

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 anteroposterior (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 branchiomic 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 skull base and, together with the anteriorly adjoining prechordal mesoderm, delivers the genuine 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 (Kioussi 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)

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(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 various signalling systems regulate each other (Brondani et al., 2002; Park et al., 2008; Rycebusch et al., 2008; Sirbu et al., 2008; Zhao et al., 2009) (this study). Thus, onset, dynamics and control of head mesoderm patterning is still unclear.

Here, we show that head mesoderm patterning relies on interconnected molecular networks that operate in a time and context dependent fashion. Three discrete phases can be distinguished: the first phase is characterised by the activation of *Pitx2* in the anterior and *Tbx1* in the posterior head mesoderm. This depends on the clearing of RA in the anterior head mesoderm, and 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 branchiomic 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 bead 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., 2001). The embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951).

Head mesoderm explant cultures

The cultures were set up as described by Tzahor et al. (Tzahor et al., 2003) with the following modifications: cranial tissues were separated from the trunk, by making a transverse cut anterior to the node (HH5-6) or the first somite (HH7-9). The dissection was performed in ice-cold PBS; for head mesoderm-only cultures, surrounding tissues were removed with the aid of collagenase. Explants were washed in ice-cold PBS, embedded in soluble collagen (BD Bioscience), cultured for 4 hours to let the collagen settle or for 1-3 days, i.e. the time it takes the wild-type embryo in ovo to develop

to HH8, HH10, HH14, HH20 or HH24 stages. Cultures were maintained in DMEM containing 10% fetal calf serum and 2% chicken embryo extract in a humidified incubator at 37°C with 5% CO₂.

Preparation and implantation of carrier beads loaded with signalling molecules

Affi-Gel blue agarose beads (BioRad) were soaked in 33 µg/ml (1.3 µM) recombinant human Bmp2, Bmp4 or Bmp7 or 200 µg/ml (7.8 µM) noggin at 4°C overnight. Heparin acrylic beads (Sigma) were incubated in 50 µg/ml (1.6 µM) recombinant human Fgf8b at 4°C overnight. All proteins were from R&D Systems and dissolved in 0.1% bovine serum albumin (BSA) in PBS. Control beads were soaked in 0.1% BSA in PBS. AG-1 X2 negatively charged beads (BioRad) were incubated in 5.9 µg/µl (20 µM) SU5402 (Calbiochem) or 50 µg/µl (166 µM) all-trans retinoic acid (RA, Sigma) dissolved in dimethyl sulfoxide (DMSO) for 1 hour at room temperature in the dark; for the RA dilution series, 5 µg/µl (16.6 µM), 0.5 µg/µl (1.66 µM) and 0.05 µg/µl (0.166 µM) RA in DMSO was loaded. DMSO beads were used as controls (see Table 1).

Beads were washed in PBS and grafted into the left head mesoderm of embryos at stages HH4-5, HH6 and HH8 using flame-sharpened tungsten needles (Dietrich 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)/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; Mootosamy and Dietrich, 2002). Details of probes have been published previously: *Alx4*, *chordin*, *Cyp26C1*, *Isl1*, *Myf5*, *MyoR*, *Nkx2.5*, *paraxis*, *Pax3*, *Pitx2*, *Raldh2*, *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 Eblaghie et al., 2003).

Photomicroscopy and image analysis

All specimens were photographed on a Zeiss AxioScope2 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 at 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 *Tbx1* and *Twist* also labelled the cranial endoderm, *Twist* the prechordal mesendoderm (prechordal plate) and *Pitx2* the left posterior heart field and lateral mesoderm.

Table 1. Numbers of bead implantation experiments

Marker	Indicative of:	Treatment [in µg/ml (µM)]											Total
		Control		RA				Bmp2	Noggin	Fgf8	SU5402	Bmp2 + Fgf8	
		DMSO*	BSA*	50 (166)	5 (16.6)*	0.5 (1.66)*	0.05 (0.166)*	33 (1.3)	200 (7.8)*	50 (1.6)	5.9 (20)*	1.3 + 1.6†	
Pitx2	Anterior head mesoderm	5	13	13	3	3	3	26	12	7	10	–	95
Alx4	Anterior head mesoderm	4	7	6	–	–	–	12	12	5	2	–	48
MyoR	Initially anterior, later all branchiomic head mesoderm	2	2	14	–	–	–	21	9	20	16	3	87
Tbx1	Initially posterior, later all branchiomic head mesoderm	3	7	23	3	2	4	18	9	10	2	3	84
Twist	Head and somitic mesoderm	3	3	5	–	–	–	22	2	4	3	–	42
Myf5	Myogenic commitment	3	6	4	–	–	–	2	2	2	3	–	22
Paraxis	Somite	4	2	5	–	–	–	3	13	5	2	–	34
Pax3	Somite and somitic muscle precursors	6	4	4	–	–	–	4	7	5	2	–	32
Isl1	Second heart field	6	5	7	–	–	–	13	3	6	2	–	42
Nkx2.5	First heart field	5	3	13	–	–	–	12	4	3	2	–	42
Cyp26C1	Anterior head mesoderm	4	3	11	–	–	–	14	24	6	3	–	65
Hoxb1	Active RA signalling	3	2	10	–	–	–	5	3	4	2	–	29
Noggin	Active Bmp signalling	3	4	8	–	–	–	12	4	13	2	–	46
Mkp3	Active Fgf signalling	3	2	7	–	–	–	8	2	3	7	–	32
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.

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).

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.

