 expression in both the mesoderm and endoderm medially lined the cardiac crescent from HH4 onwards (Yuan and Schoenwolf, 2000, and not shown). At HH10, expression was found in the endoderm and ectoderm of the oral plate and in the ventral pharyngeal endoderm, overlapping with signals for *Gata3, Nkx2.5*, and *Tbx1* (Figs. 3I,4Ii–iv, Hi–iv, Ji–iv, Di–iv). At hindbrain levels, *Isl1* labelled the splanchnic/cardiac mesoderm, dorsally overlapping with the expression domains of *Pitx2, Alx4, MyoR*, and *Tbx1* (Fig. 4Ii–v, A–Di–v; red arrowheads), and ventrally coinciding with *Nkx2.5* (Fig. 4Ji–v; red arrowheads). In addition, *Isl1* expression was found in the neural tube and in the emerging neural crest cells, overlapping with signals for *Pax3* (Fig. 4Ii–vi, Fi–vi).

Nkx2.5.

Nkx2.5 labeled cardiac cells from HH4 onwards (Schultheiss et al., 1995, and not shown). At HH10, expression encompassed the tubular heart and the splanchnic mesoderm posterior to it, which belongs to the primary heart field but was not yet incorporated into the inflow tract (Figs. 3J,4Jv). Notably, lateral and anterior to the heart, expression continued in the splanchic mesoderm overlapping with signals for *Isl1* and reaching as far dorsally as the lateral pockets of the foregut (Fig. 4 J,I, I–iv; red arrowheads). Moreover, expression was found in the endoderm and ectoderm of the oral membrane (Fig. 3J,4Ji), and in the ventral pharyngeal endoderm, overlapping with the expression of *Tbx1, Gata3, Isl1* (Fig. 4D,H,I,J,i–iii).

In summary (see Supplementary Table 1), the known (*Pitx2, MyoR, Twist*,) or putative (*Alx4, Tbx1*) promoters of cells proliferation/repressors of muscle differentiation were all expressed in the head mesoderm, with *Pitx2, Alx4, and MyoR* signals dominating more anterior territories and *Tbx1* and *Twist* signals dominating more posterior territories. Moreover, *Alx4, Tbx1*, and *Twist* labelled more medial territories, and *Pitx2* and *MyoR* labeled more lateral territories. None of the positive regulators of myogenesis (*Pax3, Myf5*) was expressed in the head mesoderm. In contrast, the somites harboured all positive regulators of myogenesis, while amongst the negative regulators, only *Twist* was present, its expression largely confined to the sclerotome. Significantly, expression of *Pitx2, Alx4, MyoR*, and *Tbx1* extended into the region expressing cardiac markers and actively contributing to the heart.

DISCUSSION

In a number of muscular dystrophies, head and trunk muscles are differentially affected (reviewed by Emery, 2002; Spence et al., 2002). However, the cause of this phenomenon and its implication for therapeutic approaches is not known. This is due to the fact that, while muscle formation in the trunk, i.e., from the segmented paraxial mesoderm known as somites, is fairly well characterised, little is known about muscle formation in the head (reviewed in Buckingham et al., 2003).

To investigate the molecular setup of the head mesoderm that may be responsible for the distinct differentiation programmes and susceptibility to muscular dystrophies, we investigated the distribution of markers for mesoderm segmentation and boundary formation, markers thought to distinguish head and somitic mesoderm, and markers for the repression or promotion myogenic differentiation. We found that, indeed, a host of markers is differentially expressed. Most importantly, the head mesoderm is loaded with negative regulators for myogenic differentiation while positive regulators are absent. Expression of the myogenic repressors overlaps with the expression of cardiac markers and reaches into the region that secondarily contributes to the heart. Thus, it is possible that myogenesis in the head may be delayed to guarantee the availability of cells for the heart.

