Title:

Cardiac competence of the head mesoderm fades concomitant with a shift towards the head skeletal muscle programme

Running title:

Shifting cellular competence in the PHM

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Summary statement:

The head mesoderm has generic cardiac competence until head fold stages. Thereafter, cardiac competence fades in the paraxial region, and Bmp activates head skeletal muscle programmes instead of cardiac programmes.

Abstract

The vertebrate head mesoderm provides the heart, the great vessels, smooth and most head skeletal muscle, and parts of the skull base. The ability to generate cardiac and smooth muscle is thought to be the evolutionary ground-state of the tissue, and initially the head mesoderm has cardiac competence throughout, even in the paraxial region that normally does not engage in cardiogenesis. How long this competence lasts, and what happens as cardiac competence fades, is not clear.

Using a wide palette of marker genes in the chicken embryo, we show that the paraxial head mesoderm has the ability to respond to Bmp, a known cardiac inducer, for a long time. However, Bmp signals are interpreted differently at different time points. Bmp triggers cardiogenesis up to early head fold stages; the ability to upregulate smooth muscle markers is retained slightly longer. Notably, as cardiac competence fades, Bmp activates the head skeletal muscle programme instead.

Keywords

Vertebrate embryos, head mesoderm, cardiogenesis, head muscles, developmental competence, Bmp.

Introduction

The vertebrate head mesoderm is the first embryonic mesoderm type to emerge during gastrulation (Camp et al., 2012; Garcia-Martinez and Schoenwolf, 1993). It delivers the heart (reviewed in (Meilhac and Buckingham, 2018; Spater et al., 2014), the great vessels (Paffett-Lugassy et al., 2013; Wang et al., 2017), the smooth muscle collar of the cardiac outflow (Waldo et al., 2005), genuine craniofacial

and oesophageal skeletal muscles (reviewed in (Schubert et al., 2018) and parts of the skull base ((Jandzik et al., 2015); reviewed in (Kuratani, 2005)). The head mesoderm has received quite some attention since in a subpopulation of cardiogenic cells, the secondary heart field (SHF) cells, differentiation is delayed. When these cells are recruited into the heart to deliver the mature in- and outflow tract, they integrate perfectly and beat according to the rhythm set by the pre-existing cells. This is in stark contrast to cells transplanted into an adult heart to replace cells lost after a heart attack: here, cells struggle to integrate, and their autonomous beating may cause potentially fatal arrhythmias (Cui et al., 2018; Scuderi and Butcher, 2017; Spater et al., 2014).

Transcriptome analyses and grafting experiments showed that the head mesoderm is distinct from the trunk mesoderm which lacks cardiac competence and forms skeletal muscle using distinct programmes. Comparative analyses between vertebrates and invertebrate chordates indicated that this distinction is evolutionarily conserved. Moreover, since originally, chordates lacked mesoderm-derived cartilage and bones, cranial muscle was pharyngeal smooth muscle, and skeletal muscle was for the purpose of locomotion and formed from trunk mesoderm only, it is thought that the evolutionary default programme of the head mesoderm was to deliver cardiac and smooth muscle (Schubert et al., 2018).

Cardiogenic cells are specified but not determined as they leave the primitive streak (Lopez-Sanchez et al., 2009; Wang et al., 2013). Migration to their target area lateral of the developing neural plate is controlled by bone morphogenetic proteins (Bmp; (Song et al., 2014)). Moreover, Bmps are essential for cardiogenic cells to realise their potential and differentiate (Cui et al., 2018; Spater et al., 2014; van Wijk et al., 2007). Bmps drive cardiogenesis indirectly by suppressing the proliferation-promoting function of Fibroblast growth factors (Hutson et al., 2010; Tirosh-Finkel et al., 2010). They also control cardiogenesis directly, with Smad1/5/8-Smad4 complexes transactivating genes encoding key cardiac transcription factors such as Isl1, Nkx2.5, Gata4 and Tbx2 (Hami et al., 2011; Liberatore et al., 2002; Lien et al., 2002; Shirai et al., 2009; Si et al., 2014). Mesodermal cells expressing the pioneer factor Isl1 still have non-cardiogenic options, however the expression of Nkx2.5 drives cells towards a cardiomyocyte fate (Gao et al., 2019; Jia et al., 2018). Nkx2.5 is not sufficient for cardiomyocyte differentiation, but the combinatorial expression of cardiogenic transcription factors allows beating cardiomyocytes to form, both in vivo and in vitro (Ieda et al., 2010; Luna-Zurita et al., 2016; Zhou et al., 2012).