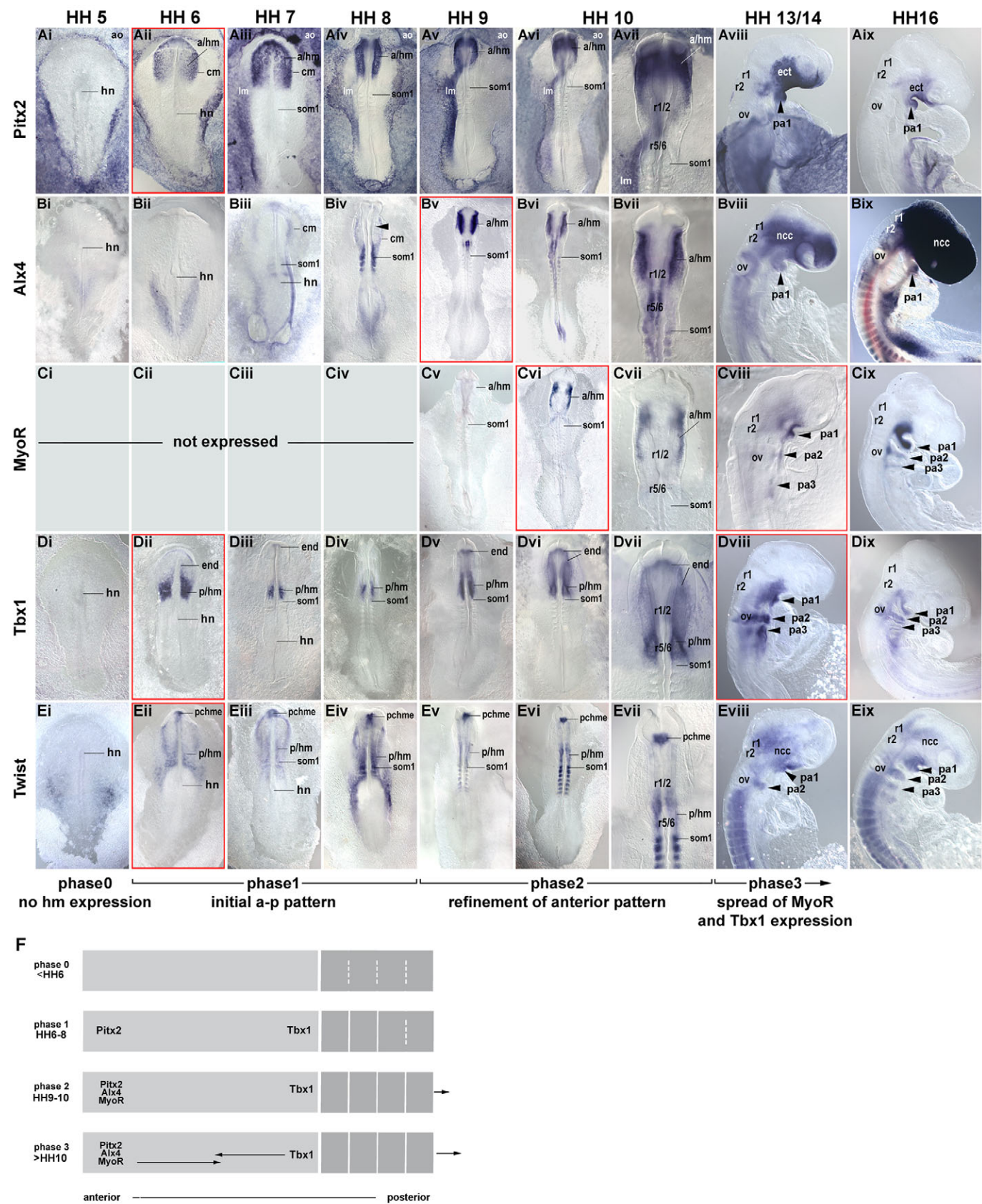


Fig. 1. See next page for legend.

Fig. 1. Onset and dynamics of head mesoderm marker expression. (Ai-Eix) Dorsal views of whole chicken embryos (i-vi) and dorsal (vii) or dorsolateral (viii,ix) views of chicken heads at the developmental stages indicated, showing expression of *Pitx2* (Ai-Aix), *Alx4* (Bi-Bix), *MyoR* (Cv-Cix), *Tbx1* (Di-Dix) and *Twist* (Ei-Eix). The stages at which marker gene expression is established in the head mesoderm or shows significant changes are indicated by red boxes. The phases characterised by a particular pattern of markers are indicated at the bottom. Note that the head mesoderm initially does not express any of the head mesoderm markers (shown for HH5; phase 0 of head mesoderm patterning). At HH6, *Pitx2* and *Tbx1* expression commences, with *Pitx2* labelling the anterior and *Tbx1* the posterior head mesoderm (phase 1). Activation of *Alx4* at HH8⁺-9⁻ and of *MyoR* at HH9⁺-10⁻ refines specifically the anterior pattern (phase 2); markers reach as far posterior as rhomomere 1/2. After HH10 (phase 3), expression of *MyoR* spreads posteriorly and that of *Tbx1* anteriorly until at HH13/14 they encompass the entire branchiomic head mesoderm [arrowheads; the most anterior demarcates the developing mandibular arch (first pharyngeal arch)]. By contrast, *Pitx2* and *Alx4* expression remains confined to the anterior region, encompassing the mesoderm of the periocular region and the mandibular arch. This pattern is maintained at HH16 when differentiation is under way. a/hm, anterior head mesoderm; ao, area opaca; cm, cardiac mesoderm; ect, surface ectoderm; end, endoderm; hn, Hensen's node; lm, lateral mesoderm; ncc, neural crest cells; ov, otic vesicle; pa 1-3, first to third pharyngeal arches; pchme, prechordal mesendoderm; p/hm, posterior head mesoderm; r1-6, rhombomeres 1-6; som1, first somite. (F) Summary of the dynamics of marker gene expression with a light grey block representing the head mesoderm and dark grey blocks the somites; anterior to the left. Black arrows pointing away from a gene name indicate the direction into which marker gene expression is spreading. Black arrows at the right of the somites indicate the progression of somite formation.

Dependence of head mesoderm marker gene expression on extrinsic cues

To investigate whether the head mesoderm might express any of the markers by default, head mesoderm at phase 0, phase 1 and phase 2 was explanted with or without surrounding tissues and cultured for the time it takes a wild-type embryo in ovo to reach HH8, HH10, HH14, HH20 or HH24. The data are shown in Fig. S1 in the supplementary material and indicate that induction, maintenance and dynamics of head mesoderm marker gene expression all depend on extrinsic cues.