The Head Mesoderm Is a Distinct Type of Mesoderm

In the adult, all skeletal muscles harbour the same contractile apparatus (reviewed by Alberts et al., 1983). However, evidence is accumulating that craniofacial muscles arising from the head mesoderm employ a head-specific developmental programme (Mootoosamy and Dietrich, 2002; Tzahor et al., 2003). Our expression analysis reinforces the notion that the head mesoderm is a distinct tissue. The markers investigated here showed different expression patterns in the head mesoderm and the adja-

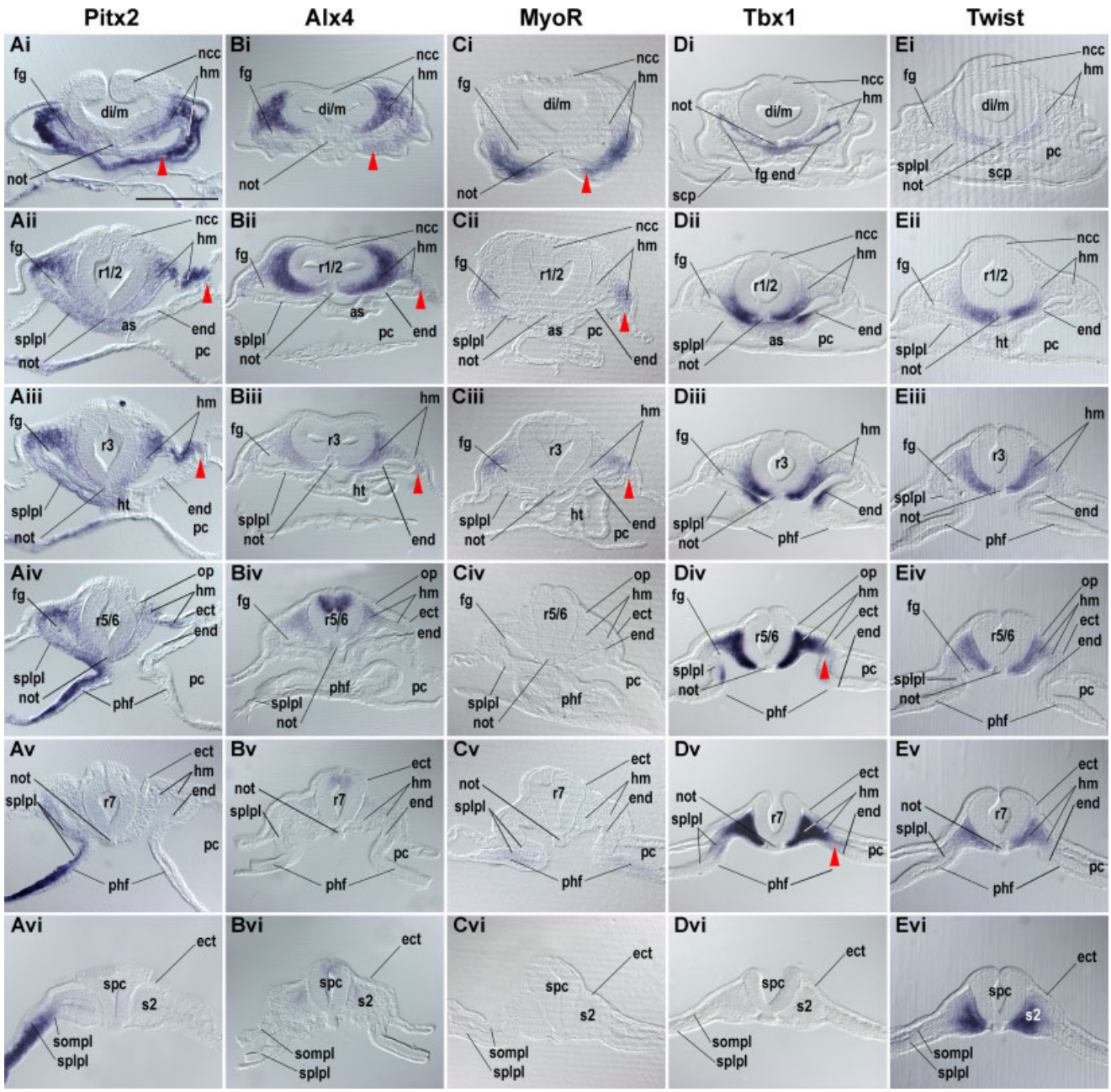

Fig. 4. Vibratome cross-sections of the embryos shown in Figure 3. **Ai–Avi:** *Pitx2*; **Bi–Bvi:** *Alx4*; **Ci–Cvi:** *MyoR*; **Di–Dvi**: *Tbx1*; **Ei–Evi:** *Twist*; **Fi–Fvi:** *Pax3*; **Gi–Gvi:** *Myf5*; **Hi–Hvi:** *Gata3*; **Ii–Ivi:** *Isl1*; **Ji–Jvi:** *Nkx2.5*. Dorsal to the top; scale bar in Ai = 250 μm for all panels. For abbreviations, see list. Level of the sections is indicated in Figure 3 with: (i) posterior diencephalon/anterior mesencephalon; (ii) metencephalon (future rhombomeres 1 and 2); (iii) rhombomeres 3–4; (iv) rhombomeres 5–6 (otic level); (v) rhombomere 7 (posterior end of head mesoderm); (vi) anterior spinal cord (formerly r8)/2nd somite. Note that the known or putative repressors of myogenesis/promoters of cell proliferation show distinct