Review

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Mesoderm patterning by a dynamic gradient of retinoic acid signalling

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Retinoic acid (RA), derived from vitamin A, is a major teratogen, clinically recognized in 1983. Identification of its natural presence in the embryo and dissection of its molecular mechanism of action became possible in the animal model with the advent of molecular biology, starting with the cloning of its nuclear receptor. In normal development, the dose of RA is tightly controlled to regulate organ formation. Its production depends on enzymes, which have a dynamic expression profile during embryonic development. As a small molecule, it diffuses rapidly and acts as a morphogen. Here, we review advances in deciphering how endogenously produced RA provides positional information to cells. We compare three mesodermal tissues, the limb, the somites and the heart, and discuss how RA signalling regulates antero-posterior and left-right patterning. A common principle is the establishment of its spatio-temporal dynamics by positive and negative feedback mechanisms and by antagonistic signalling by FGF. However, the response is cell-specific, pointing to the existence of cofactors and effectors, which are as yet incompletely characterized.

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1. Introduction

The development of the embryo requires coordination between cells to generate specific organ shapes and to position organs relative to each other. A key aspect of this coordination is the molecular patterning of cells according to their position in the embryo [1,2]. Information about the antero-posterior, dorso-ventral, left-right position is established while the vertebrate embryo undergoes morphogenesis and growth. Thus, it is encoded by gradients of morphogens that are dynamically defined by a combination of positive and negative feedback mechanisms. One of the first morphogens identified as essential for mouse embryo patterning was retinoic acid (RA), a metabolite of vitamin A [3,4]. RA is a small lipophilic molecule that can diffuse across cell membranes. The dose of RA, regulated by synthesizing (ALDH1A1/2/3, RDH5/10, CYP1B1) and degrading (CYP26A1/2/3, DHRS3) enzymes [3,5], modulates gene transcription. RA binding to nuclear receptors (RARA/B/G, which form a heterodimer with RXRA/B/G) was initially shown to convert them from transcriptional repressors to activators of genes containing *RARE* (retinoic acid response elements) regulatory sequences [6]. The transcriptional complex of RA nuclear receptors includes histone deacetylases or methyltransferases and NCOR cofactors for gene repression by chromatin compaction [7]. In reverse, histone acetylases or NCOA are recruited as co-activators of the transcriptional complex. Cell-specific response may be mediated by interaction with other regulatory cofactors, such as NKX2-5 in cardiac cells [8]. Since the source of RA is of dietary origin, fluorescently tagged versions of RA cannot be genetically engineered. Thus, the best strategy to visualize RA signalling in the mouse is a transgenic reporter, *RARE-lacZ*, in which a triplet of concatenated *RARE* sequences of the *Rarb* gene, along with a minimal *hsp68* promoter,

drive *lacZ* expression [9]. This is a sensitive reporter able to detect RA signalling in the nanomolar range [10,11].

A mechanism of global cell patterning by a morphogen gradient has been theorized as a solution to the French flag problem, in which morphogen concentration specifies position and threshold values spatial patterning into regions [12]. However, models of gradients, involving morphogen production, diffusion and degradation, cannot fully account for the observed dynamics, as shown for the fly bicoid gradient [13]. Pattern formation becomes possible if a short-range self-enhancing reaction is coupled with an antagonistic reaction that acts at a longer range, potentially based on more rapidly diffusing properties [14]. Quantifications of anti-parallel gradients of BMP and SHH signalling coupled to a maximum likelihood strategy to decode positional information have shown how dorso-ventral patterning of the neural tube is encoded at a much higher precision than the single noisy gradients would allow [15]. Opposite gradients of morphogens are resolved into transcriptional networks defining positional information [2]. Advances in quantitative measurements of gene expression, analysed within a mathematical framework based on information theory, have demonstrated how expression levels of the four gap genes in the fly embryo can be jointly decoded into position specification of 15 regions with 1% accuracy, whereas single transcription factor expression can at best encode an on/off response [16]. How does RA encode positional information and establish regional boundaries? Recent technical developments of fluorescent RA sensors [17] have opened up the possibility of applying such quantitative frameworks to the case of RA signalling. Intracellular binding of RA to CRABP was shown to reduce noise in RA levels, an important parameter underlying fish hindbrain patterning [18]. Since these quantitative approaches have become more widely used, feedback mechanisms of RA signalling have been demonstrated: positive upon activated expression of the nuclear receptors RARB and NR2F1/2, and negative upon activation of the degrading enzymes CYP26 and DHRS3 (see below). In addition, RA signalling is antagonized by an anti-parallel gradient of fibroblast growth factor (FGF) [19].