Active signalling cascades during the establishment of head mesoderm marker gene expression

Previous studies have implicated retinoic acid (RA), Bmp and Fgf signalling in the control of head mesoderm marker gene expression (Abu-Issa et al., 2002; Roberts et al., 2005; Ryckebusch et al., 2008; Sirbu et al., 2008; Tirosh-Finkel et al., 2006; Tzahor et al., 2003; von Scheven et al., 2006a). However, typically, the experiments were carried out during phases 2-3 of marker gene expression and analysed at pharyngula stages between HH18 and HH20. Descriptive studies suggested that cranial tissues are exposed to RA, Bmp and Fgf signalling much earlier (Blentic et al., 2003; Bothe and Dietrich, 2006; Faure et al., 2002; Hochgreb et al., 2003; Lunn et al., 2007). We therefore investigated these signalling systems at phases 0-3 of chicken head mesoderm marker expression (Fig. 2). The results are summarised in Fig. 2M.

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. 2Ci-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. 2Diii-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 Tgfb antagonists: at all stages, the notochord expressed *noggin* (Fig. 2Gi-iv); Hensen's node expressed *chordin* (Fig. 2Hi-iv, red staining); and at HH4-5, *follistatin* (Fig. 2Hi, blue staining) (Chapman et al., 2002). *follistatin* was also expressed in the forebrain, neural folds, head and somitic mesoderm (Fig. 2Hii-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-Donenfeld and Kalcheim, 2002) (this study). We found that *noggin* expression commenced in anterior head mesoderm during phase 2 (Fig. 2Giii). 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-Ki); in phase 1, *Fgf4* labelling the notochord, endoderm and posterior head mesoderm (Fig. 2Iii), *Fgf8* the anterior neural folds and the

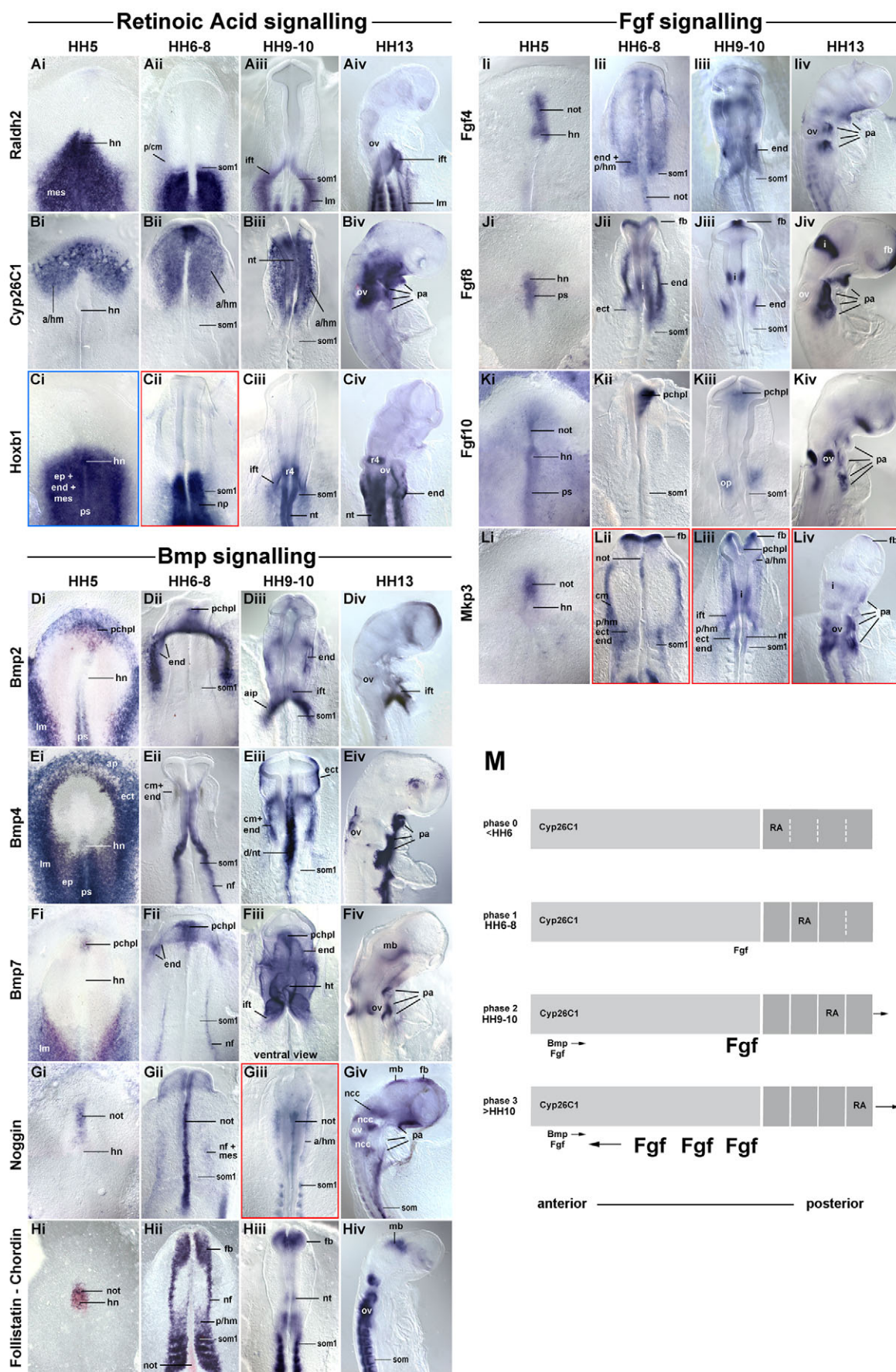


Fig. 2. See next page for legend.