but anteroposteriorly and dorsoventrally overlapping expression domains. Significantly, expression reaches into ventral regions that contribute to the heart, demarcated by *Tbx1, Gata3, Isl1, Nkx2.5* expression in the endoderm and *Isl1, Nkx2.5* expression in the splanchnic mesoderm. The region of overlap for the head mesoderm and cardiac markers is indicated by a red arrowhead. Also note that *Pax3* and *Isl1* show overlapping expression in the developing neural crest cells and in somites, with the somitic expression tapering into the head mesoderm as seen for *Paraxis*.

cent occipital somites, which in turn shared their expression profiles with somites in the trunk. Head mesoderm markers were *Cyp26C1, Pitx2, MyoR*, and *Tbx1*, while *Notch1, Dll1, EphA4, Raldh2, Paraxis, Pax3*, and *Myf5* were restricted to the presomitic or somitic mesoderm. Importantly, Cyp26C is a retinoic acid (RA) inactivating enzyme while Raldh2 is an enzyme involved in generating active RA (Blentic et al., 2003; Reijntjes et al., 2004, and references therein). Since RA initiates the Hox/HOM system, which in turn confers "trunk" axial identities, it is conceivable that the head mesoderm is set up as a RA/Hox-free territory (Burke, 2000; Kmita and Duboule, 2003). In the trunk, Hox genes control the choice between the programmes for migratory and non-migratory mus-