Here, we survey how RA signalling patterns different mesodermal tissues. We begin with the historical model of the limb, in which RA controls forelimb budding. We then review the well-characterized role of RA in patterning presomitic mesoderm. Finally, we present current knowledge about RA-mediated patterning of heart precursors, and compare two animal models, fish and mouse. Based on comparisons between different mesodermal tissues, we discuss general principles of patterning and tissue-specific implementations.

2. Formation of the limb

The developing limb is a historical model of pattern formation in vertebrates. Limbs emerge from the trunk at E8.5 in the mouse, as bilateral buds of mesenchymal cells from the lateral plate mesoderm covered by an ectodermal epithelium. An important organizer is the apical ectodermal ridge, corresponding to an ectodermal thickening at the distal tip of the bud, which expresses *Fgf4/8/9/17* [20–22] and is required for bud elongation and proximo-distal patterning into upper arms, forearms and digits [23]. RA, which was known to have teratogenic effect on limb formation, was proposed to act as a morphogen, when measured as a gradient in the

chick limb bud [24]. However, further studies showed that RA is not required as a morphogen in this tissue [25,26]. The synthesizing enzyme ALDH1A2 is expressed in the trunk, whereas the degrading enzyme CYP26B1 or its regulator POR is expressed in the distal limb [27–29]. The expression of *Aldh1a2* is transient in the lateral plate mesoderm, then in the somites [30,31].

Gene inactivations in the mouse have shown that *Aldh1a2* and *Rdh10* are required for the initiation of the forelimb, but not the hindlimb [32,33] (table 1). The part of RA has been controversial; initially it was thought to play a part in proximo-distal patterning [53]. Indeed, CYP26B1-mediated RA clearance is required in the distal limb for cell survival, in both fore- and hindlimbs [28,54]. However, upon RA deficiency, the expression of genes marking proximo-distal limb regions, respectively *Meis2* and *Hoxa11*, is normal, thus ruling out a role of RA in proximo-distal limb patterning [55,56]. RA rather provides a permissive environment, prior to limb bud initiation, for *Fgf8* repression and *Tbx5* activation, which is essential for the initiation of the forelimb and accounts for variations in severity between *Aldh1a2* and *Rdh10* mutants [11,55]. The fact that the RARE regulatory sequences of *Tbx5* are not required for limb formation [57] argues for an indirect activation by RA signalling. This may be mediated by HOX transcription factors, which are known regulators of *Tbx5* [44] and targets of RA, or, as in cardiac cells, by NR2F2 [46].

TBX5 is required for the bilateral symmetry of forelimbs, as indicated by left–right differences in malformations of the Holt–Oram syndrome. The most severe side (left) can be reversed in *Invs* mutants, which disrupt the activity of the left–right organizer. Thus, TBX5 buffers an inherent asymmetry between left–right limb-forming regions (potentially NODAL signalling) to ensure symmetric forelimb formation [58]. Whether this depends on RA signalling, by analogy with somitogenesis, remains an open question.

Later, at E13.5, RA signalling (*Rara/b/g*, *Aldh1a2*, RARE-*lacZ*) is detected in interdigital mesenchyme [59], thus along the antero-posterior axis. Here, RA was shown to play an instructive role for digit formation in both fore- and hindlimbs, by controlling apoptosis of the interdigital mesenchyme [51,56]. RA signalling is thus required for induction of the limb bud and digit formation but its mechanism of action remains incompletely understood. The identification of its direct targets should provide important insight. This has been better deciphered in the paraxial mesoderm.