Fig. 2. Candidate signalling cascades for the control of head mesoderm markers. Dorsal (i-iii) and lateral (iv) views of chicken heads at phase 0 of head mesoderm development (before HH6), phase 1 (HH6-8), phase 2 (HH9-10) and phase 3 (after HH10) displaying expression of components of the retinoic acid (RA; Ai-Ci), Bmp (Di-Hiv) and Fgf (Ii-Liv) signalling systems. Exposure of the head mesoderm to any of the signals in phase 0 is indicated by a blue box, in phases 1-3 by red boxes. **(Ai-Civ)** *Raldh2* is expressed in the somitic mesoderm with expression receding posteriorly as development proceeds (Ai-Aiv). *Cyp26C1* labels the anterior head mesoderm (Bi-Biv). Expression of the RA-dependent *Hoxb1* gene reaches further anterior than that of *Raldh2*, suggesting that at the start of head mesoderm patterning, the anterior head mesoderm receives no RA and the posterior head mesoderm low, declining levels of RA (Ci-Civ). **(Di-Hiv)** Expression of *Bmp2* (Di-Div), *Bmp4* (Ei-Eiv), *Bmp7* (Fi-Fiv), and the Bmp/Tgfb antagonists noggin (Gi-Giv), chordin (Hi-Hiv, red) and follistatin (Hi-Hiv, blue); noggin also serves as BMP-read out. The head mesoderm is surrounded by Bmp-expressing tissues, yet noggin expression in the anterior head mesoderm suggests onset of active BMP signalling not before HH9/phase 2, possibly facilitated by the receding chordin- and follistatin-expressing node and by follistatin being cleared from the anterior head mesoderm. **(Ii-Liv)** Expression of *Fgf4* (Ii-liv), *Fgf8* (Ji-Jiv), *Fgf10* (Ki-Kiv) and the Fgf-responsive Fgf antagonist *Mkp3* (Li-Liv). The head mesoderm is surrounded by Fgf signalling tissues, but *Mkp3* expression commences in the posterior head mesoderm not before phase 1/HH6-8 (Lii). In the anterior head mesoderm, Fgf signalling begins in phase 2/HH9-10 (Liii). During phase 3, Fgf signalling spreads such that at HH13 all branchiomic head mesoderm is *Mkp3* positive. **(M)** Summary of the dynamics of retinoic acid, Bmp and Fgf signalling to the head mesoderm. The light grey block represents the head mesoderm and dark grey blocks the somites; anterior to the left; dorsal to the top. Black arrows pointing away from the name of a signal indicate the direction into which this signal is spreading. Black arrows right of the somites indicate the progression of somite formation. Note that, starting with phase 1, the source of RA signalling relocates to consecutively more posterior territories. In phase 1, the posterior head mesoderm becomes exposed to Fgf signalling, with levels rising as development proceeds. In phase 2, the anterior head mesoderm becomes exposed to Bmp and low levels of Fgf signalling. In phase 3, high-level Fgf signalling spreads anteriorly along the pharynx. a/hm, anterior head mesoderm; aip, anterior intestinal portal; ap, area pellucida; cm, cardiac mesoderm; d/nt, dorsal neural tube; ect, surface ectoderm; end, endoderm; ep, epiblast; fb, forebrain; hn, Hensen's node; lm, lateral mesoderm; ht, heart; ift, inflow tract of the heart; mb, midbrain; mes, mesoderm; ncc, neural crest cells; nf, neural folds; not, notochord; nt, neural tube; ov, otic vesicle; pa, pharyngeal arches; pchpl, prechordal mesoderm; p/cm, posterior cardiac mesoderm; p/hm, posterior head mesoderm; ps, primitive streak; r1-6, rhombomeres number 1-6; som1, first somite.

endoderm (Fig. 2Jii), and *Fgf10* the prechordal plate (Fig. 2Kii). During phase 2, additional *Fgf* expression commenced at the midbrain-hindbrain boundary and the otic placode (Fig. 2I-Kiii). In phase 3, strong expression was found in the pharyngeal arches (Fig. 2I-Kiv). The Fgf inhibitor *Mkp3* responds to Fgf signals and, in the head, is expressed in tissues that show phospho-extracellular signal-regulated kinase (pErk)1/2 activity, i.e. active Fgf signal transduction (Lunn et al., 2007). Importantly, *Mkp3* expression commenced in the posterior head mesoderm in phase 1 (Fig. 2Lii), and in the anterior head mesoderm in phase 2 (Fig. 2Liii). Expression levels in the posterior head mesoderm were higher than anteriorly, but in phase 3, significant *Mkp3* activity was found along the floor of all pharyngeal

arches (Fig. 2Liv). This suggests that first the posterior, and later the anterior, head mesoderm receives Fgf signals, followed by Fgf signalling spreading along the pharynx.

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,R; green 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,I,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.

