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Abstract

Branchiomeric muscles of the head and neck originate in a population of cranial mesoderm termed cardiopharyngeal mesoderm that also contains progenitor cells contributing to growth of the embryonic heart. Retrospective lineage analysis has shown that branchiomeric muscles share a clonal origin with parts of the heart, indicating the presence of common heart and head muscle progenitor cells in the early embryo. Genetic lineage tracing and functional studies in the mouse, as well as in *Ciona* and zebrafish, together with recent experiments using single cell transcriptomics and multipotent stem cells, have provided further support for the existence of bipotent head and heart muscle progenitor cells. Current challenges concern defining where and when such common progenitor cells exist in mammalian embryos and how alternative myogenic derivatives emerge in cardiopharyngeal mesoderm. Addressing these questions will provide insights into mechanisms of cell fate acquisition and the evolution of vertebrate musculature, as well as clinical insights into the origins of muscle restricted myopathies and congenital defects affecting craniofacial and cardiac development.

Introduction

Dissecting the embryological origin of skeletal muscle diversity is an important approach towards understanding how certain myopathies affect specific sets of muscles. Despite this medical significance, mechanistic insight into the development of head and neck skeletal muscles has lagged behind knowledge of the pathways regulating trunk and limb muscles derived from somitic paraxial mesoderm [1]. Here we will focus on the development of a subset of head and neck muscles, termed branchiomeric muscles as they originate in the mesodermal core of bilateral pharyngeal or branchial arches. The pharyngeal or branchial arches are transient embryonic structures that appear as a series of swellings on both sides of the developing pharynx. Five bilateral arches form in an anterior to posterior sequence, each of which contains a mesodermal core surrounded by neural crest derived mesenchyme and endodermal and ectodermal epithelia [2]. Branchiomeric muscles include muscles of mastication derived from the first arch, muscles of facial expression from the second arch and muscles of the pharynx as well as laryngeal, oesophageal and superficial neck muscles from posterior pharyngeal arches. We will discuss recent advances emerging from the discovery that branchiomeric muscles share a clonal origin and upstream regulatory program with cardiac progenitor cells within a developmental field of cardiopharyngeal mesoderm (CPM) [3]. Supporting evidence for common heart and head muscle progenitor cells comes from genetic tracing studies and analysis of myogenesis in different chordate species, including the tunicate *Ciona*, where bipotent cardiac and pharyngeal muscle progenitor cells have been characterized in detail at the cellular and molecular levels. Stem cell studies have circumvented the challenges of looking at myogenic cell fate choices in large progenitor cell populations in vertebrate embryos and provide additional evidence for the existence of bipotent head and heart muscle progenitor cells. Identifying such cells in the mammalian embryo and understanding how distinct heart and head muscle fates arise within CPM remain major ongoing research objectives.

Branchiomeric myogenesis

Skeletal muscles of the head and neck include branchiomeric muscles, extraocular muscles derived from cranial paraxial mesoderm, and somite-derived muscles of the tongue and neck, progenitor cells of which migrate secondarily into the head and neck region. These muscle groups originate in distinct mesodermal lineages that converge on a common

regulatory program through activation of the myogenic determination factor genes *Myf5* and *MyoD* [1]. Comparison of cranial and somitic myogenesis at the molecular level has revealed distinct upstream mechanisms activating transcription of *Myf5* and *MyoD* [1, 4-7]. The transcriptional regulators TCF21 (Capsulin), MSC (MYOR), PITX2, LHX2 and TBX1 are expressed in head mesoderm and cooperatively regulate myogenesis in the head and neck [8-14], whereas PAX3 is required for trunk but not head myogenesis [4, 15]. The signaling pathways regulating myogenesis also differ between head and trunk [6, 16, 17]. Indeed, dynamic signaling events have been defined that establish myogenic territories in early cranial mesoderm [18, 19]. Despite converging on a common myogenic program, molecular differences between branchiomeric and somite-derived muscles are retained in satellite cells in adult mice [11, 20, 21]. Branchiomeric muscles are also subject to different genetic regulation from extraocular muscles. While PITX2 is required for both extraocular and first arch-derived muscles [10], TBX1, TCF21 and MSC do not appear to play a role in extraocular muscle development [8, 9] and the hierarchy of myogenic determination factors of the MYOD family differs between extraocular and branchiomeric muscles [11]. Branchiomeric muscles of the head and neck thus have distinct molecular features to other skeletal muscle progenitor populations [1, 22-25]. These differences reflect the origin of branchiomeric muscles in mesoderm associated with the developing pharynx, or pharyngeal mesoderm.