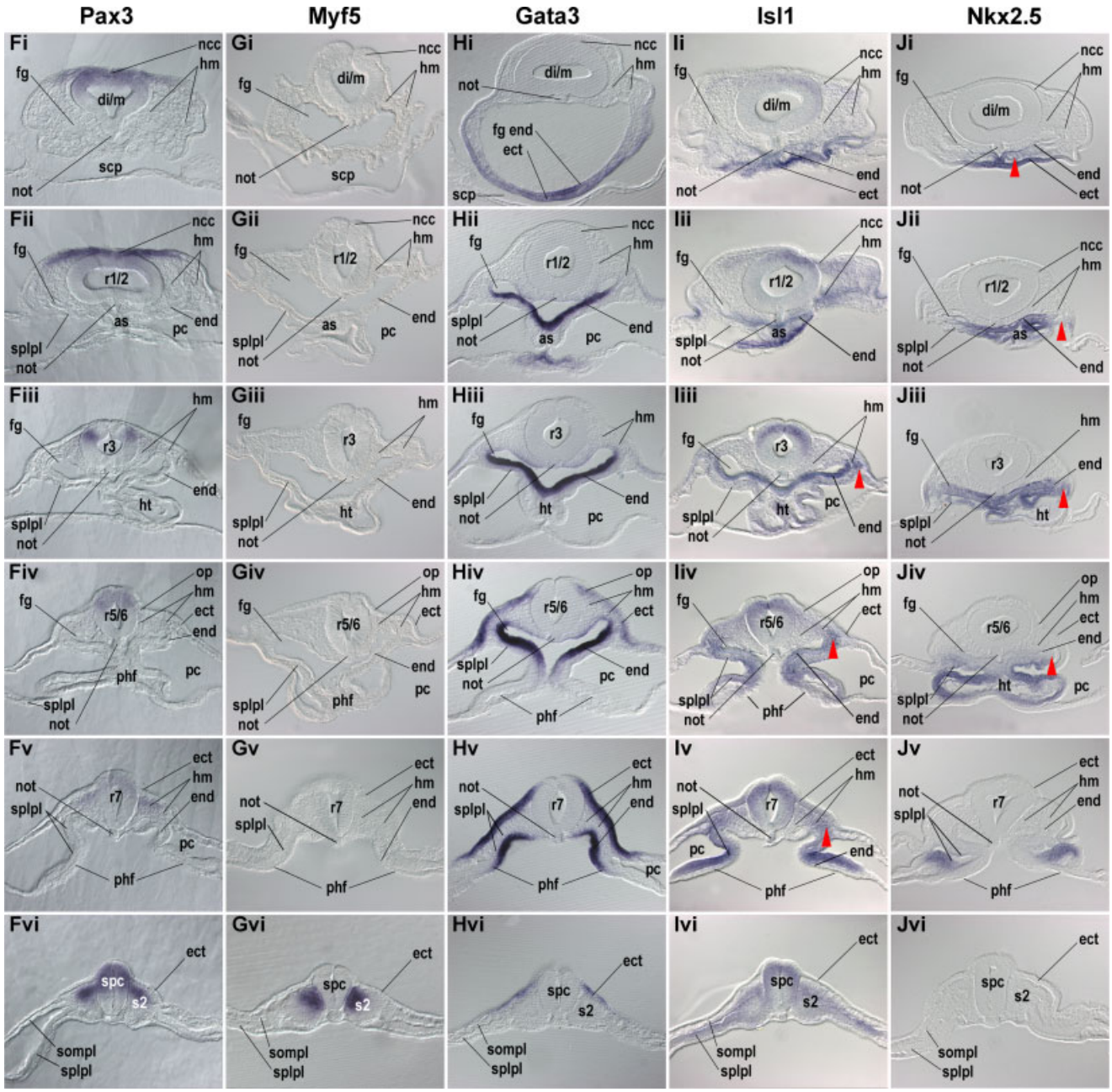

Fig. 4.

cle precursors (Alvares et al., 2003). Thus, it is possible that the absence or presence of the Hox system may, respectively, regulate the choice between head and trunk programmes of myogenesis.

Although the markers analysed here were largely confined to either head or somitic mesoderm, many showed ill-defined expression boundaries. Moreover, these boundaries were not always aligned with the border between head mesoderm and first somite (our study). For example, *Paraxis* and *Pax3* expression tapered from the somtic region into the head mesoderm, *Cyp26C1* expression faded away posteriorly, not quite reaching the first somite, *Raldh2* expression began weak, showing robust expression at about the level of the second somite. Moreover, the border between head mesoderm and first somite itself is morphologically poorly defined: the anterior wall of the first somite incompletely epithelialises and, hence, the first somite is partially attached to the head mesoderm (Hamilton and Hinsch, 1956; Huang et al., 1997). As a consequence, it cannot be excluded that cells molecularly set up as somitic may venture into the head mesoderm and vice versa.