Before and during the formation of the primitive heart, the Bmp ohnologs Bmp2 and 4 are expressed in the lateral and extraembryonic mesoderm, its overlying ectoderm and underlying pharyngeal endoderm and the neural folds (Supplementary Material S2; for Bmp phylogeny see (Hinck et al., 2016; Huminiecki et al., 2009)). Bmp2 continues to be expressed in the cardiac inflow tract, Bmp4 in the SHF

adjacent to the heart. The heart expresses Bmp7 throughout, the ventricle expresses Bmp10. Thus, the lateral head mesoderm and the interface to the paraxial head mesoderm (PHM) is under the influence of Bmp all the time. On the other hand, the Bmp inhibitors Noggin and Chordin are expressed in Hensen's node and in the notochord in the midline of the embryo. Therefore, the PHM that remains close to the midline and dorsal to the pharynx is normally not recruited into cardiogenesis.

Using the ability of Bmps to induce cardiac markers as experimental paradigm, various studies showed that when it is first laid down, the entire head mesoderm including the PHM has cardiac competence (Andree et al., 1998; Bothe et al., 2011; Schlange et al., 2000; Schultheiss et al., 1997; Tirosh-Finkel et al., 2006; von Scheven et al., 2006a; Yamada et al., 2000). However, how long this generic cardiac competence, specifically the ability to generate cardiomyocytes, lasts, and what happens as and when cardiac competence fades, is unclear. This is because the earlier studies used different Bmps, different methods for Bmp application, different Bmp concentrations, different exposure times and different marker genes (detailed in Supplementary material S1). Thus, some studies ascribed cardiac competence to the PHM of head-fold stage embryos only, in the chicken at HH5/6 (Bothe et al., 2011; Schlange et al., 2000). Other studies suggested that the generic cardiac competence of the head mesoderm lasts until HH10 or even until HH13/14, an early pharyngula stage (Tirosh-Finkel et al., 2006).

To clarify this problem, and to lay the foundations for studies on the molecular regulation of cardiac competence in developing and therapeutic cardiogenic cells, we systematically investigated the ability of the PHM to respond to Bmp at different time points of development, using the chicken embryo as model. We supplied Bmp proteins on carrier beads, because Bmp concentrations produced by tissue culture cells are not known, and to avoid a possible contribution of additional factors released from the cells. We show that six-hour-exposure to beads loaded with 6.41µM Bmp is sufficient to trigger robust expression changes for direct Bmp-response genes such as *Noggin* at all developmental stages, and responses were independent of the type of Bmp or bead used. Yet the full set of cardiac markers could only be activated at HH5/6, and only at this stage, completion of cardiomyocyte differentiation was observed upon prolonged Bmp treatment. As development proceeded and cardiac competence faded, distinct sets of marker genes were activated. These newly expressed markers suggested a shift away from cardiac/cardiomyocyte and smooth muscle programmes to the programme for craniofacial myogenesis.

Results and Discussion

The key parameter affecting the response to Bmp treatment is the Bmp concentration.

In previous studies, different bead types, different Bmp concentrations and different Bmp paralogs were used (Supplementary material S1). We therefore explored first, which of these parameters would differentially affect cardiac induction in the PHM at stage HH5/6, the stage known for its generic cardiac competence throughout the head mesoderm. We focused on *Isl1,* a Bmp-dependent marker for the cardiogenic head mesoderm and underlying pharyngeal endoderm, and *Tbx1*, a gene expressed in the pharyngeal endoderm, early caudal PHM and branchiomeric muscles, and repressed by Bmp ((Bothe and Dietrich, 2006; Bothe et al., 2011; Meireles Nogueira et al., 2015) and references therein). Results for all experiments are summarised in Supplementary material S2, n-numbers are in Supplementary material S3.

We found that at 0.192µM, the concentration said to be used by (Schultheiss et al., 1997), Bmp did not upregulate *Isl1*. At 1.28µM used by (Bothe et al., 2011), *Isl1* was mildly, at 6.41µM, *Isl1* was robustly upregulated (Supplementary Figure SF2A-D). *Isl1* upregulation was slightly more widespread with Affi-Gel "blue" beads than with heparin-coated acrylic "white" beads, possibly because heparin is able to retain the loaded protein (Ori et al., 2009). However, because there was no qualitative difference between marker gene responses, and because bead retention in the tissue was better for white beads, we continued with white beads. Beads loaded with 6.41µM of the Bmp ohnologs Bmp2 and Bmp4, the more distantly related paralog Bmp10 and the even further distant Bmp7 all upregulated *Isl1* (Fig.S1E-I) and suppressed *Tbx1* (Fig.S1J-N). Thus, in our 6 hours assay, all Bmp paralogs had the same effect, likely because all trigger the pSmad1/5/8 pathway (reviewed in (Hinck et al., 2016)). Consequently, the key parameter to study the duration of cardiac competence of the head mesoderm is the concentration of the Bmp, not the Bmp or bead type.

Only the HH5/6 PHM is able to activate the full set of cardiogenic transcription factors.

