Dynamic control of head mesoderm patterning

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SUMMARY
The embryonic head mesoderm gives rise to cranial muscle and contributes to the skull and heart. Prior to differentiation, the tissue is regionalised by the means of molecular markers. We show that this pattern is established in three discrete phases, all depending on extrinsic cues. Assaying for direct and first-wave indirect responses, we found that the process is controlled by dynamic combinatorial as well as antagonistic action of retinoic acid (RA), Bmp and Fgf signalling. In phase 1, the initial antero-posterior (a-p) subdivision of the head mesoderm is laid down in response to falling RA levels and activation of Fgf signalling. In phase 2, Bmp and Fgf signalling reinforce the a-p boundary and refine anterior marker gene expression. In phase 3, spreading Fgf signalling drives the a-p expansion of MyoR and Tbx1 expression along the pharynx, with RA limiting the expansion of MyoR. This establishes the mature head mesoderm pattern with markers distinguishing between the prospective extra-ocular and jaw skeletal muscles, the branchiomiceric muscles and the cells for the outflow tract of the heart.

KEY WORDS: Head mesoderm, Discrete phases of patterning, Head muscle, Heart, Pitx2, Alx4, MyoR, Tbx1, Fgf, Bmp, Retinoic acid, Combinatorial and antagonistic signalling, Molecular network, Chick

INTRODUCTION
The vertebrate head mesoderm is the unsegmented paraxial mesoderm lateral to the developing brain, stretching from forebrain to otic levels (for reviews, see Bothe et al., 2007; Noden and Francis-West, 2006). It generates key elements of the craniofacial musculature (Couly et al., 1992; Jacob et al., 1984; Noden, 1983; Wachtler et al., 1984) (for reviews, see Bothe et al., 2007; Noden and Francis-West, 2006). This includes the eye, jaw, face and upper throat muscles that are crucial for food uptake and eye movement, contribute to respiration and, in humans, facilitate speech. Recent studies established that the head mesoderm is laterally continuous with the cardiac mesoderm and delivers cells to the outflow tract of the heart (Tirosh-Finkel et al., 2006) (for reviews, see Rochais et al., 2009; Tzahor, 2009). Moreover, defects in head mesoderm development have been associated with craniofacial muscle and heart defects in humans and mice (for reviews, see Baldini, 2002; Bothe et al., 2007; Noden and Francis-West, 2006; Rochais et al., 2009). The head mesoderm has, therefore, moved into the focus of biomedical research.

As the head mesoderm resides anterior to the somites, the segmented paraxial mesoderm of the trunk, it was thought to represent a type of somitic mesoderm that has lost its segmental organisation (for a review, see Kuratani et al., 1999). However, factors that control somite formation in the trunk are absent in the head (Bothe and Dietrich, 2006). The key regulator of somitic myogenesis, Pax3, is not expressed in the head mesoderm either (Bothe and Dietrich, 2006; Hacker and Guthrie, 1998; Mootoosamy and Dietrich, 2002). Within the MyoD family of muscle determination factors, Mrf4 can compensate for the absence of Myf5 or MyoD in somitic, but not head, muscle formation (Kassar-Duchossoy et al., 2004). Head mesoderm myogenesis depends on the head environment, and signalling molecules that trigger somitic myogenesis suppress muscle formation in the head (Mootoosamy and Dietrich, 2002; Tzahor et al., 2003). Thus, evidence is accumulating that the head mesoderm is a distinct mesodermal tissue.

Recent studies revealed that the head mesoderm expresses a unique set of marker genes (Bothe and Dietrich, 2006). These genes, Pitx2, Alx4, MyoR (musculin) and Tbx1, label and pattern the tissue prior to the onset of differentiation; they also distinguish head-derived muscle satellite cells (adult muscle stem cells) from satellite cells in the trunk (Harel et al., 2009; Sambasivan et al., 2009). Pitx2, MyoR and Tbx1 have been shown to keep cells in an undifferentiated, proliferative precursor state, but contribute to the onset of myogenesis in a similar fashion as Pax3 in the somite (Kiousi et al., 2002; Lu et al., 1999; Martinez-Fernandez et al., 2006; Sambasivan et al., 2009; Xu et al., 2004). Mutations of the genes cause specific defects in the head musculature and outflow tract of the heart (Ai et al., 2006; Dong et al., 2006; Gage et al., 1999; Kelly et al., 2004; Kitamura et al., 1999; Liu et al., 2002; Lu et al., 2002; Nowotschin et al., 2006; Shih et al., 2007; Vitelli et al., 2002a; Vitelli et al., 2002b; Xu et al., 2004) (for reviews, see Baldini, 2002; Bothe et al., 2007; Noden and Francis-West, 2006; Rochais et al., 2009). Thus, the head mesoderm genes are crucial upstream regulators, and their correct deployment is an essential step in head muscle and heart formation.

Several studies have addressed the control of head marker gene expression, but reached controversial interpretations. Retinoic acid (RA) has been proposed to negatively regulate Tbx1 expression and to set the posterior boundary of the heart field (Keegan et al., 2005; Roberts et al., 2005; Ryckebusch et al., 2008; Sirbu et al., 2008). Yet, RA has also been shown to be required for the development of the posterior, sinoatrial region of the heart (Hochgreb et al., 2003)
(for a review, see Rochais et al., 2009). Bone morphogenetic (Bmp) molecules have been shown to recruit head mesoderm cells to the heart (Tirosh-Finkel et al., 2006) (for reviews, see Rochais et al., 2009; Tzahor, 2009). Yet, Bmp molecules can repress myogenic differentiation without the activation of cardiac markers (von Scheven et al., 2006a). Fibroblast growth factors (FgfS) have been placed upstream of both Tbx1 and MyoR (Abu-Issa et al., 2002; Mitsiadis et al., 2008; von Scheven et al., 2006a). However, initially Tbx1 and MyoR are expressed in different anteroposterior domains (Bothe and Dietrich, 2006). Most of the experiments addressing the control of head mesodermal gene expression have been performed at late neurulation (HH8-9) or early organogenesis stages (HH10-12), and phenotypes were analysed at pharyngula stages of development (HH18-20) when the formation of the heart as well as individual muscle anlagen is well under way. However, head mesoderm development begins with gastrulation at HH3-4. During the entire period, the topology of tissues and signalling centres changes dramatically. Moreover, evidence is emerging that the reduction of RA plus initiation of Fgf signalling in the posterior head mesoderm. In the second phase, the anterior pattern is refined when Alx4 and MyoR are activated in response to rising Bmp and Fgf levels; Bmp also sets the anterior boundary of Tbx1 expression. In the posterior domain, increasing Fgf levels reinforce Tbx1 expression and determine the posterior boundary of Pitx2 and Alx4 expression. In the third phase, Fgf signals spread along the pharynx, driving the anterior extension of Tbx1 and the posterior extension of MyoR expression, the rate of the latter set by the further reduction of RA. This leads to the final pattern of combinatorial marker gene expression with Pitx2 labelling the precursors of the extra-ocular and mandibular arch musculature, MyoR and Tbx1 labelling the precursors of all branchiomeric muscles, and all three markers labelling the region that also contributes to the outflow tract of the heart.