Studies on violators of segmental boundaries in the hindbrain suggested that cells that trespassed into an alien rhombomere either adapt to their environment or apoptose (Fraser et al., 1990; Birgbauer and Fraser,

TABLE 1. Head Mesoderm Expressed Genes Controlling Skeletal Myogenesis and Heart Development

	Positive regulators of cell proliferation/ negative regulators of myogenic differentiation								Positive regulators of myogenic differentiation				Regulator of 2nd heart field development	
Makers	*Pitx2*		*Alx4(+)*		*MyoR*		*Tbx1*		*Twist*		*Pax3*		*Myf5*		*Isl1*	
territory	medial	lateral	medial	lateral	medial	lateral	medial	lateral	medial	lateral	medial	lateral	medial	lateral	medial	lateral
axial level																
di- and mesencephalon	+	+++	+++	+		+++			+							
metencephalon: r1 + r2	+	++	+++	+		++	++		++							
myelencephalon: r3 + r4	+	++	+	+		++	++		++							++
myelencephalon: r5 + r6 (i.e. otic level)	+	++	+	+			+++	+	++	+						++
myelencephalon: r7 (i.e. posterior end of head mesoderm)			+	+			+++	++	++	+						++
occipital somites			+	+					+++(*)		+++	++	+++		+	+

(+) suspected negative regulator of myogenesis as expression is cleared from differentiating somitic myoblasts
(*) expression primarily in the developing sclerotome
Note that the head mesoderm is loaded with negative regulators of myogenic differentiation. Expression overlaps with the expression of the secondary heart field marker *Isl1*.
Strong expression of positive regulators for myogenesis is confined to the occipital somites.

1994). Similarly, individual neural crest cells introduced into the mesoderm at a heterotopic axial location integrate or perish (Trainor and Krumlauf, 2000; Schilling et al., 2001). Notably, the head mesoderm remains mesenchymal, despite some posterior expression of *Paraxis*, the key regulator of somite epithelialisation (Burgess et al., 1996). Likewise, although expression of the master regulator for somitic myogenesis, *Pax3* (Tajbakhsh et al., 1997; Tremblay et al., 1998), reaches into the head mesoderm, the head mesoderm does not follow the somitic differentiation profile (see below). Moreover, expression boundaries for *Paraxis* and *Pax3* sharpen over time (unpublished observations). Thus, it is conceivable that somitic cells that find themselves in a head mesodermal environment or, alternatively, head mesodermal cells in the somite, will eventually correct this mismatch.

The Head Mesoderm Is Unsegmented

Our expression analysis suggested that, in principal, head and somitic mesoderm are set up as distinct types of tissues. Moreover, it is now generally accepted that although some vertebrate species may develop head cavities, these are not somites (reviewed in Wachtler and Jacob, 1986; Kuratani, 2005). However, this does not exclude an organisation of the head mesoderm into reiterated but morphologically concealed segments, reminiscent of the incompletely epithelialised segments found in murine *Paraxis* mutants (Burgess et al., 1996); indeed, the organisation of the head mesoderm into seven somitomeres has been proposed (Meier, 1979; Anderson and Meier, 1981; Meier and Tam, 1982). In the occipital region and in the trunk, segmentation is brought about by a molecular clock, which, under the control of the Fgf8 maturation gradient, orchestrates the regular formation of boundaries at the anterior end of the presomitic mesoderm (reviewed by Dubrulle and Pourquie, 2004). Notably, components of this clock, most prominently genes acting in the Notch-Delta signalling system, are expressed in the head mesoderm precursors when they are gen-

erated in the primitive streak: *Hairy1* and *Lfgn* expression oscillates twice during head mesoderm formation (Jouve et al., 2002), while *Dll1* and *Notch1* are stably expressed in these cells (Henrique et al., 1995; this study). It, therefore, has been proposed that the head mesoderm subdivides into two segments, the prechordal and paraxial head mesoderm (Jouve et al., 2002). However, while in the presomitic mesoderm cycling gene expression continues, this is not the case for the head mesoderm (this study). Moreover, upon clock arrest, the expression of Notch-Delta signalling components becomes allocated to either the anterior or posterior territory of the developing segments, which, together with the activation of additional factors, controls boundary formation and maintenance (Jouve et al., 2002; reviewed in Dubrulle and Pourquie, 2004). In the head mesoderm, segmentation genes are either absent (*Notch, Dll1, EphA4*) or ubiquitously expressed at low levels (*Lfng*). This suggests that the earlier expression of segmentation genes in the primitive streak is not translated into segmental boundaries.