3. Antero-posterior patterning and bilateral symmetry of the presomitic mesoderm

Somitogenesis is a segmentation process of the paraxial mesoderm, resulting in the formation, in the mouse, of 65 pairs of somites, which include precursors of vertebrae and rib skeleton, dermis and intercostal skeletal muscles. Between E8.5 and E14.5, they form sequentially every 2 h, by budding off the posterior presomitic mesoderm, on either side of the neural tube. At a molecular level, somitogenesis is modelled by a segmentation clock and wavefront. The segmentation clock corresponds to an oscillatory mechanism, driving the periodic activation of the NOTCH, WNT and FGF pathways in the presomitic mesoderm and setting the periodicity of the segments along the antero-posterior axis. The wavefront or determination front is a travelling WNT/FGF signalling gradient providing threshold

Table 1. Phenotypes of mouse mutants with defective RA signalling. ASD, atrial septation defects; DORV, double outlet right ventricle; n.a., not available; VSD, ventricular septation defect.

genotype	somitogenesis	tail	heart	limb	references
RA deficiency					
<i>Rdh10</i> ^{−/−}	first six somites under-developed	shorter	ventricle misalignment, truncus arteriosus	stunted forelimbs	Sandell <i>et al.</i> [33]; Rhinn <i>et al.</i> [101]; Cunningham <i>et al.</i> [67]
<i>Dhrs3</i>	n.a.	n.a.	ASD, VSD, DORV	n.a.	Billings <i>et al.</i> [43]
<i>Aldh1a2</i> ^{−/−}	asymmetric and under-developed somites	shorter	no looping, hypoplastic atria and outflow tract	no limb buds	Niederreither <i>et al.</i> [32,99]; Ryckebusch <i>et al.</i> [110]
<i>Aldh1a2</i> ^{−/−} rescued by RA supplementation until E10.5	n.a.	n.a.	truncus arteriosus, VSD	n.a.	Niederreither <i>et al.</i> [99]; El Robirni <i>et al.</i> [50]
<i>Rere</i> ^{−/−} ; <i>Aldh1a2</i> ^{−/+}	asymmetric and under-developed somites	shorter	n.a.	n.a.	Vilhals-Nieto <i>et al.</i> [76]
<i>Rxra</i> ^{−/−}	n.a.	normal	thin-walled ventricles, VSD	normal	Sucov <i>et al.</i> [45]; Kastner <i>et al.</i> [47]
<i>Rara</i> ^{−/−} ; <i>Rarb</i> ^{−/−}	n.a.	n.a.	truncus arteriosus, VSD	normal	Lee <i>et al.</i> [49]; Ghyselinck <i>et al.</i> [59]; Mendelsohn <i>et al.</i> [52]
<i>Rarb</i> ^{−/−} ; <i>Rarg</i> ^{−/−}	n.a.	n.a.	normal	digit defects	Ghyselinck <i>et al.</i> [59]; Mendelsohn <i>et al.</i> [52]
<i>Rara</i> ^{−/−} ; <i>Rarg</i> ^{−/−}	n.a.	n.a.	truncus arteriosus, VSD	digit defects	Lohnes <i>et al.</i> [125]; Mendelsohn <i>et al.</i> [52]
<i>Rara</i> ^{−/−} ; <i>Rxra</i> ^{−/−}	n.a.	n.a.	truncus arteriosus, DORV	n.a.	Lee <i>et al.</i> [49]; Li <i>et al.</i> [121]
<i>Rxra</i> ^{−/−} ; <i>Rxrb</i> ^{−/−}	n.a.	shorter	looping defects	n.a.	Wendling <i>et al.</i> [126]
<i>Cyp26a1</i> ^{−/−}	normal	shorter	under-developed endothelium	sirenomelic hindlimbs	Abu-Abed <i>et al.</i> [73]; Sakai <i>et al.</i> [62]; Ribes <i>et al.</i> [127]
<i>Cyp26b1</i> ^{−/−}	n.a.	normal	n.a.	stunted fore- and hindlimbs	Yashiro <i>et al.</i> [28]
<i>Par</i> ^{−/−} ; <i>Aldh1a2</i> ^{−/+}	normal	shorter	abnormal	smaller forelimb buds, sirenomelic hindlimbs	Ribes <i>et al.</i> [29]
<i>Par</i> ^{−/−}	under-developed symmetric somites	shorter	under-developed endothelium, looping defects	no limb buds	Ribes <i>et al.</i> [29]