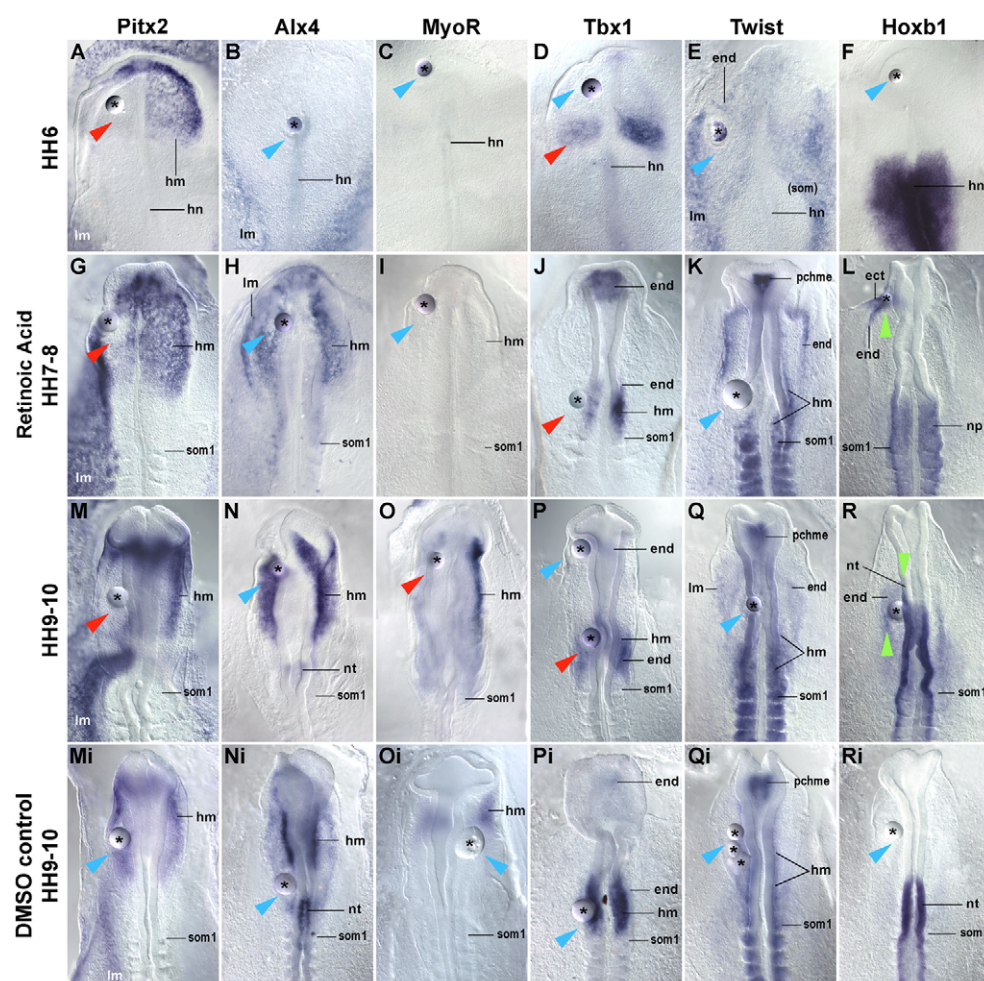


Fig. 3. Influence of retinoic acid (RA). (A–Ri) Dorsal views of chicken embryo heads treated with RA at HH4–5 (A–F), HH6–7 (G–L) and HH8–9 (M–R), or with DMSO (as control) at HH8–9 (Mi–Ri). The carrier beads are indicated by asterisks. Green arrowheads indicate upregulation, red arrowheads downregulation and blue arrowheads unchanged marker gene expression. The embryos were cultured for 5 hours to reach HH5–6 (early phase 1), HH7–8 (late phase 1), HH9–10 (phase 2). Markers are indicated on top of the panels. RA suppressed *Pitx2* (A,G,M, red arrowheads) and *MyoR* expression (O, red arrowhead), suggesting that the head mesoderm has to be RA-free to allow establishment of the anterior pattern. RA mildly downregulated *Tbx1* when placed into the posterior head mesoderm (D,J,P, red arrowheads); beads placed anteriorly had no effect (blue arrowheads). Thus, the head mesoderm has to be RA-low to allow expression of *Tbx1*. (E–Q) RA treatment did not change the expression of *Twist* (blue arrowheads). (L,R) As expected, RA upregulated *Hoxb1* (green arrowheads) albeit not at HH6 (F, blue arrowhead). ect, surface ectoderm; end, endoderm; hm, head mesoderm; hn, Hensen's node; lm, lateral mesoderm; np, neural plate; nt, neural tube; pchme, prechordal mesendoderm; som1, first somite.

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). Bmp had no effect on *Pitx2* (Fig. 5A,G,M,Mi, blue arrowheads) or *Twist* expression (Fig. 5E,K,Q,Qi, blue arrowheads). 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/branchiomic 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. 6Ri, 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 upregulated (Fig. 5Mi) *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

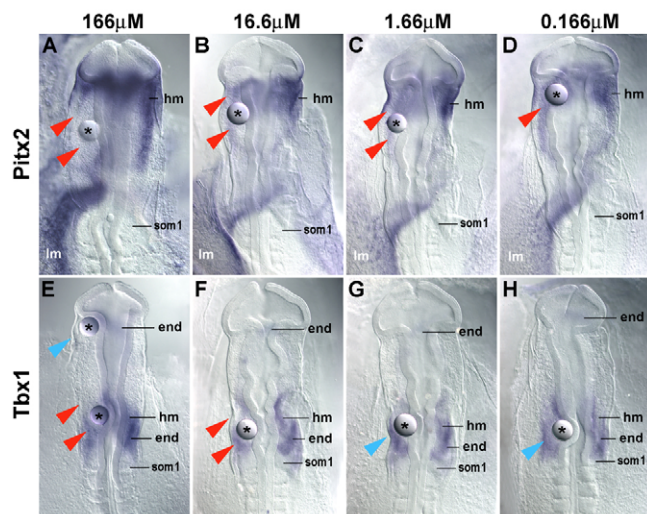


Fig. 4. Concentration-dependent effects of retinoic acid (RA). Dorsal views of chicken embryo heads treated in late phase 1 (HH8) with RA at the concentrations indicated on top of the panels; carrier beads are indicated by asterisks. The embryos were analysed 5 hours later at the start of phase 2 (HH9-10) 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 μ M and 16.6 μ M (red arrowheads), but not with 1.66 μ M and 0.166 μ M RA (blue arrowheads). end, endoderm; hm, head mesoderm; lm, lateral mesoderm; som1, first somite.

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 phase2, 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.

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).

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/somitic boundary and to recruit cells into the cardiac lineage (Diez 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,I, red arrowheads). However, *Hoxb1* was not upregulated (Fig. 8F,J, 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).