Next, we investigated whether cardiac markers can be activated in the PHM at stages HH5/6, HH7/8, HH9/10, HH13/14, using 6.41µM Bmp2 on white beads in the same 6-hour assay as before. As markers, we selected *Isl1*, *Nkx2.5* and *Tbx2*, expressed in the cardiogenic mesoderm and underlying endoderm, and *Gata4*, *Hand2*, *Tbx5*, at the relevant stages largely confined to mesodermal tissues (Supplementary material S2 and references therein). All of these genes encode transcription factors that are necessary for cardiac development and, in combination, drive induced pluripotent stem cells into cardiogenesis ((Ieda et al., 2010; Luna-Zurita et al., 2016; Zhou et al., 2012), reviewed in (Cui et al., 2018; Spater et al., 2014). Moreover, *Isl1*, *Nkx2*.5, *Gata4* and *Tbx2* are direct Bmp targets (Brown et al., 2004; Chi et al., 2005; Hami et al., 2011; Liberatore et al., 2002; Lien et al., 2002; Pal and Khanna,

2006; Shirai et al., 2009; Si et al., 2014)). At HH13/14, we also implanted Bmp beads between flank somites and the neural tube as control, reasoning that *Hand2*, *Gata4*, *Tbx2*, genes with widespread lateral/extraembryonic expression, would be upregulated there; as further control, we assayed for the down-regulation of somitic *Myf5*/*MyoD* expression (Berti et al., 2015; von Scheven et al., 2006a). Finally, we allowed HH13/14 embryos to develop overnight to test for possible delayed responses (Supplementary materials S2, S3, and data not shown).

Expression of *Isl1* was upregulated in the mesoderm and endoderm at stages HH5/6 as shown previously (Fig.1A; (Bothe et al., 2011). Yet we also obtained an upregulated expression at HH7/8 and 9/10, because we now used higher Bmp concentrations. At HH13/14, both in the 6-hour and in the overnight experiments, *Isl1* expression occasionally expanded from the SHF towards the bead, similar to the results by Tirosh-Finkel (2006); however, an ectopic expression around the bead was not observed (Fig.1Di,ii). Similar to *Isl1*, both *Nkx2.5* (Fig.1E-H) and *Tbx2* expression (Fig.1I-L) was upregulated by Bmp at HH5/6 to HH9/10 (Fig.1E-G; I-K). At HH13/14 however, none of these markers changed expression in the head (Fig.1Hi,ii; Li,ii). In the trunk *Tbx2* was upregulated, in line with its association with trunk lateral mesoderm development (Fig.1Liii). *Hand2* expression was upregulated around the Bmp beads in the HH5/6 to 9/10 PHM and in the HH13/14 trunk (Fig.1M-O,Piii). In the HH13/14 head, again only an expansion of the expression domain towards the bead was observed, and only in 1 of 4 embryos (Fig.1Pi,ii). *Gata4* was upregulated in the PHM at HH5/6 and 7/8 (Fig.1Q-R), not however at HH9/10 or HH13/14 (Fig.1S, Ti,ii); in the trunk, ectopic expression was induced (Fig.1Tiii). *Tbx5* had been described as a Bmp-unresponsive gene (Yamada et al., 2000), but we were able to obtain upregulated expression at HH5/6 (Fig.1U), possibly because we used a higher Bmp concentration compared to the previous study. Nonetheless, beyond HH5/6, *Tbx5* never responded to Bmp (Fig.1V-Xiii). Taken together, the full set of marker genes used here was only ever activated at HH5/6, thereafter the set of Bmp-responsive cardiac genes became successively more limited.

Only the HH5/6 PHM is able to complete cardiomyocyte differentiation

Our data suggest that the chicken PHM may retain cardiac competence until HH5/6. Yet the head mesoderm is laid down by the primitive streak at HH3 (Camp et al., 2012; Garcia-Martinez and Schoenwolf, 1993). Thus at HH5/6, the cardiac mesoderm and the PHM had already some 10-12 hours of Bmp-dependent or Bmp–independent development, respectively. To test whether and when the PHM may be able to complete cardiomyocyte differentiation, we again implanted Bmp beads at stages HH5/6 to HH13/14, this time assaying for the expression of the *Mef2c* and *Myocd*, two transcription factors associated with the activation of genes encoding muscle structural proteins and often part of cardiomyocyte-inducing gene cocktails (reviewed in (Cui et al., 2018; Gordon, 2018; Spater et al., 2014). We also investigated expression changes of *Myom1*, *Myh15*/sarcomeric Myosins, *Tnnt2*, all

indicating terminal differentiation of striated (cardiac and skeletal) muscle (see Supplementary Material S2). When embryos were exposed to Bmp for 6 hours, none of these markers was induced at any stage, even when the Bmp concentration was further increased to 19.23µM (Fig.2A-F, Supplementary Figure SF2). When the embryos were incubated overnight, then sarcomeric Myosin expression was found around beads with 19.23µM Bmp, but only in HH5/6 host (Fig.2L); no ectopic expression was ever seen at later stages (Fig.2M-R).