Figure 1. RA/FGF signalling antagonism patterning the presomitic mesoderm. (a) Representation of the opposite gradients of RA (blue) and FGF (green) signalling along the antero-posterior axis in the mouse posterior mesoderm at E8.5. (b) Molecular network of RA and FGF pathway components (arrow, positive regulation; T-connector, negative regulation). (c) Left–right asymmetries in NODAL signalling in the left lateral plate mesoderm (orange) and in RA signalling in the paraxial mesoderm (blue). (d) In *Aldh1a2* mutants, FGF signalling is more anterior and somitogenesis is delayed on the right side. Equivalent levels in wild-type (c) and *Aldh1a2* mutants (d) are highlighted by grey dotted lines. A, anterior; L, left; LPM, lateral plate mesoderm; P, posterior; PSM, presomitic mesoderm; R, right; RA, retinoic acid.

conditions for the formation of somite boundaries by activation of *Mesp1/2* genes [60].

RA forms an antero-posterior gradient, anti-parallel to that of FGF (figure 1a). The synthesizing enzyme ALDH1A2 is expressed in the trunk somites and most anterior presomitic mesoderm, whereas the degrading enzyme CYP26A1 is expressed in the tailbud, thus establishing RA signalling in the trunk as detected by *RARE-lacZ* [9,61,62]. RA and FGF signalling are antagonistic (figure 1b). *Fgf8* expression is higher in the tailbud and cardiopharyngeal mesoderm, i.e. on either side of the *RARE-lacZ* pattern [53,63]. The FGF8 gradient is formed by mRNA decay rather than protein diffusion [64]. This was shown by comparing intronic and exonic probes of *Fgf8*, the former being restricted to the tailbud and the latter extending more rostrally, indicating *Fgf8* transcription in the tailbud only and mRNA decay when cells incorporate the presomitic mesoderm. Upon RA genetic deficiency, *Fgf8* expression is increased in neuromesodermal progenitors of the tailbud, whereas *Cyp26a1* expression is unchanged [65–67]. The identification of a *RARE* regulatory sequence upstream of *Fgf8*, which binds RAR receptors and is required to restrict *Fgf8* expression, demonstrates direct repression of *Fgf8* by RA [68], in association with NCOR1/2 cofactors [69]. In reverse, FGF signalling in the posterior mesoderm is required for *Cyp26a1* expression in the mouse [35], whereas it represses *Aldh1a2* in the chick and *Xenopus* [19]. The antagonism between FGF and RA signalling was proposed to be key for establishing the threshold of the wavefront: bistability (exclusive FGF or RA driven fates) arising from mutual inhibition provides a molecular mechanism for the all-or-none transitions at somite boundaries [70].

The role of the morphogen gradient is shown in analyses of mutants. In the chick, ectopic FGF8 has indicated a key role in controlling the position of segment formation along the antero-posterior axis [71]. In the mouse, gene inactivation corresponding to RA deficiency, including of *Aldh1a2*, the co-repressors *Ncor1/2*, the nuclear receptors and RA targets *Nr2f1/2*, or deletion of the *RARE* regulatory sequence of *Fgf8*, leads to smaller somites and shortening of the trunk

[65,69,72]. *Rdh10* mutants, which have a less severe RA deficiency compared with *Aldh1a2* mutants, display segmentation defects restricted to the first six somites [67]. The role of RA in somitogenesis is transient, as shown by the rescue of *Aldh1a2* mutants when RA is supplemented until the six-somite stage [31]. Excess of RA in *Por* or *Cyp26a1* mutants also leads to smaller somites and shortening of the trunk [29,62]. Similarities between the phenotypes of deficiency and excess of RA suggest a threshold rather than a linear response to RA dosage, and indicate that CYP26 degrading enzymes protect against deleterious effects of ectopic RA signalling. Vertebrae, which are somite derivatives, display homeotic transformations in the cervical region of *Cyp26a1* mutants, with a more posterior phenotype [62,73]. This is potentially mediated by the HOX homeoproteins, which specify antero-posterior identity. In keeping with the antagonism between RA and FGF signalling, the small somites in *Aldh1a2* mutants can be rescued by treatment with an FGF receptor antagonist [67]. Double mutants have shown that *Fgf8* is redundant with *Fgf4*, also expressed in the tailbud, to regulate somitogenesis and axial elongation [35,40]. Cell tracking in the chick suggests that FGF/MAPK controls a gradient of cell motility underlying axial elongation [74]. Since *Fgf8* is not transcribed in the presomitic mesoderm but FGF8 is produced by the caudal neuromesodermal progenitors [64], the effect on cell motility is a cell non-autonomous process, rather reflecting collective cell behaviour. As cellular density increases anteriorly, the resistance to expansion also increases, thus biasing tissue expansion towards the posterior end of the embryo. RA signalling is thus important to pattern somitogenesis and to promote the elongation of the embryo.