Cardiopharyngeal mesoderm

Pharyngeal mesoderm also contributes to cardiac muscle. Twenty years ago, three papers demonstrated that the vertebrate heart grows by progressive addition of extra-cardiac cells to the elongating poles during looping morphogenesis [26-28]. This progenitor field, termed the second heart field (SHF), was mapped to pharyngeal mesoderm contiguous with early differentiating cardiomyocytes of the first heart field (FHF) that gives rise to the linear heart tube and left ventricle [29]. Cells within the developing pharyngeal arches were shown to progressively contribute to the right ventricle and outflow tract of the heart by dye labeling and genetic tracing experiments [26, 30-32]. The FHF and SHF have distinct genetic programs and correspond to different lineages that arise sequentially in gastrulation and independently activate the gene encoding the upstream cardiovascular transcriptional regulator MESP1 [33]. The SHF shares transcriptional regulators, including TBX1, PITX2 and ISL1, with branchiomeric muscle progenitor cells and gives rise to right ventricular and

outflow tract myocardium at the arterial pole of the heart, as well as atrial myocardium at the venous pole [9, 10, 13, 30, 34]. Perturbation of this regulatory program thus leads to congenital heart defects, as well as head muscle defects. Discovery of this shared genetic program suggested that head and heart muscle progenitor cells are derived from a common field of CPM [22, 32, 35]. This conclusion has important clinical implications given links between craniofacial and cardiovascular defects in human genetic syndromes, such as 22q11.2 deletion (or DiGeorge) syndrome, in which *TBX1* is haploinsufficient [36]. Whether heart and head muscle progenitor cells are adjacent progenitor populations that share a common genetic program or whether bipotent progenitor cells contribute to both head and heart muscle was initially unclear and has been addressed by a variety of approaches, including retrospective and genetic lineage analyses in the mouse, functional studies in *Ciona* and zebrafish and the use of multipotent stem cells.

Evidence for common head and heart muscle progenitor cells from clonal analysis in the mouse

Clonal analyses in the mouse, where the progeny of a single cell is tracked to follow its later contribution, have revealed that common progenitor cells give rise to certain branchiomeric head and neck muscles and specific SHF-derived regions of the myocardium [3, 37, 38]. The first such evidence came from a retrospective clonal analysis using a knock-in mouse line with an *nlaacZ* reporter gene inserted at the *alpha-cardiac actin* locus, expressed in the myocardium and developing skeletal muscle. *nlaacZ* encodes an inactive form of nuclear β-galacotosidase due to an intragenic duplication. Upon a rare and random intragenic recombination event, the duplication within *nlaacZ* is removed, generating a functional *nlacZ* reporter gene that is stably inherited on cell division. With a low frequency of recombination, approximately one in 10^5 cell divisions, occurring randomly at any time during mouse development prior to the time of observation, ß-galactosidase positive cells can be correlated to a single clone and lineage history can be reconstituted retrospectively from a collection of clones. The *nlaacZ* cassette being inserted in the *alpha-cardiac actin* locus, only skeletal muscle and myocardial derivatives will express ß-galactosidase after recombination [39, 40]. While this approach is powerful and unbiased, the read-out precludes analysis of *alpha-cardiac actin* negative non-muscular derivatives. Additionally, clonal recombination events can occur independently in different cells of an embryo and thus a low frequency of recombination and statistical analysis are critical to infer clonality. Finally, the stage at which

recombination occurs is unknown. Using this approach to analyse the distribution of clonally related muscle cells in the developing embryo revealed sequential phases of dispersive and coherent growth during myocardial development as well as the presence of two cardiac lineages corresponding to the FHF and SHF [39]. A first study addressing links between head muscle and heart development shed light on the existence of common progenitors for skeletal muscles derived from the first pharyngeal arch that contribute to the masticatory muscles and myocardial cells of the right ventricle, while skeletal muscles derived from the second pharyngeal arch (contributing to facial expression muscles) share common progenitors with myocardial cells at the base of the pulmonary artery or of the aorta (Figure 1A, B). Clones with co-labeling of branchiomeric head and heart muscles represented 59% (23/39) of all clones with extensive labeling in head muscles and 11% (23/208) of those in the outflow tract or right ventricle [38]. Interestingly, these experiments revealed that branchiomeric head muscles of the first pharyngeal arch also share common progenitors with extraocular muscles. A retrospective clonal analysis of the venous pole of the heart, which includes the atrial and venous myocardium, has shown that the myocardium at the base of the pulmonary artery also has a common origin with myocardial cells found in the left atrium, left caval and pulmonary veins (Figure 1C) [41]. Interestingly, a subset of the clones with large labeling of the venous pole (approximately 10% of clones, 18/174) also showed co-labeling with branchiomeric neck-muscles, including the trapezius and sternocleidomastoid muscles. This reveals the existence of common progenitors for venous pole myocardium and non-somitic neck muscles. Clones with co-labeling of branchiomeric neck muscles and the venous pole of the heart represented about 60% (18/30) of clones with extensive labeling in branchiomeric neck muscles, similar to the situation for head muscles [37]. The low fraction of clones in the heart with labeling in branchiomeric head or neck muscles suggests that there is a higher proportion of progenitors restricted to a cardiac fate than common progenitors for skeletal and cardiac muscle lineages.