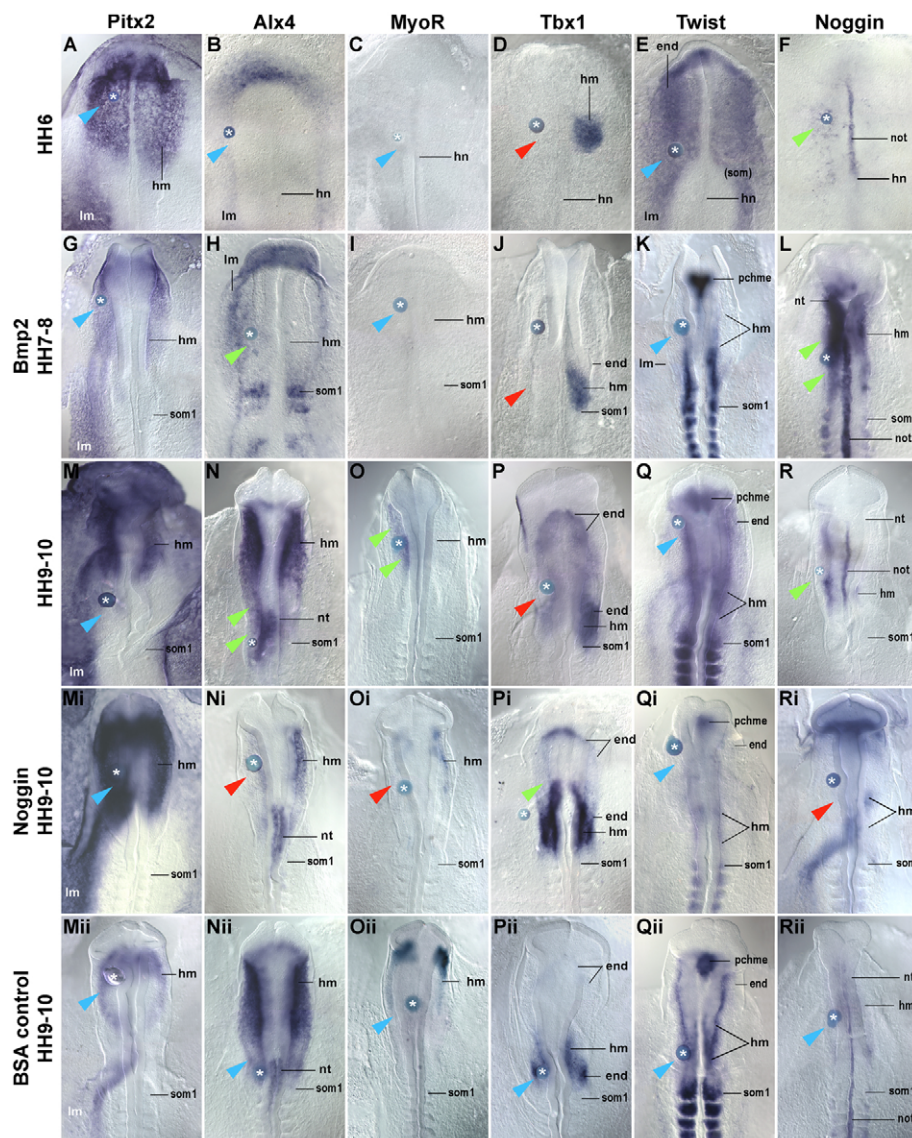


Fig. 5. Influence of Bmp and noggin.

(A-Rii) Dorsal views of chicken embryo heads treated with Bmp2 at HH4-5 (A-F), HH6-7 (G-L) and HH8-9 (M-R), with the Bmp antagonist noggin at HH8-9 (Mi-Ri), or as control with BSA at HH8-9 (Mii-Rii). The carrier beads are indicated by asterisks. Green arrowheads indicate upregulation, red arrowheads downregulation and blue arrowheads unchanged marker gene expression. Embryos were cultured for 5 hours to reach HH5-6 (early phase 1), HH7-8 (late phase 1) or HH9-10 (phase 2). Markers are indicated on top of the panel. Bmp advanced the onset of *Alx4* expression in the anterior head mesoderm to HH7-8 (late phase 1) and expanded *Alx4* expression posteriorly at HH9-10 (phase 2; H,N, green arrowheads); noggin downregulated *Alx4* (Ni, red arrowhead). Bmp elevated *MyoR* levels at the time expression commences, not before (O, green arrowheads); noggin downregulated *MyoR* (Oi, red arrowhead). Thus, Bmp is necessary and sufficient to initiate *Alx4* expression, and necessary but not sufficient to upregulate *MyoR*. Bmp suppressed *Tbx1* at all stages (D,J,P, red arrowheads), yet noggin only mildly upregulated the gene (Pi, green arrowhead), suggesting that suppression of Bmp is necessary but not sufficient to allow *Tbx1* expression. (E,K,Q,Qi) Neither Bmp nor noggin treatment affected the expression of Twist (blue arrowheads). (F,L,R,Ri) As expected, Bmp upregulated (green arrowheads) and noggin downregulated (red arrowhead) expression of noggin at all times. (F,L,R, green arrowheads). end, endoderm; hm, head mesoderm; hn, Hensen's node; lm, lateral mesoderm; not, notochord; nt, neural tube; pchme, prechordal mesendoderm; som1, first somite.

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; Tirosh-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