Another aspect of somite patterning regulated by RA signalling is their bilateral symmetry (figure 1c,d). Deficiency in RA generates asymmetric somitogenesis, despite normal signalling from the major left determinant NODAL [66,75]. This effect is restricted to somites numbers 8–16. In *Aldh1a2* mouse mutants, *Fgf8* expression is expanded anteriorly, more strikingly on the right side, indicating an anterior shift of the determination front on the right [31,66,76]. In addition, the oscillation clock is accelerated on this side, generating

Figure 2. RA/FGF signalling antagonism patterning the heart field. (a) Regionalization of the lateral plate mesoderm in the zebrafish along the antero-posterior axis at the eight-somite stage, reflecting the specification of vascular/myeloid (orange), cardiac (red) and fin (brown) progenitors (a1). This depends on opposite gradients of RA (blue) and FGF (green) signalling (a2). (b) Representation of the opposite gradients of RA and FGF signalling in the mouse anterior mesoderm, in wild-type embryos at E8 (b1) and E9.5 (b3). The cardiac crescent, containing differentiated cardiomyocytes, is the primordium of the heart (red, b1–b2). In *Aldh1a2* mutants, the heart field expressing *Fgf8/10* and *Isl1* is expanded posteriorly (green, b2). At E9.5, FGF and RA signalling pattern the heart field (dotted lines) into an anterior and posterior domain, respectively. The arrows indicate the recruitment of precursor cells underlying heart tube elongation. A, anterior; L, left; LV, left ventricle; OFT, outflow tract; P, posterior; R, right; RA, retinoic acid; RV, right ventricle; SHF, second heart field.

shorter somites. Thus, RA deficiency uncovers a lack of coordination between the right and left somitogenesis. Interestingly, the somitogenesis defect is on the right side in the mouse [66] but on the left side in the chick [75]. This is consistent with the differential role of FGF8 signalling in the two species, as a right determinant in the chick but not in the mouse. The lateralization of shorter somites can be reversed in *Dnah11* mutants (also known as *iv*) with defective left–right patterning. RA signalling is thus important to buffer the lateralizing influence of FGF8 [77], providing synchronization of somitogenesis bilaterally. The molecular complex mediating RA signalling for somite symmetry has been recently identified [76,78]. It is referred to as WHHERE, because of its components Wdr5, Hdac1, Hdac2 and rERE, corresponding to chromatin modifiers and a cofactor of transcription, binding the RA receptor RAR and the nuclear receptor and RA target NR2F2. The complex recruits the polymerase II to function as a transcription co-activator, and act together with the histone methyltransferase Ehmt2. *Nr2f2* and *RARE-lacZ* are asymmetrically expressed, more posteriorly in the right presomitic mesoderm, whereas *Rere*, *Fgf8* and *Aldh1a2* are symmetrical [76]. *Rere*, as well as *Wdr5* and *Hdac1* mutants phenocopy *Aldh1a2* mutants. In *Rere* mutants, *Fgf8* expands rostrally particularly on the right side, whereas *Aldh1a2* expression is downregulated posteriorly on the right side and *RARE-lacZ* is downregulated. *Aldh1a2*;*Rere* double mutants have more severe defects than single mutants, showing genetic synergy. RERE upregulates the expression of the RA target gene *Rarb* [76], whereas RERE is removed by RA from the RARE regulatory sequences of *Fgf8*, a gene repressed by RA signalling [68]. The WHHERE complex, which is usually associated with transcriptional repression, thus acts as an activator of target genes

in the context of RA signalling. The combination of different factors binding DNA might explain why the presence of *RARE* regulatory sequences is not enough to qualify a gene as a RA- target. ChIP-seq of RA-regulated chromatin modifications (H3K27me3, H3K27ac) near *RARE* regulatory sequences is an approach to validate the functionality of *RARE* sequences. Several genes involved in RA signalling (*Nr2f1/2*, *Dhrs3*, *Rarb*, *Crabp2*) or in the antagonistic FGF signalling (*Fgf8*, *Spry4*) fit these criteria, thus highlighting feedback mechanisms [72].