Clones giving rise to first arch muscles, second arch muscles and neck muscles, together with different parts of the heart, are not only distinct from somitic muscle labeling events, but are also independent from each other, suggesting the reiteration of a lineage module contributing to branchiomeric muscles and myocardium (Figure 2). Three distinct bipotent progenitor cell populations are thus distributed along the anterior posterior axis of the pharyngeal region, giving rise to specific sets of branchiomeric muscles and SHF-derived regions of the heart. These findings provide important insights into the spatiotemporal

sequence of morphogenetic events accompanying pharyngeal development. The SHF is situated between head muscle progenitors and the FHF along the medial to lateral axis of cranial mesoderm. The clonal units identified in these studies reflect the caudal displacement of the arterial pole of the heart in the embryo as pharyngeal arches form in an anterior to posterior sequence. The early, FHF-derived, heart tube forms from anterior lateral mesoderm prior to pharyngeal arch development and gives rise to left ventricular myocardium. Subsequently, as right ventricular progenitors from the SHF are added to the elongating heart tube, the first arch forms. Outflow tract progenitor cells are then added from the level of the second arch that also gives rise to muscles of facial expression. At the end of pharyngeal arch morphogenesis, the arterial pole of the heart is attached to arches 3-6, where progenitors for neck muscles as well as distal outflow tract and atrial myocardium are located. Craniofacial and cardiovascular development are orchestrated by close interactions between pharyngeal mesoderm and neural crest cells [36]. Strikingly, three populations of neural crest cells have been defined in the pharyngeal region contributing to the first, second and posterior pharyngeal arches [42]. While classically identified cardiac neural crest cells migrate through the posterior pharynx into the arterial pole of the heart, neural crest cells from more anterior arches have recently been shown to contribute to the heart, giving rise to smooth muscle cells in the right ventricle [43], thus mirroring the myocardial contribution of anterior CPM.

In addition to anterior-posterior patterning of CPM, retrospective clonal analysis has revealed differential left/right contributions, such that left and right facial expression muscles share common progenitors with subpulmonary and subaortic myocardium, respectively [38]. Similarly, the left trapezius and sternocleidomastoid neck muscles are related to the subpulmonary myocardium, left atrium, left caval vein and pulmonary vein myocardium, while their right counterparts are related to the right venous pole myocardium (right atrial and right caval vein myocardium) [37] (Figure 1 and 2). The significance of these clonal relationships remains as yet poorly understood and may relate to rotation of the arterial pole of the heart during late stages of cardiac looping [44].

Genetic lineage tracing of cardiopharyngeal mesoderm

While these experiments provide evidence for common heart and head muscle progenitor cells in mice, where, when and how many such cells are present in the embryo is unclear. Interestingly, studies performed in the late 1990s have shown that cranial and cardiac

mesoderm ingress into the primitive streak at the same stage of gastrulation [45-47], indicating their close spatial and temporal proximity as early as gastrulation. Retrospective clonal analysis using the *nlaacZ* system is based on a random recombination event, without information as to the time of creation of a clone. Based on clone size and unilaterality, it has been suggested that segregation between skeletal muscle and myocardium occurs after gastrulation [48]. However, only temporally controlled clonal analysis can precisely address the timing of segregation. *Mesp1,* encoding the earliest transcription factor expressed in the cardiovascular lineage, is transiently expressed in the first mesodermal cells as they exit the primitive streak. Genetic lineage tracing has shown that *Mesp1* expressing cells contribute to most of the heart as well as to masticatory, facial expression, neck and esophageal muscles, in addition to anterior somite-derived muscles [14, 20, 37, 49, 50]. Clonal analyses of *Mesp1* expressing cells have shown that these early progenitors are already restricted in their cardiac contribution to the first or second myocardial lineage at the onset of gastrulation [33, 51]. A temporally controlled clonal analysis of *Mesp1*+ progenitor cells has also shown that there is a pool of about 250 progenitors that will build the heart with *Mesp1* being sequentially expressed in FHF and later in SHF progenitors. Late expressing *Mesp1* SHF progenitors include bipotent progenitors for head muscles and myocardium indicating that the segregation between skeletal muscle and myocardium occurs after the onset of *Mesp1* expression, providing further support that it occurs after gastrulation [33, 52]. Moreover, using the MADM system (mosaic analysis with double markers) Devine et al. have observed some co-labeling of the outflow region with the neck and one co-labeling of the esophagus/lung region with the interventricular septum [51]. While these co-labeling events could not be linked statistically it indicates that *Mesp1*+ bipotent progenitors might contribute to non-somitic neck muscles and the venous pole of the heart and outflow region. Moreover, the esophagus, which was not analyzed in the retrospective clonal analysis, may also share common *Mesp1*+ progenitors with the heart.