MATERIALS AND METHODS

Chicken embryos

Fertilised chicken eggs (Gallus gallus) were obtained from Winter Farm (Royston, UK) and Henry Stewart & Co. (Lincolnshire, UK) and incubated at 38.5°C in a humidified incubator (LMS) to the desired stages. For head implantation experiments, embryos were recovered from the eggs, placed on filter rings (ventral side up) and cultured on albumen-agar dishes at 38.5°C in a humidified incubator as described by Chapman (Chapman et al., 1998; Dietrich et al., 1997). Beads were placed next to the anterior end of the notochord to target the anterior head mesoderm, or anterior of the node (HH4-6) or anterior to the anterior-most somite (HH8) to target the posterior head mesoderm. The embryos were incubated for 5 hours to reach HH6, HH7-8 and HH9-10 and to allow for the response of direct and the first wave of indirect targets of the signalling pathways. Timing and position of bead implantations is summarised in Fig. S5 in the supplementary material.

In situ hybridisation and vibratome sectioning

Whole-mount in situ hybridisation and vibratome sectioning was carried out as previously described (Dietrich et al., 1998; Dietrich et al., 1997; Mootooasy and Dietrich, 2002). Details of probes have been published previously: Alx4, chordin, Cyp26C1, Iis1, Myf5, MyoR, Nkx2.5, paraxis, Pax3, Pitx2, Radh2, Tbx1 and Twist (see Bothe and Dietrich, 2006); Bmp2, Bmp7, Fgf4, Fgf8 and Fgf10 (see Lours and Dietrich, 2005; von Scheven et al., 2006a); Bmp4 (see Francis et al., 1994); follistatin and noggin (see Chapman et al., 2002); Hoxb1 (see Bell et al., 1999); Mkp3 (Pyst) (see Eblagie et al., 2003).

Photomicroscopy and image analysis

All specimens were photographed on a Zeiss AxiosScope2 microscope using Nomarski optics. Images were captured with a Zeiss AxioCam digital camera with AxioVision 3.0 software and processed using Adobe Photoshop 6.0 or CS.

RESULTS

Dynamics of head mesoderm marker gene expression

To determine the onset and dynamics of head mesoderm patterning, we systematically investigated marker gene expression in the chicken embryo from the stage a which the head mesoderm is laid down at HH4 to mid-pharyngula stages of development at HH16 when myogenic differentiation is underway (Noden et al., 1999) (Fig. 1).

Initially, the head mesoderm did not express any of its marker genes (data not shown for HH4; Fig. 1Ai-Ei for HH5). At HH6, the anterior head mesoderm expressed Pitx2 (Fig. 1Aii), the posterior head mesoderm Tbx1 (Fig. 1Dii). Twist was also expressed, with elevated levels in the posterior head mesoderm and the developing somites (Fig. 1Eii). Overall, expression was more complex as the pharynx, driving the anterior extension of Tbx1 and the posterior extension of MyoR expression, the rate of the latter set by the further reduction of RA. This leads to the final pattern of combinatorial marker gene expression with Pitx2 labelling the precursors of all branchiomeric muscles, and all three markers labelling the region that also contributes to the outflow tract of the heart.

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expression of MyoR spread posteriorly and that of Tbx1 anteriorly such that at HH13-14, the mesoderm of all prospective pharyngeal arches co-expressed both markers (Fig. 1Cviii,Dviii, arrowheads). By contrast, Pitx2 and Alx4 remained confined to the anterior head mesoderm (Fig. 1Aviii,Bviii, arrowheads). This expression pattern was maintained at later stages (shown for HH16, Fig. 1A-Eix) and represents the mature, pharyngula-stage pattern described previously for the chicken (Roberts et al., 2005; Tirosh-Finkel et al., 2006; von Scheven et al., 2006a; von Scheven et al., 2006b) with corresponding expression in the mouse (Kelly et al., 2004; Lu et al., 2002; Shih et al., 2007).

Taken together (Fig. 1F), four phases of head mesoderm marker gene expression can be distinguished: phase 0/HH4-5, no marker expression; phase 1/HH6-8, anteroposterior subdivision of the head mesoderm by Pitx2 and Tbx1 or elevated Twist expression; phase 2/HH9-10, refinement of anterior pattern by Alx4 and MyoR; phase 3/HH10-HH14, anteroposterior spread of MyoR and Tbx1 expression to establish the final pattern.

Alx4 expression commenced at HH6 in the lateral mesoderm, followed by the cardiac mesoderm and the occipital somites at HH7 (Fig. 1Bii-iv). In the head mesoderm, expression was barely detectable at HH8 (Fig. 1Biv, arrowhead), but established at HH9 (Fig. 1Bv).