In conclusion, the antagonism between RA and FGF signalling is essential to coordinate somitogenesis, ensuring bilateral symmetry and controlling the size and number of somites along the antero-posterior axis. Abutting the paraxial mesoderm, the lateral plate mesoderm is another source of RA signalling for the morphogenesis of the heart, a non-segmented and asymmetric organ.

4. Patterning of the cardiac mesoderm and cardiac chambers in the fish

In the fish, heart precursors originate in the lateral plate mesoderm (figure 2a1), between anterior vascular/myeloid progenitors and more posterior fin progenitors [79–81]. The heart forms as a tube by the fusion of bilateral fields of progenitors from the 21-somite stage (20 hours post-fertilization (hpf)). The tube, which is initially straight, becomes asymmetrical during the process of jogging (until 28 hpf), when the venous pole is shifted to the left side, and that of looping (between 30 and 48 hpf), when the tube acquires a flat S-shape [82]. Along the axis of the tube, the atrial and ventricular chambers are specified,

such that ventricular cells first incorporate the tube and are positioned posteriorly at 24 hpf. Thus, the formation of the heart depends on both antero-posterior and left–right patterning.

Reminiscent of its role in somitogenesis, RA is involved in the left–right patterning of fish heart precursors. *aldh1a2* is asymmetrically expressed (stronger on the left) in the lateral plate mesoderm from the one- to two-somite stage, i.e. before asymmetric Nodal signalling, and this is maintained in *spaw* (*nodal*) mutants, indicating independence of Nodal signalling [83]. Upon the downregulation of a RA receptor, anomalies of jogging are observed despite normal Nodal signalling [84]. This effect is specific to *rargb* morphants, but not observed with other RA receptors (Raraa, Rarab, Rarga). Contrary to Fgf signalling in somitogenesis, asymmetric RA signalling in the lateral plate mesoderm has been shown to affect Bmp signalling. *rargb* morphants display increased P-Smad1/5/8 and can be rescued by inhibition of Bmp. Whereas in control embryos *bmp4* is asymmetrically expressed (higher on the left) in the heart cone at the 20–22 somite stages, *bmp4* expression and Bmp signalling are symmetrical in *rargb* morphants, predictive of jogging defects. The cell behaviour, such as cell migration, modulated by asymmetric RA signalling in the lateral plate mesoderm remains speculative.

In the lateral plate mesoderm, RA signalling displays an antero-posterior gradient (figure 2a2). At eight somites, expression of the synthesizing enzymes *Aldh1A2* and *Rdh10A* is posterior in the lateral plate and paraxial mesoderm, overlapping with the pectoral fin field, where the RA targets *hoxb5b* and *nr2f1a/2* are also expressed. The degrading enzymes *Cyp26a1/c1* are instead expressed anteriorly, in the region of vascular progenitors [80,85–87]. *fgf8/24* is expressed in between, in cardiac precursors [88,89]. In *nls* (*aldh1a2*) mutants, the pectoral fins are lost, while the heart is enlarged [80]. This is associated with a posterior expansion of *gata4*, labelling the anterior lateral plate mesoderm including the cardiac field, and a reduction of *tbx5* in the pectoral fin field [80,90]. The fate map of the mutant lateral plate mesoderm is consistent with the posterior expansion of cardiac progenitors at the expense of pectoral fin progenitors. RA signalling is modulated by negative feedback mechanisms such as induction of *cyp26a1* and *dhfr3* and repression of *aldh1a2* and *rdh10a* [91–93]. As in somitogenesis, an antagonism between RA and Fgf signalling takes place: RA signalling in the pectoral fin field is required to restrict Fgf8A to the cardiac field, which allows the pectoral fin field to form just posterior to a cardiac field of normal size [11]. Upon pharmacological deficiency in RA signalling, Fgf signalling is expanded, so that downregulation of *fgf8a* can rescue fin formation and resize the heart field [94]. Excess of Fgf until the eight-somite stage, generated with a constitutively active Fgfr1 receptor, phenocopies RA deficiency. In this case, the expansion of the cardiac field, labelled by *nkx2–5*, is not only posterior but also anterior, at the expense of the vascular/myeloid progenitors, labelled by *etv2* and *tal1* [94]. Excess of RA in *cyp26a1/c1* double morphants increases cardiac progenitors at the expense of vascular progenitors [86]. Thus, RA signalling is important to establish the boundaries of the heart field, anteriorly adjacent to vascular/myeloid progenitors, and posteriorly adjacent to the pectoral fin field. Analysis of double mutants for the nuclear receptors and RA targets *nr2f1a;nr2f2* uncovers another equilibrium between ventricular progenitors and adjacent pharyngeal muscle progenitors in the cranial/lateral plate mesoderm, both expressing the T-box transcription factor Tcf21 at the eight-somite stage [87].