After HH5/6 Bmp upregulates smooth muscle and skeletal muscle precursor markers

Our data indicate that generic cardiac competence in the head mesoderm fades after HH5/6. Yet *Isl1*, which marks cells with myocardial but also vascular, smooth or head skeletal muscle potential (Jia et al., 2018; Nathan et al., 2008), and *Hand2*, a marker for head and trunk lateral mesoderm (Firulli et al., 2005; Lopez-Sanchez et al., 2009), could be activated in the PHM as late as HH10 and its expression dorsally expanded at HH13/14. This suggests that the PHM remains Bmp responsive. Moreover, Bmp is expressed in the pre-chordal plate, the pharyngeal pouches, the otic vesicle and the dorsal neural tube, suggesting that some regions of the PHM are exposed to Bmp (Bothe et al., 2011; von Scheven et al., 2006a). This then infers that, as cardiac competence fades, the PHM may respond to Bmp by activating non-(myo)cardial programmes. To test this, we first confirmed Bmp-responsiveness in the head, assaying for the expression of *Noggin*, a Bmp inhibitor activated in response to exogenously applied Bmp (Bothe et al., 2011; Sela-Donenfeld and Kalcheim, 2002). We also investigated the expression of *Gata3*, a marker for the pharyngeal endoderm and ectoderm and, in the trunk, a marker for the mesonephric duct, all sites subject to Bmp signalling (Begbie et al., 1999; Bothe and Dietrich, 2006; James and Schultheiss, 2005; Sheng and Stern, 1999). Both markers were activated within 6 hours, at all stages and sites investigated here (Fig.3A-Hiii, Supplementary Table1).

We next used *Nfatc1*, a maker with high expression levels in the endocardium and low expression levels in blood vessels, the notochord and, at HH13/14, the pharyngeal endoderm (unpublished observations and (Wu et al., 2011)). This marker did not respond to Bmp at HH5/6 to HH9/10, suggesting that Bmp did not move cells towards an endocardial fate (Fig.3I-K). At HH13/14, however expression was upregulated in the endoderm (Fig.3Hi,ii).

Acta2 is a marker for the early cardiogenic cells, and remains expressed in embryonic cardiac (and skeletal) muscle for some time (Lopez-Sanchez et al., 2009). However, the gene has a widespread expression in the extraembryonic mesoderm, demarcating developing vascular smooth muscle (Colas et al., 2000; Lopez-Sanchez et al., 2009). Likewise, the gene is expressed in the collar of smooth muscle that demarcates the junction between the heart and the great vessels (Waldo et al., 2005), and it is smooth muscle that retains *Acta2* in the adult (e.g. oesophageal smooth muscle, (Gopalakrishnan et

al., 2015). Interestingly, *Acta2* was upregulated by Bmp between at stages HH5/6 to HH9/10 (Fig.3M-O), suggesting a prolonged competence of the PHM to produce smooth muscle.

Finally, we assayed for the expression of *Msc* (*MyoR*). This gene is first activated in the rostral PHM in response to Bmp and then spreads caudally to eventually label all craniofacial skeletal muscle precursors and to participate in the activation of *MyoD* (Bothe and Dietrich, 2006; Bothe et al., 2011; Meireles Nogueira et al., 2015; Moncaut et al., 2012; von Scheven et al., 2006b). Using a higher Bmp concentration than in our original study (Bothe et al., 2011), we were able to upregulate *Msc* at stages HH7/8 rather than HH9/10 (Fig.3R,S). Moreover, within the 6 hour time window, *Msc* was readily upregulated at HH13/14 (Fig.3Ti,ii). The response was specific to the PHM since in the trunk, *Msc* was not activated (Fig.3Tiii). This suggests that as cardiac competence fades, competence to engage in craniofacial myogenesis becomes established in the PHM.

Conclusion

Our study reconciles previous studies, showing that different Bmp concentrations lead to different outcomes in cardiac induction assays. Nonetheless, using a large set of marker genes, we can show that the head mesoderm has generic cardiac competence from the time of its gastrulation to early head fold stages only; smooth muscle competence is maintained slightly longer. Yet as cardiac competence ceases, Bmp signals promote the programme for head skeletal muscle formation (Fig.4). Our study allows to now depict the correct time point to analyse the cellular competence of natural and engineered cardiogenic cells.

Material and methods

Chicken embryo culture and staging

Fertilised chicken eggs were obtained from Henry Stewart & Co. (Lincolnshire, UK) and incubated at 38.5°C in a humidified incubator (LMS) to the desired stages, following the staging system of Hamburger and Hamilton (Hamburger and Hamilton, 1951). For bead implantation experiments at HH5-10, embryos were cultured on filter rings as described by Chapman and Bothe (Bothe et al., 2011; Chapman et al., 2001). Experiments at HH13/14 were performed in ovo.

Recombinant proteins and bead preparation

Recombinant Bmp proteins from R&D Systems/Biotechne (Bmp2: 355-BM-10, Bmp4: 314-BP-10, Bmp7: 354-BP-10, Bmp10: 2926-BP-025) were reconstituted in 4mM HCl, supplemented with 0.1% bovine serum albumin, at a concentration of 1mg/ml, and then diluted in sterile water to obtain 0.19µM, 1.28µM, 6.41µM or 19.23µM. Affi-Gel blue agarose beads (BioRad) and heparin-coated acrylic beads (Sigma) were soaked in these solutions together for an hour, then washed in saline before grafting. Control beads were soaked in 0.1% BSA in saline.