MyoR expression began at HH9+/HH10– (Fig. 1Cvi,vii). Alx4 and MyoR labelled the anterior head mesoderm only. During their activation, the distribution of Pitx2, Tbx1 and Twist did not change. Thus, the HH10 head mesoderm is characterised by Pitx2, Alx4, MyoR and low-level Twist expression anteriorly, reaching as far posterior as the territory of rhombomeres r1-2 (prospective metencephalon) and the prospective mandibular arch. Tbx1 and higher-level Twist expression labelled the posterior head mesoderm flanking the prospective myelencephalon, as shown previously (Bothe and Dietrich, 2006).

After HH10, the genes were expressed at numerous additional sites, with Pitx2 labelling the oral ectoderm and Alx4 the neural tube and neural crest-derived cranial mesenchyme, the latter also expressing Twist. Significantly, in the head mesoderm, the expression of MyoR spread posteriorly and that of Tbx1 anteriorly such that at HH13-14, the mesoderm of all prospective pharyngeal arches co-expressed both markers (Fig. 1Cviii,Dviii, arrowheads). By contrast, Pitx2 and Alx4 remained confined to the anterior head mesoderm (Fig. 1Aviii,Bviii, arrowheads). This expression pattern was maintained at later stages (shown for HH16, Fig. 1A-Eix) and represents the mature, pharyngula-stage pattern described previously for the chicken (Roberts et al., 2005; Tirosh-Finkel et al., 2006; von Scheven et al., 2006a; von Scheven et al., 2006b) with corresponding expression in the mouse (Kelly et al., 2004; Lu et al., 2002; Shih et al., 2007).

Table 1. Numbers of bead implantation experiments

<table>
<thead>
<tr>
<th>Marker</th>
<th>Indicative of:</th>
<th>Treatment [in µg/ml (µM)]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DMSO*</td>
<td>BSA*</td>
</tr>
<tr>
<td>Pitx2</td>
<td>Anterior head mesoderm</td>
<td>5</td>
</tr>
<tr>
<td>Alx4</td>
<td>Anterior head mesoderm</td>
<td>4</td>
</tr>
<tr>
<td>MyoR</td>
<td>Initially anterior, later all branchiomeric head mesoderm</td>
<td>2</td>
</tr>
<tr>
<td>Tbx1</td>
<td>Initially posterior, later all branchiomeric head mesoderm</td>
<td>3</td>
</tr>
<tr>
<td>Twist</td>
<td>Head and somitic mesoderm</td>
<td>3</td>
</tr>
<tr>
<td>Myf5</td>
<td>Myogenic commitment</td>
<td>3</td>
</tr>
<tr>
<td>Paraxis</td>
<td>Somite</td>
<td>4</td>
</tr>
<tr>
<td>Pax3</td>
<td>Somite and somitic muscle precursors</td>
<td>6</td>
</tr>
<tr>
<td>Isl1</td>
<td>Second heart field</td>
<td>6</td>
</tr>
<tr>
<td>Nkx2.5</td>
<td>First heart field</td>
<td>5</td>
</tr>
<tr>
<td>Cyp26C1</td>
<td>Anterior head mesoderm</td>
<td>4</td>
</tr>
<tr>
<td>Hoxb1</td>
<td>Active RA signalling</td>
<td>3</td>
</tr>
<tr>
<td>Noggin</td>
<td>Active Bmp signalling</td>
<td>3</td>
</tr>
<tr>
<td>Mkp3</td>
<td>Active Fgf signalling</td>
<td>3</td>
</tr>
</tbody>
</table>

Total: 54 | 63 | 130 | 6 | 5 | 7 | 172 | 106 | 93 | 58 | 6 | 700 |

BSA, bovine serum albumin; RA retinoic acid. Beads were implanted in phase 0 (HH4-5), early phase 1 (HH6-7) and late phase 1 (HH8), with the exception of: *tested in late phase 1 at HH8; †tested in early phase 1 at HH6. Embryos were cultured for a further 5 hours to reach early phase 1 (HH6-7), late phase 1 (HH8) and phase 2 (HH9-10), thus testing for the initiation of Pitx2 and Tbx1, the maintenance of Pitx2 and Tbx1, and the initiation of Alx4 and MyoR expression, respectively.
Fig. 1. See next page for legend.
Retinoic acid signalling
Raldh2, the main enzyme generating RA during early embryogenesis (Rochette-Egly and Germain, 2009), was expressed in the somitic mesoderm posterior to the developing head mesoderm at HH4-5/phase 0 (Fig. 2Ai) (Blentic et al., 2003; Bothe et al., 2007; Hochgreb et al., 2003). As development proceeded, expression receded further in the posterior direction, vanishing from the first somite in phase 1/HH7-8 (Fig. 2Aii) and the second somite in phase 2/HH9-10 (Fig. 2Aiii). By contrast, expression in the lateral mesoderm extended anteriorly, eventually encompassing the sinoatrial region of the heart (Hochgreb et al., 2003). RA is metabolised by Cyp26A1, Cyp26B1 and Cyp26C1 (Rochette-Egly and Germain, 2009). Of these, only Cyp26C1 was expressed in the head mesoderm, labelling the anterior head mesoderm from phase 0 onwards (Fig. 2Bi-iv) (Bothe and Dietrich, 2006; Reijntjes et al., 2004). Hoxb1 is an RA responsive gene and serves as a read-out for RA signalling (Bel-Vialar et al., 2002; Forlani et al., 2003). Its expression reached as far anterior as the otic region and rhombomere 4, with strong expression confined to the neural tube and endoderm (Fig. 2Cii-iv). The data suggest that from phase 1 onwards, the anterior head mesoderm is an RA-free territory. The posterior head mesoderm is exposed to some RA, with levels dropping as Raldh2 expression shifts posteriorly.