RA signalling differentially affects ventricular and atrial cells. Time-controlled pharmacological inhibition of RA signalling indicates that the specification of ventricular cells is sensitive to RA during gastrulation (stage 40% epiboly), whereas that of atrial cells is still sensitive to RA later, at the six- to eight-somite stages [80]. In differentiated cardiomyocytes, *nr2f1a* is expressed in the atrium from 24 hpf and is required for its size [95], whereas *fgf8* is expressed in the ventricle and required for its size [88,96]. Upon inhibition of Fgf signalling, ectopic atrial cardiomyocytes are detected in the smaller ventricle [37]. The RA-degrading enzymes *Cyp26a1/c1* are expressed in the pharyngeal arches when the heart tube is formed. Upon excess of RA in double *cyp26a1/c1* morphants the ventricle is reduced and *fgf8a* is downregulated [97]. The smaller ventricle results from both defective progenitor cell recruitment at the arterial pole, which depends on Fgf signalling [34], and extrusion of cardiomyocytes associated with increased expression of the matrix metalloprotease *Mmp9*. Taken together, these observations indicate a later requirement of the RA/Fgf signalling antagonism for promoting cardiac chamber growth and identity.

In the fish cardiac mesoderm, the antagonism between RA and Fgf signalling is required to define the boundaries of the heart field, and later to regulate chamber size and identity. In addition, RA signalling is required upstream of *Bmp4* for left–right asymmetry. In the next section, we will analyse whether the role of RA is conserved in the mouse, in which the heart contains four cardiac chambers (atria, ventricles) instead of two.

5. Antero-posterior patterning of the cardiac mesoderm in the mouse

Similar to that in the fish, the formation of the mouse heart depends on antero-posterior and left–right patterning. Heart precursors migrate bilaterally within the lateral plate mesoderm until they form a midline tube under the headfolds from E8. The cardiac tube grows by the addition of precursor cells from the anterior arterial pole and the posterior venous pole. Along the axis of the tube, cardiac segments are defined sequentially, from the venous to the arterial pole: the left–right atria, the atrio-ventricular canal, the left ventricle, the right ventricle and the outflow tract. While the tube grows, it undergoes asymmetric morphogenesis (heart looping), corresponding to the formation of a rightward helix [82]. Behind the heart tube at E8.5–E9.5, in the dorsal pericardial wall, cardiac precursor cells form an epithelial tissue referred to as the second heart field, which is patterned into an anterior and posterior domain, contributing cells to the elongation of the arterial and venous poles of the heart tube, respectively [98].

The left–right asymmetry of the heart is disrupted upon RA or FGF deficiency: looping defects have been reported in *Por*, *Fgf8* and *Aldh1a2* mutant embryos [29,99,100], and misaligned ventricles in *Rdh10* mutant fetuses [101]. *Fgf8;Mesp1^{Cre/+}* and *Fgf8;Mef2cAHF-Cre* conditional mutants [100] exhibit transposition of the great arteries and double outlet right ventricle, and are reminiscent of defective NODAL signalling [102–104]. The depletion of RERE, shown as a transcriptional cofactor of the RA receptor RAR in the presomitic mesoderm, also leads to looping defects [105]. Despite the presence of RARE regulatory sequences [38,106,107], NODAL pathway components do not respond to variations in RA signalling in this context, since they are correctly left-sided in *Aldh1a2* and

Fgf8 mutants [99,100]. In addition, the expression patterns of components of the RA pathway did not reveal any asymmetry in the chick [108,109]. Whether *Nr2f2* and *RARE-lacZ*, which are asymmetrically expressed in the presomitic mesoderm, or *aldh1a2*, asymmetrically expressed in the fish lateral plate mesoderm, are also asymmetric in the mouse cardiac mesoderm remains an open question. The defects in heart looping may be unrelated to left–right patterning and rather secondary to dorsal mesocardium persistence or impaired tube elongation [110,111]. Although shown in the fish, a role of RA signalling in the left–right patterning of mouse heart precursors has not yet been demonstrated.