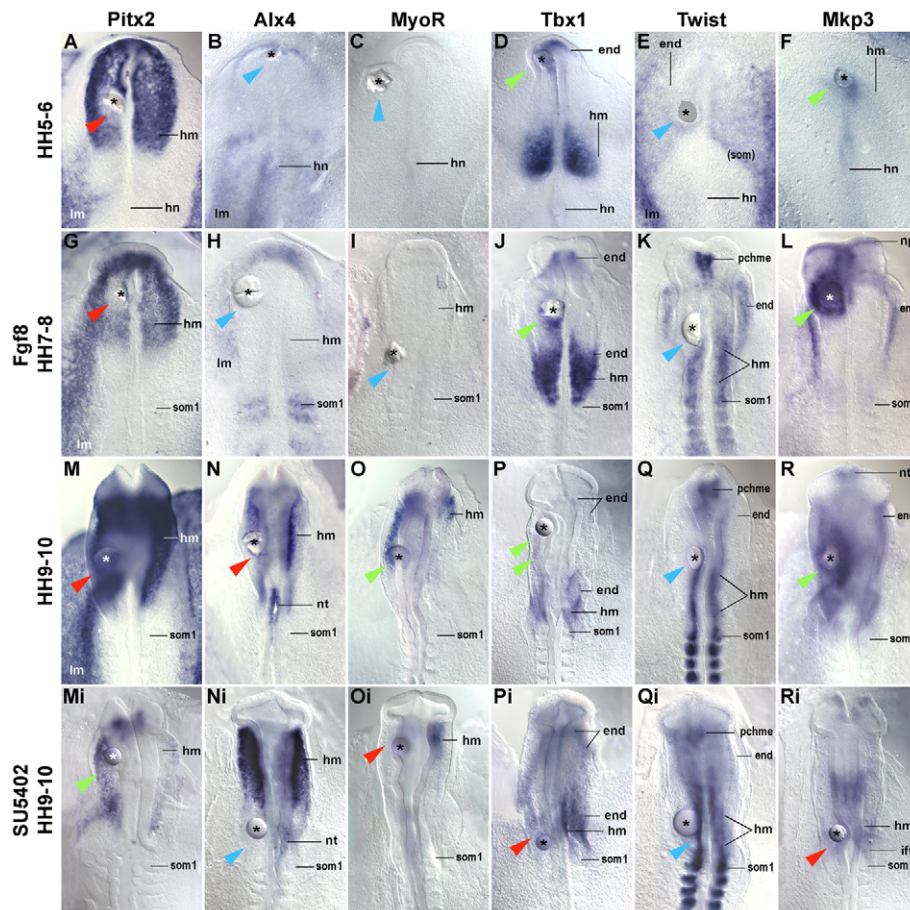


Fig. 6. Influence of Fgf and SU5402. (A-Ri) Dorsal views of chicken embryo heads treated with Fgf8 at HH4-5 (A-F), HH6-7 (G-L) and HH8-9 (M-R) or the Fgf antagonist SU5402 at HH8-9 (Mi-Ri). The BSA control for the Fgf experiment is shown in Fig. 4, the DMSO control for the SU5402 experiment in Fig. 3. The carrier beads are indicated by asterisks. Green arrowheads indicate upregulation, red arrowheads downregulation and blue arrowheads unchanged marker gene expression. Embryos were cultured for 5 hours to reach HH5-6 (early phase 1), HH7-8 (late phase 1) or HH9-10 (phase 2). Markers are indicated on top of the panel. Fgf mildly downregulated *Pitx2* and *Alx4* (A,G,M,N, red arrowheads) and strongly upregulated and expanded *Tbx1* expression (D,J,P, green arrowheads); SU5402 mildly upregulated *Pitx2* (Mi, green arrowhead) and suppressed *Tbx1* (Pi, red arrowhead). This suggests that Fgf initiates and drives the expansion of *Tbx1* expression and contributes to the restriction of the two anterior markers. Fgf upregulated and expanded *MyoR* expression at the time the gene would normally be expressed (O, green arrowhead), SU5402 downregulated *MyoR* expression (Oi, red arrowhead). Thus, Fgf is necessary but not sufficient to initiate, yet sufficient to expand, *MyoR* expression. (E,K,Q,Qi) Neither Fgf nor SU5402 treatment affected the expression of *Twist* (blue arrowheads). (F,L,R,Ri) As expected, Fgf upregulated (green arrowheads) and SU5402 downregulated (red arrowhead) expression of *Mkp3* at all times. end, endoderm; hm, head mesoderm; hn, Hensen's node; lm, lateral mesoderm; ift, inflow tract of the heart; np, neural plate; nt, neural tube; pchme, prechordal mesendoderm; som1, first somite.

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 read-out 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

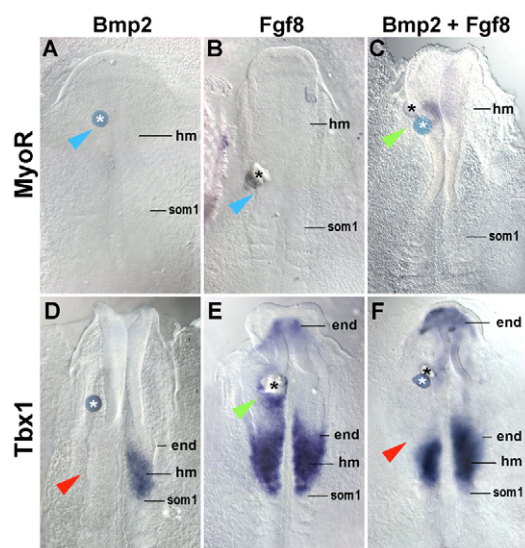


Fig. 7. Combinatorial signalling of Fgf and Bmp. (A-F) Dorsal views of chicken embryo heads treated with Bmp2 (A,D), Fgf8 (B,E), or Bmp2 and Fgf8 (C,F) at HH6-7. The carrier beads are indicated by asterisks. The embryos were cultured for 5 hours to reach HH7-8 (late phase 1) and analysed for the expression of *MyoR* (top row) and *Tbx1* (bottom row). At this stage, *MyoR* is not normally expressed yet; *Tbx1* is expressed in the posterior head mesoderm. Bmp2 and Fgf8 alone are unable to induce *MyoR* expression ahead of time (A,B, blue arrowheads). When both ligands are present, premature *MyoR* expression occurs (C, green arrowhead). Bmp suppresses *Tbx1* expression (D, red arrowhead), and Fgf alone upregulates the gene (E, green arrowhead). In the presence of both Bmp and Fgf, Bmp prevents the upregulation of *Tbx1* by Fgf and mildly downregulates *Tbx1* in its endogenous domain (F, red arrowhead). end, endoderm; hm, head mesoderm; som1, first somite.

dose-dependent fashion. 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, simultaneous 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