Additional genetic lineage tracing analyses have revealed co-labeling of the head/skeletal muscles together with derivatives of the SHF. However, these analyses, not performed at a clonal level, do not distinguish whether the gene of interest is expressed in a progenitor cell that contributes to both derivatives or is activated after lineage segregation to distinct progenitor pools contributing to skeletal muscle and myocardium. *Nkx2-5*, expressed from the cardiac crescent stage, is first expressed in progenitors in the FHF and later in the CPM [53], including SHF and head muscle progenitor cells (Figure 3) [20, 54, 55]. *Isl1*

lineage tracing has shown contribution to SHF derivatives [30] as well as to branchiomeric muscles of the head and neck (Figure 3) [56, 57]. Similarly, the *Tbx1* genetic lineage gives rise to all branchiomeric muscles and SHF-derived parts of the heart [58]. Other *Cre*-driven tracing experiments label sub-branches of the lineage tree. Inducible tracing of *Six2*+ cells indicates that *Six2* expressing cells contribute sequentially to the right ventricle and outflow region while also contributing to skeletal muscles of the head [59] (Figure 1). A transcriptional enhancer from the *Mef2c* locus is expressed in progenitor cells of the anterior SHF that contribute to the right ventricle, outflow tract as well as head and neck muscles (Figure 3) [31, 37, 38]. Lineage tracing using Cre activity driven by this enhancer also shows labeling of a subset of venous pole myocardium giving rise to the primary atrial septum and dorsal mesenchymal protrusion, critical structures for atrial and atrioventricular septation; in contrast to continued expression of this enhancer in myocardium at the arterial pole of the heart, expression in venous pole myocardial progenitors is transient [60, 61]. This is consistent with divergence in the transcriptional programs operating in the clonally distinct modules identified at different anterior posterior levels. Indeed, posterior SHF cells express *Hoxb1* and contribute to both the venous pole and arterial pole of the heart, where they contribute to myocardium at the base of the pulmonary artery [60, 62-64]. Arterial pole progenitor cells retain a CPM transcriptional program, including expression of *Tbx1* and the *Mef2c* enhancer, while venous pole progenitor cells downregulate this program and activate *Tbx5* through the activity of retinoic acid signaling [61]. The dynamic expression of CPM regulators during neck muscle development remains to be investigated.

Genetic analysis has revealed broader contributions of CPM than initially thought. Esophageal striated muscle has been shown to be a CPM-derivative [50], although clonal analysis has not yet determined whether this muscle is linked to other CPM sub-lineages along the anterior-posterior pharyngeal axis. Genetic lineage analysis also suggests that CPM is multipotent and can give rise to smooth muscle as well as non-myogenic cell types, including vascular and lymphatic endothelial cells, pharyngeal cartilage and muscle connective tissue [65-68]. CPM-derived muscle connective tissue makes complementary contributions to those of neural crest-derived connective tissue and has recently been implicated in patterning both branchiomeric and somite-derived neck muscles [67] Additional analyses are required to validate the clonal nature of multipotency within CPM and to define the relative timing of different fate choices and regulators of non-myogenic CPM fates. Other regulatory elements are specifically activated in the cardiac branches of the lineage tree. This

is the case for example of a cardiac specific enhancer of *Smarcd3*, encoding a chromatin remodeling protein, which is activated during gastrulation but is only expressed within cardiac sub-lineages [51].

Future work will provide further insights into the transcriptional program during progressive fate restriction in CPM. *Foxa2*, for example, is a good candidate for an early and anterior subset of these bipotent progenitors as lineage tracing of *Foxa2* expressing cells at the early stage of gastrulation marks progenitor cells that contribute specifically to ventricular myocardium, including anterior branches of the cardiopharyngeal lineage tree [69]. The emergence of single cell transcriptomic analysis has opened up new possibilities for uncovering dynamic lineage trajectories towards particular fates within CPM. Single cell transcriptomic analysis of mammalian organogenesis has confirmed that multiple upstream myogenic trajectories converge on a core skeletal muscle transcriptional program, including a pharyngeal mesodermal trajectory expressing *Tbx1*, *Pitx2* and *Lhx2* [70]. Similar analysis focused at the time of gastrulation has revealed the emergence of the CPM genetic program [71, 72]. Early transcriptional divergence has been observed within the pool of *Mesp1*+ progenitors, with at least 4 distinct trajectories and signatures corresponding to the endothelial/endocardial lineage, early cardiomyocytes, likely derived from the FHF, and progenitors of anterior and posterior CPM. Expression of *Tbx1* and *Tcf21* within the anterior CPM cluster indicates co-expression of markers of the anterior SHF and head myogenic program [72]. De Soysa and colleagues performed single cell RNA-sequencing at slightly later stages, and identified a cluster of multipotent progenitor cells that can be further divided into anterior SHF, posterior SHF and branchiomeric muscle progenitor sub-clusters [73]. Further mining of these and newly emerging single cell RNAseq datasets will reveal the transcriptional profile of heart and head muscle progenitors in CPM and provide insights into the regulatory changes associated with divergent trajectories towards alternate myogenic fates.