Microsurgical manipulation, bead implantation and embryo harvesting

Beads were grafted into the paraxial head mesoderm (PHM) of embryos at stages HH5/6, HH7/8, HH9/10, HH13/14, using flame-sharpened tungsten needles ((Bothe et al., 2011; von Scheven et al., 2006a), Supplementary Figure SF1). The embryos were incubated for 6 hours, during which time they may progress to the next stage of development. Six hours of incubation allows for responses of direct and the first wave of indirect targets of signalling pathways (Bothe et al., 2011). HH5/6 and HH13/14 embryos were also incubated overnight to allow for the response of indirect targets further downstream in regulatory cascades. All embryos were harvested in 4% PFA.

Generation of templates and probes for in situ hybridisation

Probes for in situ hybridisation and wildtype gene expression patterns are summarised in Supplementary Material S2. Templates for probe synthesis were generated using restriction endonucleases or by PCR with plasmid-based primers; antisense probes were made using the appropriate T3/T7/sp6 RNA polymerases and the DIG labelling mix (Roche/Sigma).

In situ hybridisation

In situ hybridisation followed the procedure described in (Bothe et al., 2011). Embryos were permeabilised first in methanol, then with a detergent mix (15 minutes), re-fixed and equilibrated in pre-hybridisation mix for 1 hour. Hybridisation was performed overnight at 70°C; probes were

detected using the alkaline phosphatase-coupled anti-DIG antibody (1:2000, Roche/Sigma) and NBT/BCIP (Roche/Sigma). After staining, embryos were fixed in 4% PFA and stored in 80% glycerol.

Immunofluorescence

Immunofluorescence followed the procedure described in (Berti et al., 2015; Meireles Nogueira et al., 2015), using the mouse MF20 (1:500, DSHB) primary antibody and the anti-mouse IgG+IgM (H+L) – Alexa fluor 594 secondary antibody (1:200, Jackson Immuno).

Photomicroscropy

Images were captured on a Zeiss Axioskop with DIC and fluorescence optics, using a Zeiss AxioCam digital camera with ZEN light software. Images were processed using Adobe Photoshop 6.0.

Acknowledgements

We thank Soraya Idris-Anderson and Ashley Haywood for their assistance to the project, the IBBS section Epigenetics and Development for comments, and Frank Schubert for critically reading the manuscript. The work was funded by a UoP grant.

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Figure legends

Figure 1. Bmp activates the full set of cardiac marker at HH5/6 only.

Beads with 6.41µM Bmp2 were implanted into the PHM or trunk of embryos at HH5/6, 7/8, 9/10 and 13/14, assaying for the expression of the cardiac transcription factors shown on the left. Images (i-iii) are from the same specimen. Dorsal views, rostral to the top in all except (i,ii) which are lateral views. Abbreviations: Supplementary material S3. The beads are marked by asterisks. Upregulated gene

expression around the bead is indicated by green arrowheads, expanded expression domains by green arrows, unchanged expression by blue arrowheads. Note the declining responses to BMP after HH5/6.

Figure 2. Only the HH5/6 PHM can complete myocardial differentiation.

Implantation of control beads and beads loaded with 6.41µM or 19.23µM Bmp2 into the HH5/6, 7/8, 9/10 and 13/14 PHM, followed by staining for sarcomeric Myosins. Embryos were left to develop for 6 hours (A-F, dorsal views) or overnight (G-R, ventral views). Note: ectopic cardiomyocyte differentiation occurs after overnight incubation with 19.23µM Bmp2, but only in HH5/6 hosts.