Bmp signalling
In phase 0, Bmp2 and Bmp4 were co-expressed in the cardiac region and primitive streak, and Bmp2 and Bmp7 expression overlapped in the prechordal region, the epiblast and the developing lateral mesoderm (Fig. 2Di,Ei,Fi) (Chapman et al., 2002; Faure et al., 2002). In phase 1, Bmp molecules were expressed in the cardiac and prechordal territory and the neural folds (Fig. 2Dii,Eii,Fii); in phase 2, the surface ectoderm expressed Bmp (Fig. 2Eiii); and in phase 3, strong Bmp expression was found in the heart and the ventromedial aspect of the pharyngeal arches (Fig. 2Eiii-Fiii). Thus, the head mesoderm is surrounded by Bmp-producing tissues at all times. Significantly, the head mesoderm is also exposed to various Bmp and Tgfβ antagonists: at all stages, the notochord expressed noggin (Fig. 2Gii-iv); Hensen’s node expressed chordin (Fig. 2Hii-iv, red staining); and at HH4-5, follistatin (Fig. 2Hii, blue staining) (Chapman et al., 2002). Follistatin was also expressed in the forebrain, neural folds, head and somitic mesoderm (Fig. 2Hi-iii). Notably, as development proceeded from phase 0 to phase 1, the chordin-producing node regressed posteriorly (Fig. 2Hi-ii), and between phase 1 and 2, follistatin expression vanished from the anterior head mesoderm, suggesting that the head mesoderm became accessible for Bmp ligands. At this stage, pSmad1, indicative of active Bmp signalling, can be detected in the head mesoderm, spreading from lateral to medial and anterior to posterior (Faure et al., 2002). In many tissues, including the head mesoderm, noggin is activated in response to Bmp, i.e. is a read-out for Bmp signalling (Sela-Donfenfeld and Kalcheim, 2002) (this study). We found that noggin expression commenced in anterior head mesoderm during phase 2 (Fig. 2Gii-iii). This suggests that from phase 2 onwards, the anterior head mesoderm receives Bmp signals.

Fgf signalling
Fgf molecules are expressed at various sites (Chapman et al., 2002; Karabagli et al., 2002; Lunn et al., 2007) with Fgf4, Fgf8 and Fgf10 labelling the developing notochord in phase 0 (Fig. 2I-Kii); in phase 1, Fgf4 labelling the notochord, endoderm and posterior head mesoderm (Fig. 2Ii), Fgf8 the anterior neural folds and the
Fig. 2. See next page for legend.
**Significance of the suppression of retinoic acid (RA) signalling**

The initially high, from phase 1 onwards declining, RA levels suggest that RA might negatively regulate the establishment of head mesoderm markers. Moreover, RA might exert a differential effect on the anterior (no RA) and posterior (some RA) head mesoderm. To test this, we implanted RA loaded beads into the head mesoderm in phase 0, early phase 1 and late phase 1. The embryos were cultured for 5 hours to allow the direct and the first wave of indirect target genes to respond, and for the embryos to reach early phase 1, late phase 1 and phase 2, as appropriate. We tested for the initiation of Pitx2 and Tbx1, the maintenance of Pitx1 and Tbx1, and the initiation of Alx4 and MyoR expression. As negative control, beads soaked in the solvent DMSO were used. As positive control, we assayed for the expression of Hoxb1. The number of specimens for this and subsequent experiments are summarised in Table 1, the results in Table S1 in the supplementary material, and the sites and timing of bead implantation in Fig. S5 in the supplementary material.

Surprisingly, Hoxb1 was upregulated when beads were implanted during early or late phase 1 (Fig. 3L; red arrowheads), but not in phase 0 (Fig. 3F; blue arrowhead). Yet, at that stage, we obtained a clear response to RA treatment: Pitx2 expression was suppressed (Fig. 3A; red arrowhead). RA treatment also prevented the maintenance of Pitx2 (Fig. 3G,M; red arrowheads) and the initiation of MyoR expression (Fig. 3C,L,O; red arrowheads). By contrast, Alx4 was not affected (Fig. 3B,H,N; blue arrowheads). Thus, RA suppresses two of the three anterior head mesoderm markers.

When assaying for the expression of Tbx1, we found that RA hindered the establishment of normal expression levels in phase 0 (Fig. 3D; red arrowheads). Later, RA beads mildly downregulated Tbx1 when implanted into the Tbx1 domain (Fig. 3J,P; red arrowheads); beads implanted anteriorly had no effect (Fig. 3J,P; blue arrowheads; data not shown). Thus, Tbx1 is negatively regulated by RA but seems to tolerate higher RA levels than the anterior markers Pitx2 and MyoR. Twist expression remained unchanged, inferring that *Twist* is a general paraxial mesoderm marker rather than a specific head mesoderm marker (Fig. 3E,K,Q; blue arrowheads).

**Concentration-dependent effects of RA**

To test directly whether the anterior markers are more sensitive to RA than the posterior marker Tbx1, a dilution series of RA in DMSO at 5 μg/μl (16.6 μM), 0.5 μg/μl (1.66 μM) and 0.05 μg/μl (0.166 μM) was prepared, beads were implanted into the head mesoderm in late phase 1, and the embryos were assayed for Pitx2 and Tbx1 expression 5 hours later at HH9-10 in phase 2 (Fig. 4). We found that, even at the lowest concentration, RA still mildly downregulated Pitx2. By contrast, Tbx1 was downregulated with 166 μM and 16.6 μM, but not with 1.66 μM and 0.166 μM RA. These data reinforce that RA needs to be cleared anteriorly to allow expression of Pitx2 and MyoR. In the posterior head mesoderm, RA levels are high enough to suppress the anterior markers but low enough to permit Tbx1 expression. Further posterior in the occipital somites, RA remains high enough to prevent ectopic expression of Tbx1. Thus, RA establishes the first pattern of the head mesoderm and sets their posterior boundaries.
Role of Bmp signalling
As the anterior head mesoderm appeared to receive Bmp signals from phase 2 onwards, we hypothesised that Bmp might refine the anteroposterior pattern after the initial Pitx2-Tbx1 divide. To test this, beads loaded with Bmp or with the Bmp antagonist noggin were implanted in phase 0, early phase 1 and late phase 1; the embryos were cultured for 5 hours as before. As negative control, BSA beads were used. As positive control, we assayed for the expression of noggin.