Formally, it cannot be excluded that a distinct, yet to be identified molecular mechanism is in operation in the head mesoderm to bring about some cryptic segmentation. If this was the case, then head mesoderm segments would not be serially homologous with somites. However, it is unlikely that during vertebrate evolution first a distinct segmentation programme was installed in the head mesoderm, which then disappeared without a trace in extant species. Moreover, it is now recognised that urochordates such as tunicates and vertebrates are the most closely related chordates, both having segmented mesoderm confined to trunk and tail while the cranial mesoderm, prior to the formation of the branchial arches and gill slits, is not overtly segmented (Goodrich, 1958; Delsuc et al., 2006). This suggests that an unsegmented cranial mesoderm is a primitive character of "olfactoria" (Delsuc et al., 2006).

In the trunk, the second somite to form aids the establishment of boundaries for the actively detaching somite in front (Sato et al., 2002). This may not be possible at the onset of somitogenesis, since at this stage only limited material has been delivered into the PSM through gastrulation (Garcia-Martinez and Schoenwolf, 1992; Schoenwolf et al., 1992; Psychoyos and Stern, 1996). More importantly even, formation and maintenance of segmental boundaries require Notch-Delta signalling across the intersomitic boundaries (reviewed by Dubrulle and Pourquie, 2004) As the components of this signalling system are absent from the head mesoderm, this reciprocal signalling is disabled. This may then account for the ill-defined boundary between the head mesoderm and the first of the occipital somites.

The Head Mesoderm Is Regionalised

While no evidence for the establishment of segmental boundaries was found, we discovered that one set of molecular markers (*Pitx2, Alx4, MyoR*) labelled the head mesoderm from di- to metencephalic levels, and the second (*Tbx1, Twist*) marked the head mesoderm adjacent to the hindbrain proper, with both sets overlapping at the level of the metencephalon. This suggests that the head mesoderm is regionalised. As discussed below, the five markers are all involved in the control of proliferation versus differentiation, and hence, may have the same biological role in head mesoderm development. Moreover, eventually, *Pitx2, MyoR*, and *Tbx1* will be expressed in muscle anlagen developing from all head mesodermal regions (Mootoosamy and Dietrich, 2002; Kelly et al., 2004; von Scheven et al., 2006a,b). However, it is remarkable that the anterior head mesoderm is fated to deliver the extraocular muscles, while the posterior region generates the muscles of the first three branchial arches; both regions overlap at the level of rhombomere 1 and 2, as does the expression of the molecular markers (Noden, 1983; Jacob et al., 1984; Wachtler et al., 1984; Couly et al., 1992; Evans and Noden, 2006; reviewed by Wachtler and Jacob, 1986; Kuratani, 2005). Thus, it is possible that regionalised head mesoderm is predisposed towards either extraocular or branchiomeric muscle development.

The Early Head Mesoderm Is in a Myo-Repressed State

Somites begin their myogenic differentiation soon after they detached from the PSM (reviewed by Buckingham et al., 2003); in the occpital somites, myogenesis is well under way at HH10 (Noden et al., 1999; this study). In the head mesoderm, in contrast, in addition to the absence of posteriorising RA signalling and segmentation, genes that positively regulate skeletal muscle formation are silent for a prolonged period (*Myf5*; Noden et al., 1999; Kassar-Duchossoy et al., 2004) or are never expressed (*Pax3*; Hacker and Guthrie, 1998; Mootoosamy and Dietrich, 2002; this study). Moreover, the known (*Pitx2, MyoR, Tbx1, Twist*; Hebrok et al., 1994, 1997; Lu et al., 1999; Kioussi et al., 2002; Xu et al., 2005) or suspected (*Alx4*; Cheng et al., 2004) stimulators of cell proliferation and repressors of differentiation were strongly expressed in the head mesoderm, but not in the somites (with the exception of *Twist* expression in the sclerotome). Anteriorly, *Alx4* labelled more dorsomedial territories and *Pitx2* and *MyoR* labeled more ventrolateral territories. Posteriorly, *Tbx1* expression extended further ventrolaterally than the expression of *Twist*. However, the markers showed significant overlap in the mediolateral extent of their expression. Thus, the net result is a massed expression of negative regulators of skeletal muscle differentiation in the head mesoderm, while the somitic mesoderm harbours promoters of myogenic differentiation (see Supplementary Table 1). This suggests that the head mesoderm is actively withheld from myogenic differentiation.