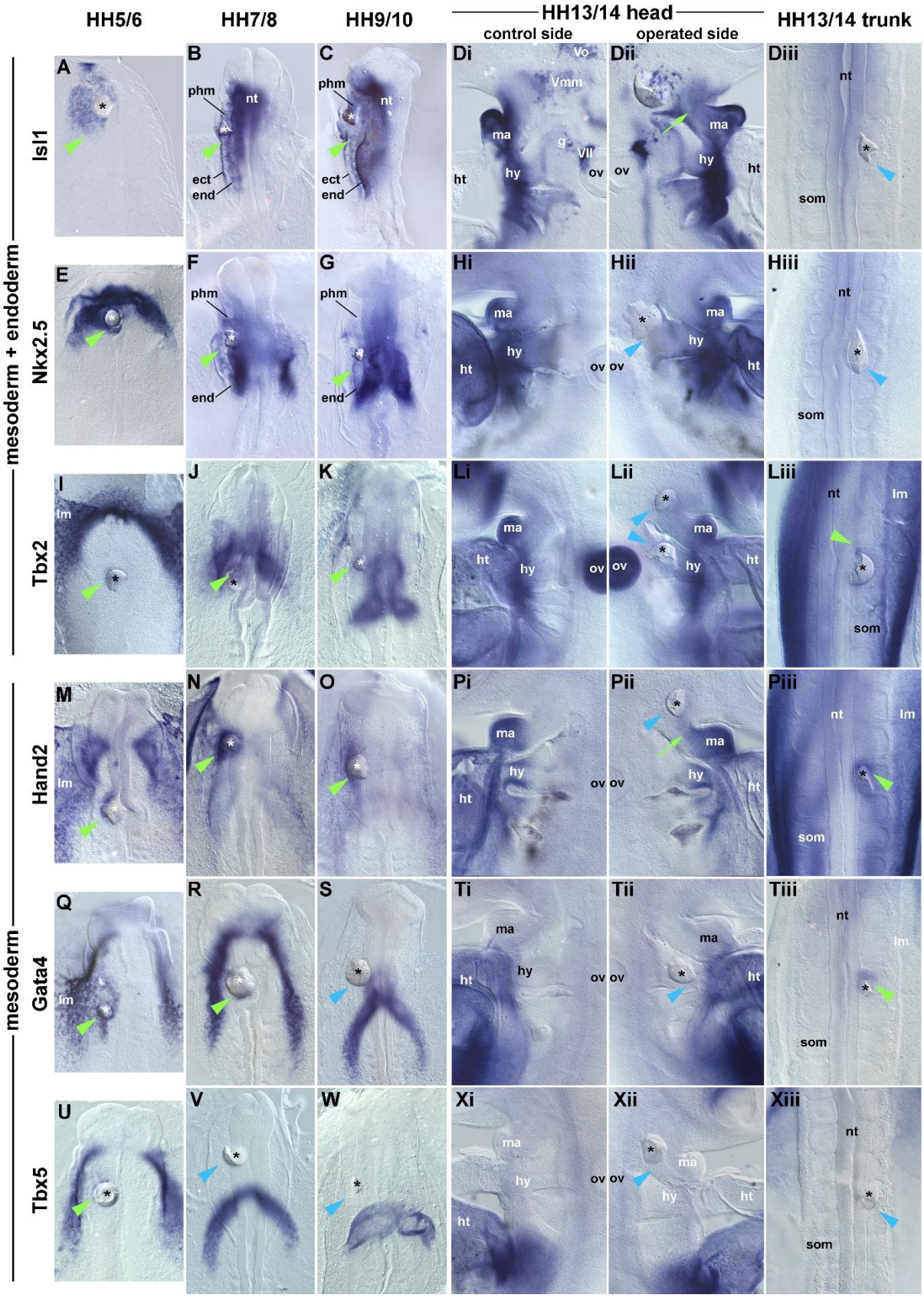
Figure 3. The head skeletal muscle programme becomes available after HH5/6.

Implantation of Bmp2 beads as in Figure 1. The upregulated expression of *Noggin* (A-Diii) indicates Bmp responsiveness in craniofacial tissues and the trunk. Bmp upregulated *Gata3* in the pharyngeal ectoderm, endoderm and trunk mesoderm (E-Hiii) as well as *Nfactc1* in the HH13/14 pharyngeal endoderm (Li,ii). (M-Pii) *Acta2*, a marker with sustained expression in smooth muscle, responds to Bmp at stages HH5/6 to 9/10. (Q) *Msc*, a marker for craniofacial skeletal muscle, is not upregulated at stage HH5/6 when the PHM has cardiac competence. Thereafter, *Msc* is robustly activated (R-Tii).

Figure 4. Summary.

The head mesoderm has full cardiac competence until HH5/6, and prolonged smooth muscle competence until HH9/10. In the PHM, the head skeletal muscle programme becomes available as cardiac competence declines.

Figure 1


HH5/6
HH7/8
HH9/10
HH13/14 head
control side
operated side
HH13/14 trunk
mesoderm + endoderm
mesoderm
Isl1
Nkx2.5
Tbx2
Hand2
Gata4
Tbx5
A
B
C
Di
Dii
Diii
E
F
G
Hi
Hii
Hiii
I
J
K
Li
Lii
Liii
M
N
O
Pi
Pii
Piii
Q
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S
Ti
Tii
Tiii
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V
W
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Figure 2