As expected, Bmp beads induced noggin expression in the head mesoderm at all stages (Fig. 5F,L,R, green arrowheads), whereas treatment with noggin downregulated endogenous noggin expression (Fig. 5Ri, red arrowhead). However, exposure to Bmp advanced the onset of Alx4 expression to late phase 1 (Fig. 5H, green arrowhead) and induced ectopic expression of Alx4 in phase 2 (Fig. 5N, green arrowhead); noggin treatment prevented expression of Alx4 (Fig. 5Ni, red arrowhead). Thus, Bmp is necessary and sufficient to initiate and maintain Alx4 expression. Bmp treatment also upregulated MyoR expression, albeit only within its usual expression domain in phase 2 (Fig. 5O, green arrowhead); noggin treatment suppressed MyoR (Fig. 5Oi, red arrowhead). Thus, Bmp is necessary but not sufficient to initiate and maintain MyoR expression. Bmp treatment downregulated Tbx1 expression at all times (Fig. 5D,J,P, red arrowheads), but noggin treatment, if at all, only mildly upregulated Tbx1 (Fig. 5Pi, green arrowhead). Thus, absence of Bmp signalling is necessary but not sufficient to allow Tbx1 expression. Taken together, this suggests that the onset of active Bmp signalling during phase 2 is required for the refinement of the initial anteroposterior pattern of the head mesoderm. However, additional factors contribute.

Role of Fgf signalling
As the posterior head mesoderm received Fgf signals in phase 1, the anterior head mesoderm in phase 2, and in phase 3 all prospective ventral/branchiomeric head mesoderm was exposed, we speculated that Fgf might carry out multiple roles during this process. To explore this, beads loaded with Fgf or the Fgf inhibitor SU5402 were implanted, using the same experimental paradigm as before. BSA beads served as negative control for the Fgf treatment (displayed in Fig. 5) and DMSO coated beads as negative control for the SU5402 treatment (displayed in Fig. 3). As positive control, we assayed for the expression of Mkp3. As expected, Fgf treatment upregulated (Fig. 5F,L,R, green arrowheads) and SU5402 treatment suppressed (Fig. 5Pi, red arrowhead) Mkp3 at all stages. 

Assaying for the head mesoderm markers, we found that Fgf mildly downregulated (Fig. 5A,G,M, red arrowheads) and SU5402 suppressed (Fig. 5Pi, red arrowhead) Pitx2. Conversely, Fgf strongly upregulated (Fig. 5D,J,P, green arrowheads) and SU5402 suppressed (Fig. 5Pi, red arrowhead) Tbx1. This suggests that during phase 1 and 2, in
Influence of the signalling systems on myogenic differentiation and the recruitment of cells into the somitic or cardiac lineage

The signalling systems investigated here have been reported to regulate myogenic differentiation, to control the head-trunk/somatic boundary and to recruit cells into the cardiac lineage (Díez del Corral et al., 2003; Tzahor et al., 2003; von Scheven et al., 2006a). We therefore investigated whether any of the observed changes in head mesoderm marker gene expression were due to premature differentiation, or to cell allocation to the somitic or cardiac lineage. The results are shown in Fig. S2 in the supplementary material (muscle), Fig. S3 in the supplementary material (somite markers) and Fig. S4 in the supplementary material (primary and secondary heart field markers). They indicate that the head mesoderm was not driven into differentiation or another cell lineage. We thus conclude that during early head mesoderm development, RA, Bmp and Fgf specifically control its patterning.

Cross talk between the signalling systems

Studies on limb development showed that Fgf activates direct response genes within 1 hour (Isaac et al., 2000). It is thus possible that during the 5 hour incubation period used here, direct and first-wave indirect response genes are de-regulated. Specifically, it is possible that treatment with one signal de-regulates another signalling system, which then changes head mesoderm marker gene expression. To explore this possibility, we implanted RA, Bmp, Fgf or control beads during late phase 1 and allowed the embryos to develop for 5 hours to reach phase 2, i.e. the stage at which in the wild type all systems are activated. We then assayed for the expression of Cyp26C1, the RA responsive gene Hoxb1, the Bmp responsive gene noggin and the Fgf responsive gene Mkp3.

RA did not change the expression of Cyp26C1 (Fig. 8A, blue arrowhead) (Reijntjes et al., 2004), but, as reported above, upregulated Hoxb1 (Fig. 8B, green arrowheads). RA also did not alter the expression of noggin (Fig. 8C, blue arrowhead), but suppressed Mkp3 in the posterior head mesoderm (Fig. 8D, red arrowhead), which is required for the onset of Tbx1 expression. Thus, the negative effect of RA on Tbx1 might, in part, be due to the suppression of Fgf signalling.

Bmp upregulated noggin (Fig. 8G, green arrowhead), and Fgf upregulated Mkp3 (Fig. 8L, green arrowhead). Interestingly, both Bmp and Fgf downregulated Cyp26C1 (Fig. 8E,J, red arrowheads). However, Hoxb1 was not upregulated (Fig. 8FJ, blue arrowheads), suggesting that the loss of Cyp26C1 in the head mesoderm did not lead to an immediate gain of RA signalling. Remarkably, in the anterior head mesoderm Bmp upregulated Mkp3 (Fig. 8H, green arrowhead) and Fgf upregulated noggin (Fig. 8K, green arrowhead). Thus, in this territory, Fgf and Bmp signalling might reinforce each other, possibly driving the sudden onset of MyoR expression at HH9+.

DISCUSSION

The head mesoderm markers Pitx2, Alx4, MyoR and Tbx1 pattern the head mesoderm prior to the formation of morphological boundaries and the onset of differentiation (Bothe and Dietrich, 2006), and mutations cause specific defects in craniofacial muscles and the outflow tract of the heart (Ai et al., 2006; Dong et al., 2006; Gage et al., 1999; Harel et al., 2009; Kelly et al., 2004; Kitamura et al., 1999; Liu et al., 2002; Lu et al., 2002; Nowotschin et al., 2006; Sambasivan et al., 2009; Shih et al., 2007; Vitelli et al., 2002a; Vitelli et al., 2002b; Xu et al., 2004) (for reviews, see Baldini, 2002; Bothe et al., 2007; Noden and Francis-West, 2006; Rochais et al., 2009).