Cardiopharyngeal mesoderm in *Ciona* and zebrafish

CPM has been investigated in a range of chordate species, including the invertebrate tunicate *Ciona*, where bilateral pairs of trunk ventral cells have been shown to define a cardiopharyngeal lineage that gives rise to both heart and pharyngeal muscles [74, 75]. Each trunk ventral cell divides asymmetrically to give rise to a first heart progenitor cell and a

secondary trunk ventral cell that in turn undergoes asymmetric cell division to generate second heart progenitor and pharyngeal muscle progenitor cells. Trunk ventral cells are thus bipotent myogenic progenitor cells and have been shown to be transcriptionally primed for both the cardiac and pharyngeal muscle lineages [76]. Antagonistic interactions between the cardiac transcription factor *nk4*, homologue of *Nkx2-5*, and *tbx1/10*, homologue of *Tbx1*, determine cardiac or pharyngeal muscle fates [77]. These factors are coexpressed in bipotent secondary trunk ventral cells and act in part through opposing effects on expression of a GATA transcription factor that cell autonomously promotes cardiac and inhibits pharyngeal muscle identity [77, 78]. Pharyngeal muscle fate potential is maintained in trunk ventral cells by Fibroblast growth factor (FGF)/ MAPK signaling [79]. A similar role for FGF signaling has been shown in avian embryos [80]. Secondary trunk ventral cells thus resemble the common progenitor cells giving rise to SHF-derived parts of the heart and branchiomeric head muscles, predicted from retrospective clonal analysis, in the mouse, defining a conserved ontogenetic motif [3]. Single cell RNAseq analysis has generated a high-resolution spatiotemporal roadmap of fate specification in *Ciona* CPM and led to identification of new regulators, such as the transcriptional co-factor Dach required for second heart precursor lineages [81]. Moreover, work on *Ciona* identified the transcription factor Ebf as an upstream regulator of pharyngeal muscle development [74]. Both *Dach1* and *Ebf* genes in mammals are expressed in CPM in patterns consistent with their roles in *Ciona* demonstrating the power of the tunicate model to discover new transcriptional players in CPM fate determination [81, 82]. Myogenic cell fate decisions in *Ciona* are made at the level of single cells in contrast to the larger populations of CPM progenitor cells present in vertebrates. This suggests there may be a proliferative step between the common progenitor and fate acquisition in vertebrates. It remains to be seen whether, as in *Ciona*, single bipotent cells are present early in the mouse embryo, potentially persisting at the interface between SHF and branchiomeric muscle progenitor cells or whether, alternatively or in addition, polarized signaling events direct skeletal and cardiac muscle cell fates within a clonally related pool of uncommitted progenitor cells [75].

Bipotent myogenic progenitor cells have not been documented in fish, although head muscle and SHF progenitor populations are closely juxtaposed in the early embryo. In the developing zebrafish, *tbx1*-dependent *nkx2-5* expression in CPM progenitor cells has been shown to define future branchiomeric muscles and cardiac outflow tract structures upstream of *gdf3*-ALK4 signaling [83]. *Tcf21* is expressed in cells contributing to head muscles and the

outflow tract of the zebrafish heart and is required for head muscle formation, thus playing a conserved role as a branchiomeric muscle regulator in fish and mice [8, 84]. Single cell approaches have recently implicated *gata5* in the acquisition of distinct fates in zebrafish CPM. Loss of gata5 function results in absence of cardiac mesoderm and an expansion of head muscle progenitors, indicating a bias towards branchiomeric over cardiac muscle fates [78]. Additional genetic experiments in zebrafish have shown that WNT and retinoic acid intercellular signaling pathways can also bias myogenic fate choices. WNT signaling in the early zebrafish embryo cell-autonomously promotes cardiac and inhibits pharyngeal muscle development [85]. WNT signaling has also been shown to inhibit head muscle development in avian embryos, suggesting conserved roles of this pathway in myogenic regulation [17]. The retinoic acid target genes *nr2f1a* and *nr2f2*, promote a skeletal muscle over ventricular myocardial fate, in particular in posterior CPM [86]. These signaling pathways may regulate cell fate choices within a pool of progenitor cells primed for heart and head muscle fates rather than acting on individual bipotent cells. Finally, work in Xenopus has shown that the transcription factor *FoxN3* is required in the anuran branchiomeric neck muscle program as well as for development of the venous pole of the heart, although the underlying mechanisms remain to be resolved [87].