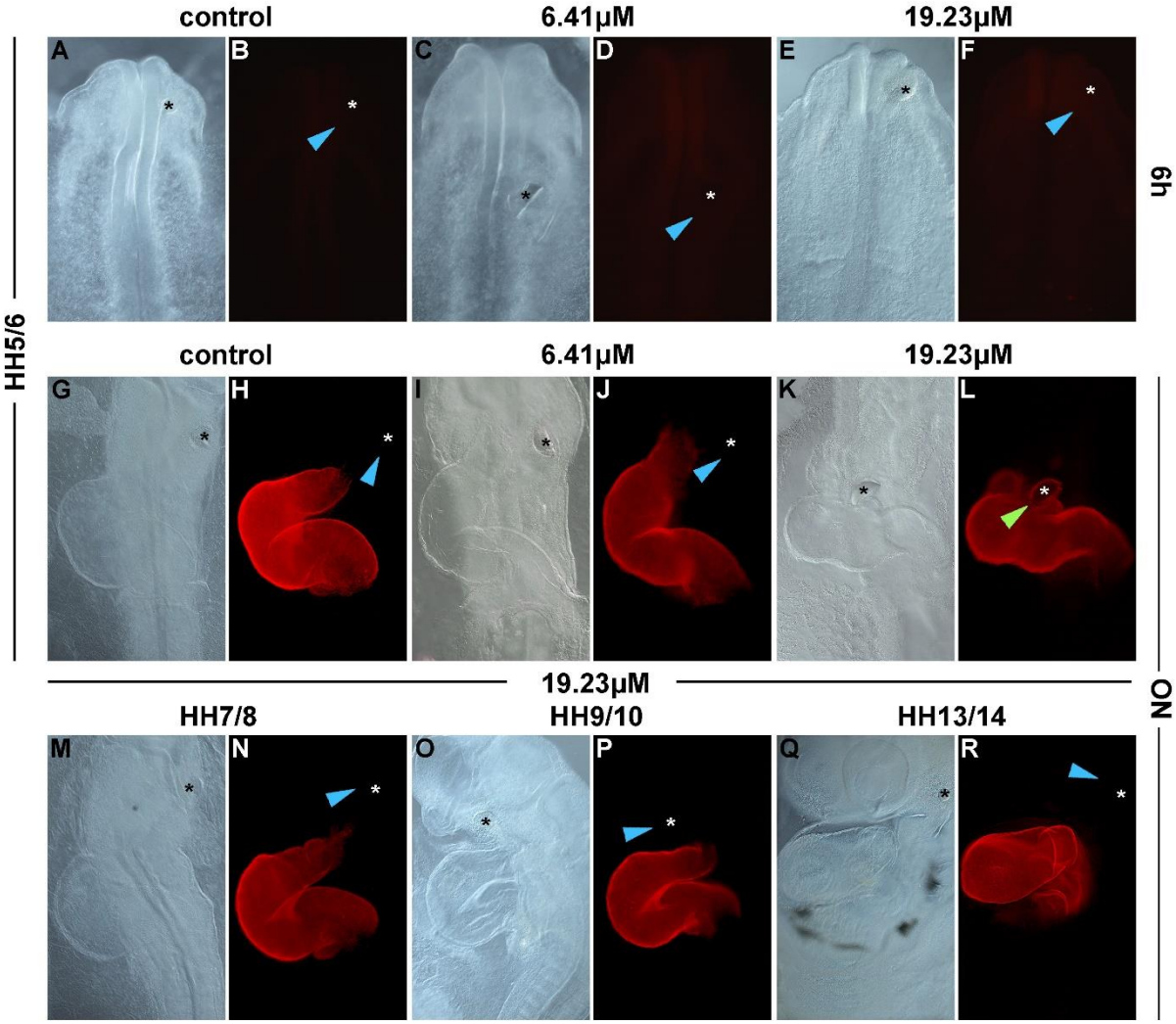
HH5/6
control
6.41µM
19.23µM
6h
A
B
C
D
E
F
control
6.41µM
19.23µM
G
H
I
J
K
L
19.23µM
ON
HH7/8
HH9/10
HH13/14
M
N
O
P
Q
R