Fig. 4. Concentration-dependent effects of retinoic acid (RA). Dorsal views of chicken embryo heads treated in late phase 1 (HH8) with RA at the concentrations indicated on top of the panels; carrier beads are indicated by asterisks. The embryos were analysed 5 hours later at the start of phase 2 (HH9-10) for the expression of Pitx2 and Tbx1 as indicated at the left of the panel. Note that Pitx2 was mildly downregulated even at the lowest concentration of RA (red arrowheads). By contrast, Tbx1 was downregulated with 166 µM and 16.6 µM (red arrowheads), but not with 1.66 µM and 0.166 µM RA (blue arrowheads). end, endoderm; hm, head mesoderm; lm, lateral mesoderm; som 1, first somite.

Combinatorial effect of Fgf and Bmp

As both Bmp and Fgf signals contributed, but were insufficient, to initiate MyoR expression, we tested for their combinatorial role, simultaneously implanting Fgf and Bmp beads. We performed this experiment in early phase 1 and allowed the embryos to develop for further 5 hours to reach late phase 1, thus testing for the premature onset of MyoR. Fig. 7 shows that Bmp and Fgf alone failed to activate MyoR (Fig. 7A,B, blue arrowheads). When applied together, however, MyoR was induced (Fig. 7C, green arrowhead). Tbx1 on the other side was suppressed by Bmp (Fig. 7D, red arrowhead) and induced by Fgf alone (Fig. 7E, green arrowhead); when both beads were implanted, Bmp prevented the upregulation of Tbx1 by Fgf (Fig. 7F, red arrowhead).

Addition to falling RA levels, rising Fgf levels in the posterior head mesoderm are required to initiate and maintain Tbx1 and contribute to restricting Pitx2 expression.

Similar to Pitx2, Alx4 was also downregulated by Fgf (Fig. 5N, red arrowhead), but SU5402 did not cause upregulation (Fig. 5Ni, blue arrowhead). Thus, Fgf contributes to limiting Alx4 expression, but additional factors, such as Bmp as shown above, are required to initiate expression. In contrast to Pitx2 and Alx4, MyoR expression expanded posteriorly upon Fgf treatment (Fig. 5O, green arrowhead) and became repressed by SU5402 (Fig. 5Oi, red arrowhead). However, similar to Bmp, Fgf was unable to induce MyoR expression prematurely (Fig. 5C,I, blue arrowheads). Thus, when Fgf signalling commences in the anterior head mesoderm in phase 2, this is necessary but not sufficient to induce MyoR. Given that Fgf treatment facilitated the anterior expansion of Tbx1 and the posterior expansion of MyoR expression, it appears that their expansion in phase 3 is in response to spreading Fgf signals.

Cross talk between the signalling systems

Studies on limb development showed that Fgf activates direct response genes within 1 hour (Isaac et al., 2000). It is thus possible that during the 5 hour incubation period used here, direct and first-wave indirect response genes are de-regulated. Specifically, it is possible that treatment with one signal de-regulates another signalling system, which then changes head mesoderm marker gene expression. To explore this possibility, we implanted RA, Bmp, Fgf or control beads during late phase 1 and allowed the embryos to develop for 5 hours to reach phase 2, i.e. the stage at which in the wild type all systems are activated. We then assayed for the expression of Cyp26C1, the RA responsive gene Hoxb1, the Bmp responsive gene noggin and the Fgf responsive gene Mkp3.

RA did not change the expression of Cyp26C1 (Fig. 8A, blue arrowhead) (Reijntjes et al., 2004), but, as reported above, upregulated Hoxb1 (Fig. 8B, green arrowheads). RA also did not alter the expression of noggin (Fig. 8C, blue arrowhead), but suppressed Mkp3 in the posterior head mesoderm (Fig. 8D, red arrowhead), which is required for the onset of Tbx1 expression. Thus, the negative effect of RA on Tbx1 might, in part, be due to the suppression of Fgf signalling.

Bmp upregulated noggin (Fig. 8G, green arrowhead), and Fgf upregulated Mkp3 (Fig. 8L, green arrowhead). Interestingly, both Bmp and Fgf downregulated Cyp26C1 (Fig. 8E,J, red arrowheads). However, Hoxb1 was not upregulated (Fig. 8FJ, blue arrowheads), suggesting that the loss of Cyp26C1 in the head mesoderm did not lead to an immediate gain of RA signalling. Remarkably, in the anterior head mesoderm Bmp upregulated Mkp3 (Fig. 8H, green arrowhead) and Fgf upregulated noggin (Fig. 8K, green arrowhead). Thus, in this territory, Fgf and Bmp signalling might reinforce each other, possibly driving the sudden onset of MyoR expression at HH9+.
Therefore, the establishment of the correct head mesoderm pattern is the prerequisite for the appropriate development of both organs. The aim of our work was to unravel how the head mesoderm pattern is established. Investigating the dynamics of marker gene expression, their short-term responses to various signalling systems and the interdigitation of these systems, we were able to identify three discrete phases of anteroposterior (a-p) head mesoderm patterning.

The process depends on the combinatorial as well as antagonistic action of Bmp and Fgf signalling, combined with the suppression of retinoic acid (RA) signalling (Fig. 9). Importantly, these signals act specifically on head mesoderm patterning without affecting cell fate or differentiation.

**Anteroposterior head mesoderm pattern is established in three distinct phases**

Our analysis revealed that when the head mesoderm is first laid down, none of the head mesoderm markers is active (phase 0). Expression commences at early neurulation stages, with Pitx2 labelling the anterior and Tbx1 the posterior territory (phase 1). Thus, there might be no pan-head mesodermal markers; the head mesoderm is set up as two anteroposterior territories. At late neurulation stages, first Alx4, then MyoR, are initiated within the Pitx2 expression domain whereas Tbx1 expression remains unchanged. Thus, phase 2 is characterised by a refinement of anterior marker gene expression. During pharyngula stages, Tbx1 signals spread along the floor of the pharynx anteriorly and MyoR signals spread posteriorly such that, eventually, all branchiomeric muscle anlagen harbour both markers. By contrast, Pitx2 expression remains anterior, overlapping with MyoR and Tbx1 signals in the first arch muscles and the cells contributing to the heart. This is the mature head mesoderm pattern described for avians as well as the mouse (Kelly et al., 2004; Lu et al., 2002; Roberts et al., 2005; Shih et al., 2007; Tirosch-Finkel et al., 2006; von Scheven et al., 2006a; von Scheven et al., 2006b). Taken together, a-p head mesoderm patterning is achieved in three discrete phases.