Stem cell approaches

A powerful approach to circumvent the study of cell fate choices in large populations of CPM is to study cell fate decisions in differentiating pluripotent cells. This system has been used to recapitulate sequential regulatory events driving somitic myogenesis [88]. An alternate pathway leading to branchiomeric myogenic differentiation has been defined using mouse and human pluripotent stem cells directed towards cardiopharyngeal fates [89, 90]. Inhibiting canonical WNT and Nodal signaling constitutes an instructive cue that drives pluripotent stem cell differentiation to head and heart muscle progenitor cells with dual myogenic potential [89]. Impaired survival of single cells precluded clonal analysis, however the observation that approximately 30% of cells following WNT and Nodal inhibition have an upstream CPM marker profile suggests that they include bipotent cells. A similar cardiopharyngeal program can be induced by MESP1 expression [33, 90]. Clonal analysis showed that single PDGFRa positive cells induced by MESP1 give rise to both cardiac and skeletal muscle lineages [90]. While BMP signaling and antagonism of TGFβ signaling were found to enhance cardiac differentiation, Rho-kinase inhibitors enhanced skeletal muscle

differentiation [90]. The recent development of organoid and gastruloid culture systems reinforces the relevance and promise of applying mouse and human pluripotent stem cell approaches to dissect fate choices in the early embryo [91]. Indeed, a number of *in vitro* models of early heart organogenesis have been recently reported. These comprise juxtaposed FHF and SHF lineages as evaluated by fluorescent reporter genes and analysis of endogenous gene expression and reveal a remarkable level of organisation within the developing organoids [92-95]. Gastruloids have been shown to recapitulate developmental decisions made in the early embryo at the transcriptional level, suggesting that this will be a powerful approach to dissect the regulation of cell fate acquisition in CPM [93]. While these models have been developed from mouse embryonic stem cells, human embryonic stem cells have also shown their ability to form gastruloids but it is still unclear whether cardiogenesis could be recapitulated in this model [96]. New protocols have recently been developed using human induced pluripotent stem cells embedded in Matrigel, that form self-organizing structures, including myocardium as well as foregut endoderm, fibroblasts and endothelial cells [97, 98]. If these models allow CPM specification towards both cardiac and branchiomeric skeletal muscle lineages, which remains to be shown, this will allow investigation of early events in CPM specification in an accessible and high throughput *in vitro* system. Moreover, using iPS cells, such fate decisions can be investigated in pathological contexts such as the 22q11.2 deletion syndrome or other congenital syndromes with cardiac and craniofacial defects.

Conclusions

CPM is the source of both branchiomeric muscles and myocardium. However how these divergent myogenic fates arise remains unclear. In particular, when and where the common progenitors identified by clonal analysis in the mouse are present in the early embryo remains to be resolved. Detailed cell lineage experiments, potentially coupled with live imaging, and single cell transcriptomic approaches will be required in different animal and *ex vivo* models to resolve these questions and to address broader cardiopharyngeal contributions to non-myogenic cardiac and pharyngeal lineages. These approaches will provide insights into cell fate determination and the evolution of vertebrate musculature as well as clinical insights into the developmental origins of muscle restricted myopathies and congenital anomalies.

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Figure legends

Figure 1. Retrospective clonal analysis of cardiopharyngeal mesoderm reveals clonal relationships between branchiomeric muscles and the heart. A, A'. Examples of clones from the retrospective clonal analysis showing a clone comprising ß-gal+ cells in masticatory muscles (te, temporalis and ma, masseter) and in the right ventricle at emrbyonic day 14.5. **B, B'**. A second clone shows labeling of the right facial expression muscles and sub-aortic myocardium, while the last example (**C, C'**) shows labeling in the left trapezius muscle and the left atrium. Arrowheads show ß-galactosidase positive cells. Asterisks indicate independent labelling of the forelimb (not statistically related to branchiomeric labeling). LV, left ventricle; RV, right ventricle; OFT, outflow tract; ao, aorta; pt, pulmonary trunk; RA, right atrium; LA, left atrium; ma, masseter muscle; te, temporal muscle; au, auricularis; bu, buccinator; fr, frontalis; oc, occipitalis; oo, orbitalis occuli; zy, zygomaticus; atrap, acromio-trapezius; strap, spino-trapezius. The identification number of the embryo is indicated in each panel [37, 38].

Figure 2. Myogenic lineage tree of mouse cardiopharyngeal mesoderm. A. Lineage history of CPM reconstructed from clonal analyses of *Mesp1* expressing cells as well as from the random clonal analysis of the muscular lineages [33, 37, 38, 41]. FHF cells are shown in red, bi-potent progenitors for branchiomeric and cardiac muscles in green, SHF-derived cardiac lineages in orange and branchiomeric muscle progenitor cells in yellow. The different branches of the lineage tree correspond to different populations of progenitor cells along the antero-posterior axis of the embryo. **B.** Scheme of mouse gastrulation showing *Mesp1*+ progenitors emerging from the primitive streak (PS) between embryonic days E6.5 and E7.5. **C.** At E8.5 CPM is located dorsally to the heart tube and in the core of the pharyngeal arches. **D.** CPM later contributes to branchiomeric muscles of the head and neck (masticatory muscles in blue, facial expression muscles in light blue and branchiomeric neck muscles in purple) as well as SHF-derived cardiac lineages. a, anterior ; p, posterior ; d, dorsal ; v, ventral ; r, right ; l, left ; ao, aorta ; pt, pulmonary trunk ; RA, right atrium ; LA, left atrium ; RV, right ventricle ; LV, left ventricle.