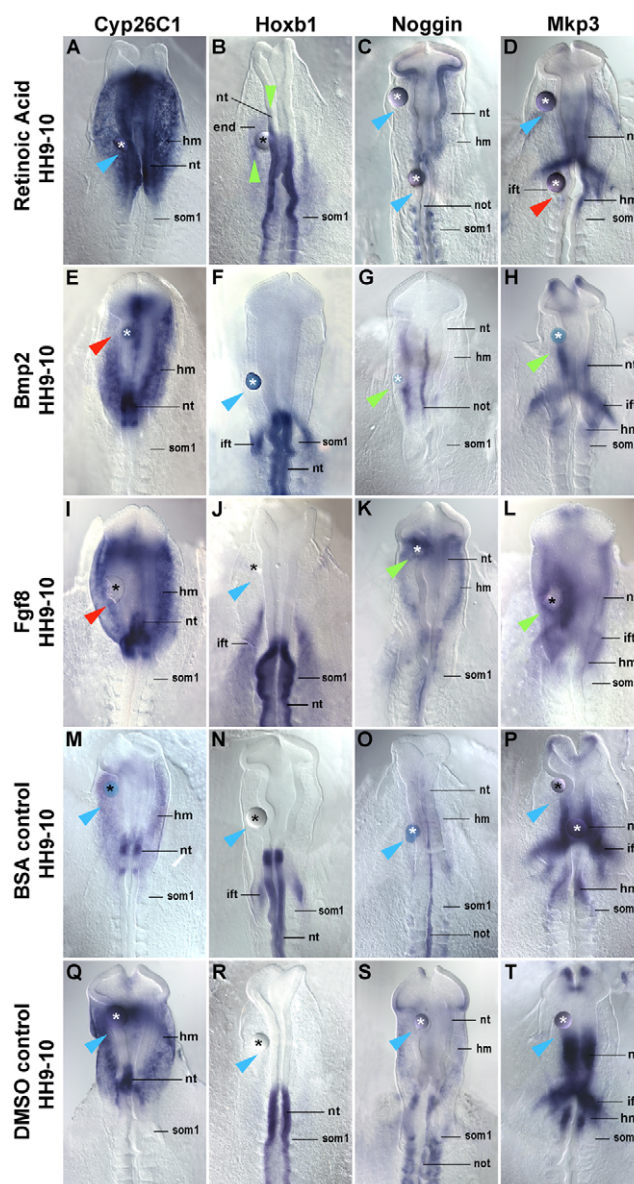


Fig. 8. Reciprocal control of the signalling systems. (A-T) To test for the cross-regulation of retinoic acid (RA), Bmp and Fgf signalling, beads loaded with ligand (A-L) or control beads (M-T) were implanted into the head mesoderm of HH8-9 embryos as indicated on the left of the panel. After 5 hours (at HH9-10), the embryos were analysed for the expression of *Cyp26C1*, the RA-responsive gene *Hoxb1*, the Bmp responsive gene *Noggin* and the Fgf-responsive gene *Mkp3* as indicated on top of the panel. Beads are marked by asterisks. Green arrowheads indicate upregulation, red arrowheads downregulation and blue arrowheads unchanged marker gene expression. RA did not affect the expression of *Cyp26C1* (A, blue arrowhead), but upregulated *Hoxb1* (B, green arrowheads). RA did not affect *Noggin*, but mildly downregulated *Mkp3* expression (C,D), suggesting that it negatively regulates Fgf signalling. Bmp2 and Fgf both downregulated *Cyp26C1* (E,I, red arrowheads). However, *Hoxb1* was not upregulated (F,J, blue arrowheads), suggesting that no significant gain of RA signalling occurred. Bmp, besides upregulating *Noggin* (G, green arrowhead), upregulated *Mkp3* expression in the anterior head mesoderm (H, green arrowhead). Likewise, Fgf, besides upregulating *Mkp3* (L, green arrowhead), upregulated *Noggin* expression in the anterior head mesoderm (K, green arrowhead). Thus, in the anterior head mesoderm, Bmp and Fgf signalling might reinforce each other. end, endoderm; hm, head mesoderm; ift, inflow tract of the heart; not, notochord; nt, neural tube; som1, first somite.

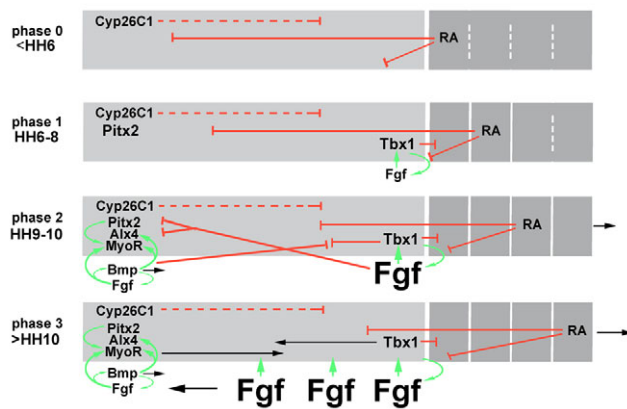


Fig. 9. Summary of the molecular network controlling the anteroposterior pattern of the head mesoderm. Schematic of head mesoderm patterning; head mesoderm is shown in light grey, somites in dark grey, anterior to the left. Red bars indicate suppression of genes, green arrows indicate activation of genes with black arrows pointing away from the name of a signal or marker indicate the direction into which this signal or marker is spreading. Black arrows on the right of the somites point at the direction of somite formation. In phase 0, head mesoderm markers are repressed by RA. In phase 1, falling RA levels and onset of Fgf signalling establish the initial a-p pattern with *Pitx2* marking the anterior and *Tbx1* the posterior head mesoderm. In phase 2, onset of Bmp signalling and low levels of Fgf signalling result in the activation of *Alx4* and *MyoR*, and refinement of the anterior pattern; the antagonism of Bmp (anterior domain) and *Tbx1*-high Fgf (posterior domain) reinforces the a-p boundary. In phase 3, the spreading of Fgf signalling along the pharynx facilitates the expansion of *MyoR* and *Tbx1* expression across the earlier a-p boundary. Further details are in the text.

et al., 2006), we conclude that the antagonism of Fgf-mediated *Tbx1* activity and RA determines the posterior boundary of the *Tbx1* domain.

In phase 2, activation of anterior BMP and Fgf signalling refines the anterior pattern

Expression of Fgf and Bmp responsive molecules indicated that the anterior head mesoderm receives Fgf and Bmp signals for the first time during phase 2 when *Alx4* and *MyoR* are upregulated (Faure et al., 2002; Lunn et al., 2007) (this study). Suppression of Bmp signalling prevented, and elevated Bmp signalling advanced, *Alx4* activation. Thus, Bmp is necessary and sufficient to control *Alx4*. *MyoR*, however, was repressed by suppression of either Bmp or Fgf signalling. Elevation of Bmp or Fgf signalling promoted *MyoR*, albeit only at the stage at which the gene is normally expressed; premature *MyoR* expression could only be achieved by combinatorial application of Bmp and Fgf. Thus, combined Fgf and Bmp activity is required to activate *MyoR*.

Our expression analysis showed that the onset of *MyoR* is rather sudden. The bead implantation experiments indicated that in the anterior head mesoderm, Fgf enhanced the expression of Bmp responsive genes and Bmp upregulated genes indicative of active Fgf signalling. This suggests that Bmp and Fgf reinforce each other, possibly creating the appropriate setting to activate *MyoR*. Studies on mouse mutants placed *Pitx2* upstream of *MyoR* (Dong et al., 2006; Shih et al., 2007). Thus, it is conceivable that, in addition to Bmp and Fgf, the earlier activation of *Pitx2* is a further prerequisite for the activation of *MyoR*.

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; Ryckebusch 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; Ryckebusch 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.

Acknowledgements

We are most grateful to L. Alvares, M. Baylies, A.-G. Borycki, M. Buckingham, J. Carvajal, P. Currie, P. Francis West, M. Gani, O. Halevy, S. Hughes, P. Ingham, K. Jagla, E. Jorge, G. Kardon, R. Kelly, R. Knight, R. Krauss, S. Kuratani, G. Marcelli, P. Maire, B. Mankoo, C. Marcelle, A. Münsterberg, D. Noden, S. Nowotschin, F. Relaix, F. Schubert, C. Sharpe, S. Tajbakhsh and E. Tzahor for inspiring discussions and invaluable support. We thank F. Schubert for critically reading the manuscript, and the EU Network of Excellence MyoRes and HFSP for funding this work.

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.062737/-DC1>

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Table S1. Summary of results

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

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