**The head mesoderm does not express any specific marker genes by default**

To test whether any of the head mesoderm markers are expressed by default, head mesoderm prior to and during phases 1-3 of patterning was explanted and cultured in vitro with or without surrounding tissues. In the presence of surrounding tissues, head mesoderm does not express any specific marker genes by default.
mesoderm markers were expressed, albeit not in their normal sequence and pattern, possibly because the cultures were unable to undertake normal morphogenetic movements and establish the appropriate topology of tissues (this study and I.B. and S.D., unpublished observations). Notably, without surrounding tissues, the markers were neither initiated nor maintained, indicating that all three phases of head mesoderm patterning depend on extrinsic cues. When performing tissue ablation experiments during phases 1-3 in vivo, however, changes in marker gene expression were rarely observed, suggesting that multiple tissues provide the same signal (I.B. and S.D., unpublished observations). Indeed, the head mesoderm neighbours the site of RA production and is surrounded by numerous tissues producing Fgf and Bmp. Importantly, markers serving as signalling readout suggest that the head mesoderm actively receives these signals at specific sites and times only.

In phase 1, falling RA levels and onset of posterior Fgf signalling control the initial anteroposterior pattern
When the head mesoderm is laid down during gastrulation, it is under the influence of RA (Blentic et al., 2003; Bothe and Dietrich, 2006; Hochgreb et al., 2003) (this study). At the time at which Pitx2 and Tbx1 expression commences, RA production has receded posteriorly into the somitic region, the anterior head mesoderm expresses the RA antagonist Cyp26C1, and the RA-responsive Hoxb1 gene is not expressed further anterior than rhombomere 4 (Bel-Vialar et al., 2002; Blentic et al., 2003; Bothe and Dietrich, 2006; Forlani et al., 2003; Hochgreb et al., 2003; Reijntjes et al., 2004). This suggests that the anterior head mesoderm is cleared of RA signalling and the posterior head mesoderm receives low-level and the somites high-level RA signalling. Implantation of RA beads prevented expression of Pitx2 and reduced expression of Tbx1 in a
A similar downregulation of Pitx2 and Tbx1 upon RA administration at day 8.5 post-coitum (8.5 dpc) has been reported in the mouse (Abe et al., 2008). This suggests that the clearance of RA anteriorly and the reduction of RA signalling levels posteriorly is a prerequisite for head mesoderm patterning. Moreover, RA is a key regulator of the Pitx2-Tbx1 expression boundary.

As none of the head mesoderm markers is expressed by default, we hypothesised that loss or reduction of RA is necessary but not sufficient for marker gene expression. Significantly, simultaneously to falling RA levels, Fgf signalling commences in the posterior head mesoderm (Lunn et al., 2007) (this study). Fgf application in phase 0-1 strongly promoted Tbx1 expression; suppression of Fgf signalling suppressed Tbx1. Thus, Fgf activates and maintains the posterior head mesoderm marker Tbx1, in line with findings in the mouse (Abu-Issa et al., 2002). Which signal might contribute to the initiation of Pitx2 is currently not known.

It is established that at many sites in the embryo, RA negatively regulates Fgf signalling (Brondani et al., 2002; Diez del Corral et al., 2003; Zhao et al., 2009). We observed that RA downregulated the Fgf signalling indicator Mkp3, in line with studies in the mouse (Abe et al., 2008; Ryckebusch et al., 2008; Sirbu et al., 2008). This suggests that the initiation of Fgf signalling, and hence the activation of Tbx1 in the posterior head mesoderm, might occur in response to the falling RA levels. As Tbx1 has been shown to negatively regulate RA production (Caterino et al., 2009; Roberts...
In the posterior head mesoderm, Bmp strongly suppressed Tbx1. Fgf signalling, however, was unaffected, suggesting that Bmp controls the anterior border of Tbx1 expression, possibly directly targeting Tbx1. Tbx1, by contrast, has recently been suggested to suppress Bmp signalling by preventing Smad1-Smad4 interaction (Fulcoli et al., 2009). This suggests that Tbx1 indirectly controls the extension of Bmp dependent markers.

When Bmp and Fgf signalling commences in the anterior head mesoderm, Fgf signalling levels increase significantly in the posterior domain, owing to the positive Fgf-Tbx1 feedback loop (Abu-Issa et al., 2002; Hu et al., 2004; Vitelli et al., 2002b; Xu et al., 2004). After applying Fgf to the anterior head mesoderm, i.e. elevating the Fgf level beyond that which is normally found there, we noticed that Pitx2 and Alx4 expression declined. Thus, although Fgf is necessary for the activation of MyoR, high Fgf levels prevent the molecular set-up of the anterior head mesoderm. This infers that, whereas Bmp controls the anterior border of the posterior head mesoderm marker, Fgf controls the posterior border of the two anterior markers Pitx2 and Alx4.

In phase 3, spreading of Fgf signalling allows the expansion of MyoR and Tbx1 expression along the floor of the pharynx

In phase 3, extension of MyoR and Tbx1 expression is concomitant with the spread of high-level Fgf signalling along the floor of the pharynx. We found that Fgf application accelerated the MyoR-Tbx1 spread, and suppression of Fgf signalling prevented it. This suggests that Fgf signalling is key to establishing the final head mesoderm pattern. Notably, MyoR remained sensitive to RA. In the embryo, however, the site of RA production continuously recedes posteriorly during phases 2 and 3 (Blentic et al., 2003; Bothe and Dietrich, 2006; Hochgreb et al., 2003; Reijntjes et al., 2004) (this study), suggesting that the posterior extension of MyoR expression occurs at a rate set by RA.