Figure 3. Genetic tracing of cardiopharyngeal mesoderm. Cartoon showing skeletal muscle labeling at fetal stages after conditional reporter gene activation by *Cre* recombinase under transcriptional control of the *Mef2c* anterior heart field enhancer, *Tbx1*, *Isl1* and *Nkx2-5*. Based on data from [14, 20, 37, 55, 58]. ad+mh, anterior digastric and mylohyoid muscles; atrap, acromio-trapezius muscle; fe, facial expression muscles; ma, masseter muscle; strap, spino-trapezius muscle; te, temporal muscle.

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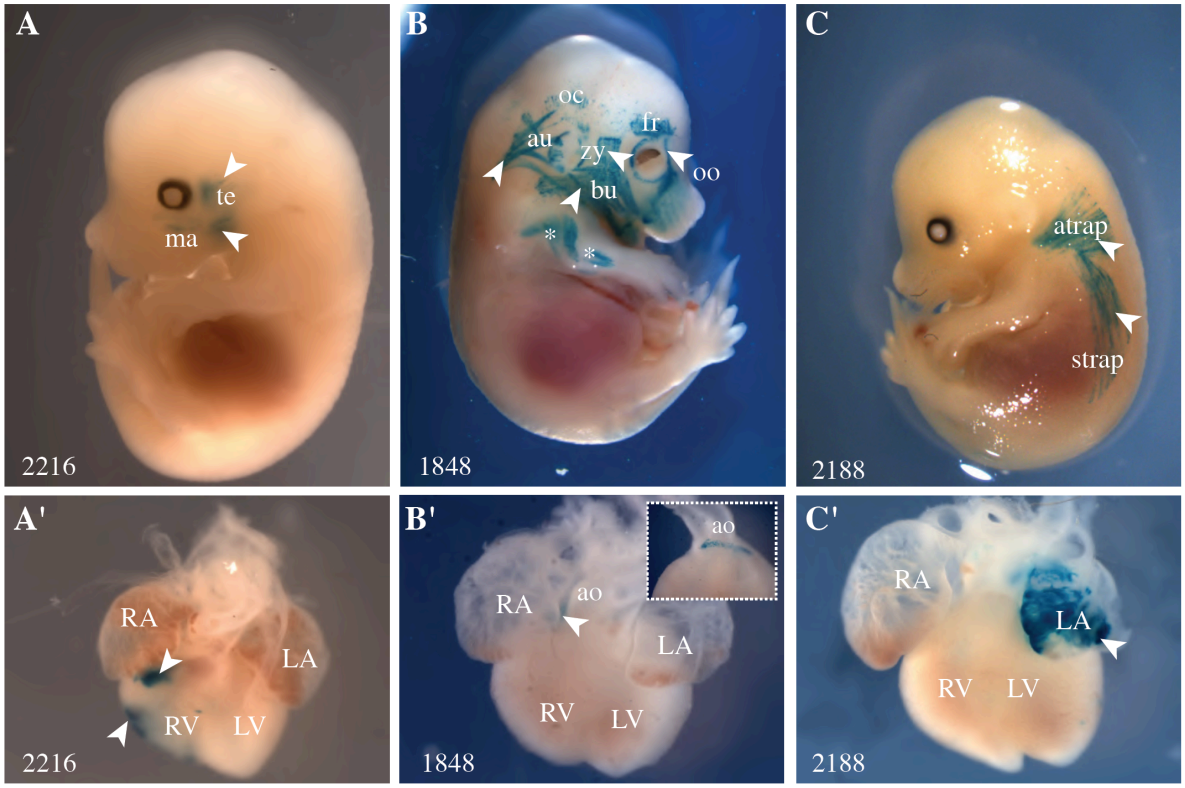
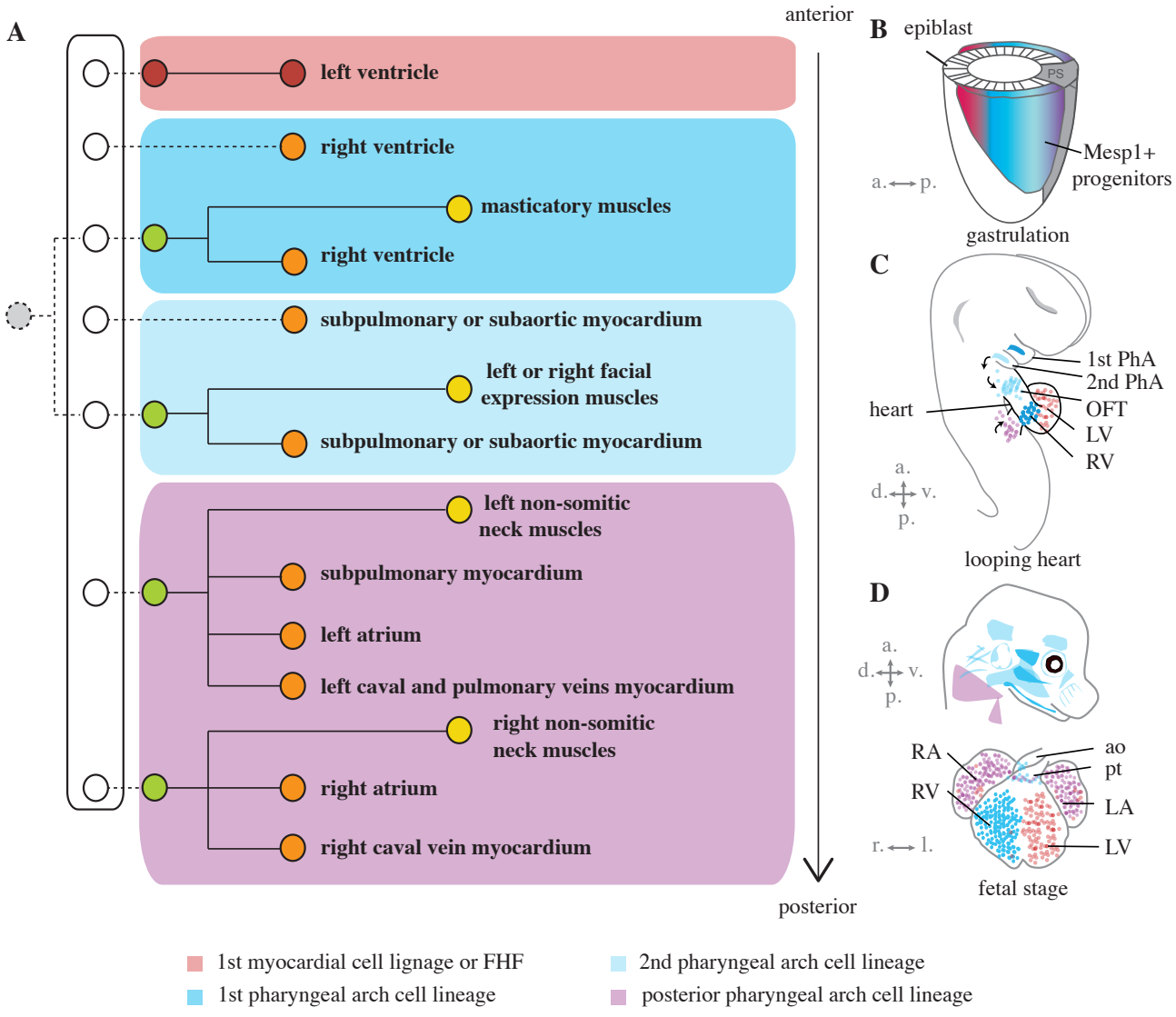
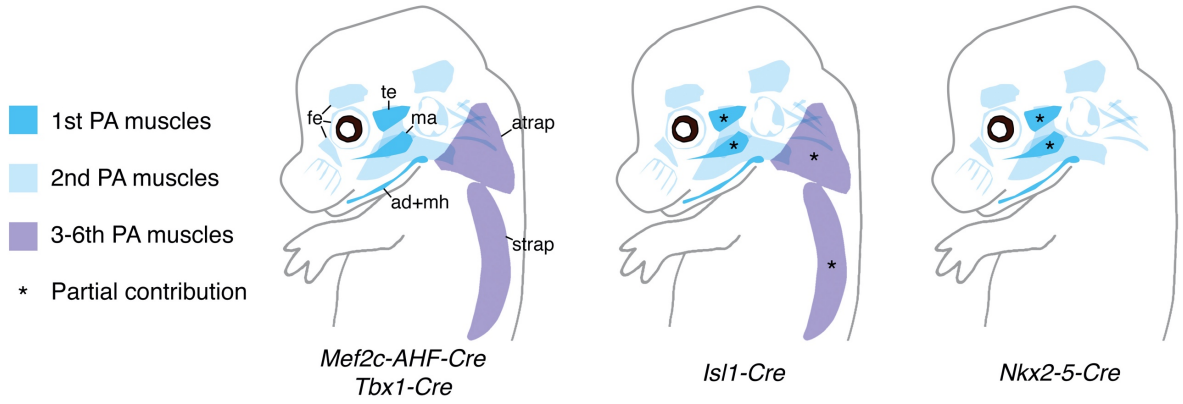


Lescroart et al Revised Figure 1



Lescroart et al Revised Figure 2



Lescroart et al Revised Figure 3