The Repression of Skeletal Myogenesis May Make Cells Available to the Heart

Recent studies have established that the primary, tubular heart contains the anlage for parts of the ventricles only (reviewed by Brand, 2003; Kelly, 2005). The inflow tract is added via the incorporation of the posteriorly adjacent splanchnic mesoderm, which is part of the primary heart field. However, the outflow tract is added via the incorporation of the anteriorly

located splanchnic and neighbouring head mesoderm, which are not part of the primary heart field. Yet the entire head mesoderm can be triggered to express cardiac markers when treated with BMP molecules, suggesting that it has cardiac potency (Tzahor and Lassar, 2001). Muscle development relies on the deployment of a sufficient number of precursor cells through cell proliferation (reviewed by Buckingham et al., 2003). Likewise, a prerequisite for secondary heart field and outflow tract development is cell proliferation (Cai et al., 2003). Here we show that the head mesoderm is loaded with positive regulators of cell proliferation and negative regulators of differentiation. Moreover, expression reaches into the secondary heart field, overlapping with the expression of *Isl1* (Yuan and Schoenwolf, 2000; Cai et al., 2003; this study). This suggests that head mesoderm and splanchnic mesoderm are continuous. Moreover, it suggests that the head mesoderm may be kept in a proliferative, myo-repressed state not only to deliver a sufficient number of skeletal muscle precursors, but also to make cells available to the heart.

EXPERIMENTAL PROCEDURES

Chick Embryos

Fertilised hen's eggs were obtained from Winter Farm (Royston) and incubated at 38.5°C in a humidified incubator. Embryos were staged according to Hamburger and Hamilton (1951).

In Situ Hybridisation

Whole mount in situ hybridisation was carried out as previously described (Dietrich et al., 1997, 1998; Mootoosamy and Dietrich, 2002). Probes are detailed in: Alx4 (Takahashi et al., 1998), Chordin (Chapman et al., 2002), Cyp26C1 (Reijntjes et al., 2004), Dll1 (Henrique et al., 1995), Gata3 (Sheng and Stern, 1999), Isl1 (Tsuchida et al., 1994), Lunatic Fringe (Laufer et al., 1997), Myf5 (Saitoh et al., 1993), MyoR (von Scheven et al., 2006b), Nkx2.5 (Schultheiss et al., 1995); Notch1 (Henrique et al., 1995), Paraxis (Šošic et al., 1997), Pax3 (Goulding et al., 1993), Pitx2 (Yoshioka et al., 1998); Raldh2 (Blentic et al., 2003), Tbx1 (Garg et al., 2001), Twist (Scaal et al., 2001). The EphA4 probe is an unpublished PCR fragment kindly provided by F. Schubert.

Sectioning

Embryos were embedded in 20% gelatin (Sigma) in PBS at 50°C, then cooled to 4°C. Subsequently, blocks were trimmed and fixed in 4% PFA for up to 2 days, then rinsed in PBS and sectioned to 50 μm on a Pelco 1000 Vibratome. Sections were collected on gelatinised slides and mounted in either 80% glycerol/PBS or Aquamount (BDH).

Photomicroscopy

After in situ hybridisation, embryos were cleared in 80% glycerol/PBS. Embryos and sections were photographed on a Zeiss Axiophot, using Nomarski optics. The identification of anatomical structures followed Schoenwolf (2001), modified according to Brand (2003) and Kelly (2005) to accommodate for the now established secondary heart field.

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