The anteriorly spreading Fgf signals will eventually reach the Pitx2-Alx4 domain. Both genes were negatively regulated by high Fgf levels in phases 1 and 2; yet, in phase 3 the genes remain expressed. Likewise, Tbx1 spreads anteriorly although this territory is controlled by Bmp. Notably, Fgf levels vary along the anteroposterior extent of the pharynx; at HH13, for example, Fgf signalling appears lower in the anterior compared with the posterior pharyngeal arches (this study). Thus, it is possible that in the anterior head mesoderm, Fgf levels might remain low enough to allow Pitx2 and Alx4 expression, but rise sufficiently to override the Bmp effect on Tbx1. Conversely, the Fgf levels in the posterior head mesoderm might be so high that MyoR expression can spread, whereas Pitx2 and Alx4 remain repressed. It cannot be excluded that additional signals restrict Pitx2 and Alx4 expression. Yet, the spread of MyoR outside of the Pitx2 territory indicates that in phase 3 MyoR expression has become independent from its former upstream regulator.

The signalling molecules patterning the head mesoderm do so without influencing cell fate or differentiation

RA, Bmp and Fgf signalling play multiple roles during development. RA, in many settings, promotes cell differentiation (for a review, see Diez del Corral and Storey, 2004); in the head, RA first suppresses cardiac markers to set the posterior limit of the heart field, but then specifies the sinoatrial region of the heart (Hochgreb et al., 2003; Keegan et al., 2005; Ryckebeusch et al., 2008; Sirbu et al., 2008). Moreover, RA has the capacity to provide...
cells with a more posterior positional identity (Brondani et al., 2002; Rycekebusch et al., 2008; Sirbu et al., 2008; Zhao et al., 2009). Bmp is a crucial regulator of cardiac development (Schultheiss et al., 1997) and has been suggested to recruit head mesodermal cells into the cardiac lineage (Tirosh-Finkel et al., 2006). Fgf promotes the secondary heart field and keeps cells proliferative and undifferentiated (Sirbu et al., 2008) for a review, see Rochais et al., 2009). We therefore tested whether the observed changes in head mesodermal marker expression occurred because of cell recruitment into cardiac lineage, premature differentiation or posteriorisation. We found that our RA or Fgf treatment did not change cell fate or differentiation status. Bmp induced cardiac marker gene expression only when applied during phase 0. When applied in phase 1, i.e. just before Bmp signalling is normally activated in the head mesoderm, Bmp did not induce cardiac markers unless we increased the dosage (this study and G.T. and S.D., unpublished observations). This suggests that, possibly, cardiac induction reported by others (Tirosh-Finkel et al., 2006) was due to exposure to higher Bmp levels and/or longer exposure times. Taken together, our study suggests that RA, Bmp and Fgf specifically control head mesoderm patterning with the cells remaining undifferentiated and competent to enter any of the possible mesodermal lineages.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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References


### Table S1. Summary of results

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pitx2</th>
<th>Alx4</th>
<th>MyoR</th>
<th>Tbx1</th>
<th>Twist</th>
<th>Myf5</th>
<th>paraxis</th>
<th>Pax3</th>
<th>Isl1</th>
<th>Nkx2.5</th>
<th>Cyp26C1</th>
<th>HoxB1</th>
<th>noggin</th>
<th>Mkp3/Pyst</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA</td>
<td>down</td>
<td>no response</td>
<td>down</td>
<td>down; response weaker than for Pitx2 and MyoR</td>
<td>down</td>
<td>no response</td>
<td>no response</td>
<td>no response</td>
<td>no response</td>
<td>no response in hm (down in cm)</td>
<td>no response</td>
<td>no response</td>
<td>up at HH8 and later</td>
<td>no response</td>
</tr>
<tr>
<td>Bmp</td>
<td>no response</td>
<td>up</td>
<td>up at HH9</td>
<td>no response</td>
<td>no response</td>
<td>down</td>
<td>no response</td>
<td>down in p/hm</td>
<td>down in p/hm</td>
<td>up in hm at HH5-6, no response later</td>
<td>up in hm at HH5-6, no response later</td>
<td>down</td>
<td>no response</td>
<td>up</td>
</tr>
<tr>
<td>noggin</td>
<td>no response</td>
<td>down</td>
<td>down</td>
<td>slightly up</td>
<td>no response</td>
<td>no response</td>
<td>slightly up</td>
<td>no response</td>
<td>slightly up</td>
<td>no response</td>
<td>no response in hm (up within endogenous Isl1 domain)</td>
<td>no response</td>
<td>no response</td>
<td>up anterior, no response posterior</td>
</tr>
<tr>
<td>Fgf</td>
<td>down</td>
<td>up at HH9</td>
<td>up</td>
<td>no response</td>
<td>no response</td>
<td>no response</td>
<td>no response</td>
<td>no response</td>
<td>no response</td>
<td>no response</td>
<td>no response</td>
<td>no response</td>
<td>no response</td>
<td>no response</td>
</tr>
<tr>
<td>SU5402</td>
<td>up</td>
<td>no response</td>
<td>down</td>
<td>down</td>
<td>no response</td>
<td>no response</td>
<td>no response</td>
<td>no response</td>
<td>no response</td>
<td>no response</td>
<td>no response</td>
<td>no response</td>
<td>no response</td>
<td>down</td>
</tr>
<tr>
<td>Fgf + Bmp</td>
<td>up already at HH7-8</td>
<td>upregulation by Fgf suppressed</td>
<td>up</td>
<td>no response</td>
<td>no response</td>
<td>no response</td>
<td>no response</td>
<td>no response</td>
<td>no response</td>
<td>no response</td>
<td>no response</td>
<td>no response</td>
<td>no response</td>
<td>down</td>
</tr>
</tbody>
</table>

a/hm, anterior head mesoderm; cm, cardiac mesoderm; hm, head mesoderm; p/hm, posterior head mesoderm.