Figure 3

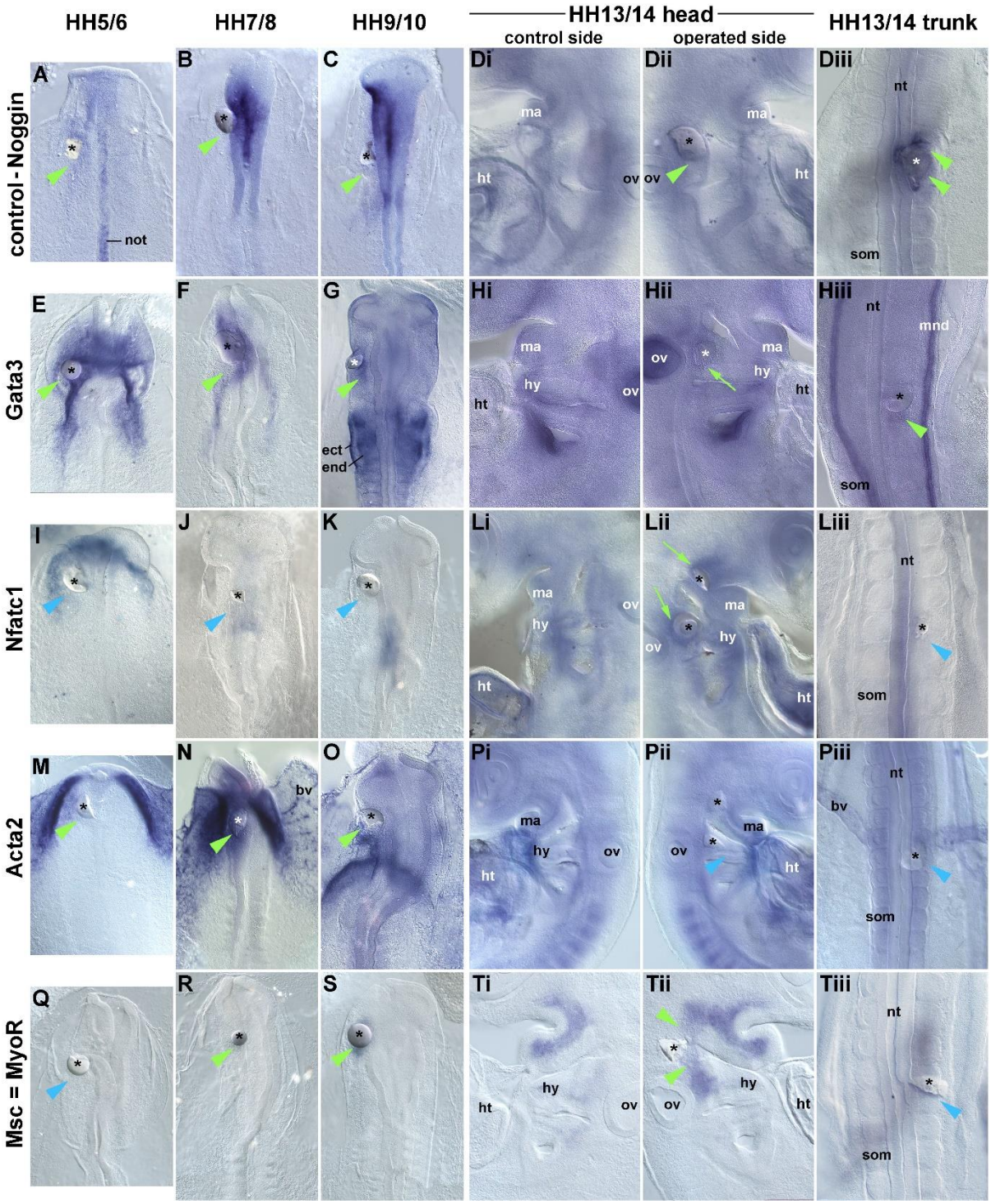
HH5/6
HH7/8
HH9/10
HH13/14 head
control side
operated side
HH13/14 trunk
control - Noggin
Gata3
Nfatc1
Acta2
Msc = MyoR
A
B
C
Di
Dii
Diii
E
F
G
Hi
Hii
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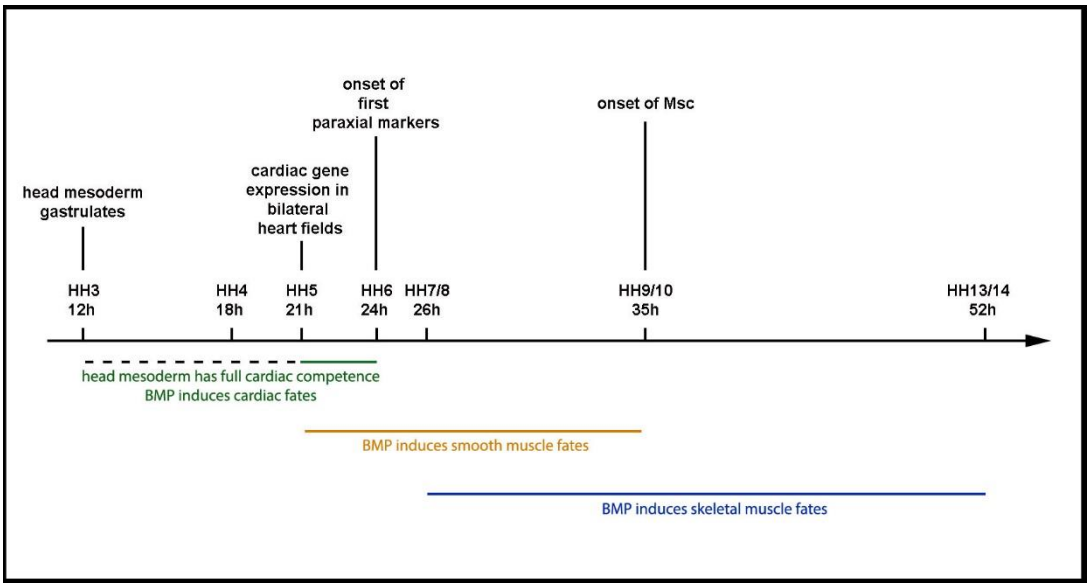
Figure 4


onset of
first
paraxial markers
onset of Msc
cardiac gene
expression in
bilateral
heart fields
head mesoderm
gastrulates
HH3
12h
HH4
18h
HH5
21h
HH6
24h
HH7/8
26h
HH9/10
35h
HH13/14
52h
head mesoderm has full cardiac competence
BMP induces cardiac fates
BMP induces smooth muscle fates
BMP induces skeletal muscle fates