RA signalling is detected along the antero-posterior axis and adjacent to FGF8/10 in the mouse lateral plate mesoderm (figure 2b). From E7.5 (late bud/early headfold stages), expression in the posterior mesoderm of the gene encoding the synthesizing enzyme ALDH1A2 expands anteriorly to the posterior boundary of the cardiac field [30]. At E8–E9.5, *Aldh1a2* overlaps with the posterior heart field, where the RA targets *Hoxa1/b1* and *Nr2f2* are also expressed [110,112]. *Rdh10* has a more restricted expression pattern within the domain of *Aldh1a2*, initiated later and extending less posteriorly [113]. In keeping with this, *Rdh10* mutants have a less severe phenotype compared with *Aldh1a2* mutants, in terms of lethality and heart malformation [101]. The degrading enzymes CYP26A1/B1 are expressed in the most anterior mesoderm at E7.5 [114]. Consistently, RA signalling, detected by the *RARE-lacZ* reporter line, mainly follows the pattern of *Aldh1a2* expression [9,110]. From E9.5, *RARE-lacZ* is also detected within the heart tube, in the sinus venosus and atria, as well as in a subdomain (inferior) of the outflow tract [115]. RA signalling in the sinus venosus reflects the expression of another synthesizing enzyme, CYP1B1 [5]. *Fgf8/10* are expressed in the anterior heart field at E8 (late headfold stage). They persist in the growing right ventricle and outflow tract, whereas they are absent in the left ventricle and inflow tract at E8.5 [100,116]. FGF and RA are thus signalling in adjacent domains. One exception is the inferior domain of the outflow tract, in which both RA (*Aldh1a2*, *RARE-lacZ* and *Hoxb1*) signalling and FGF (*Fgf8/10*, *Spry2*) signalling are overlapping [115–118]. This is reminiscent of the double origin of this region, from the anterior and posterior heart fields [48,119]. The signalling antagonism is manifested by increased and expanded FGF signalling in *Aldh1a2* mutants [110,120] and decreased in *Por* mutants [29].

Upon RA genetic deficiency, markers of the heart field, *Fgf8/10*, *Tbx1*, *Nkx2–5* and *Isl1*, are expanded posteriorly from the three- to four-somite stages (E8) [110,120] (figure 2b1,b2). Similarly to the fish, this is accompanied by the loss of limb progenitors [32]. In reverse, FGF deficiency decreases *Isl1* and *Mef2c* expression in the heart field [100]. The impact of excess RA upon *Cyp26* deficiency has not yet been studied at a molecular level. Impairment of FGF or RA signalling hinders heart tube elongation similarly, yet for distinct reasons: reduced cardiomyocyte differentiation in *Aldh1a2* and *Rara*; *Rarb* mutants [110,121] and reduced progenitor proliferation and survival in the anterior heart field of *Fgf* mutants [100,122]. In *Fgf* mutants, the right ventricle and outflow tract at the arterial pole are hypoplastic, whereas mutants with RA deficiency have hypoplastic atria and outflow tract [99,110]. In summary, antagonist FGF and RA signalling at early somite stages is required to restrict the mouse heart field and promote heart tube elongation.

RA is important to define the anterior and posterior second heart field (figure 2b3). RA signalling, which progresses anteriorly, activates *Hoxa1/a3/b1* including in the posterior heart field from E7.5 [32,118], by binding *RARE* regulatory sequences [39]. Genetic tracing indicates that this early domain contributes to both arterial and venous poles of the heart. Between E7.5 and E8.5, RA signalling activates *Tbx5* in the posterior heart field, including in some cells expressing the anterior marker *Tbx1*. Then, TBX5 downregulates the transcriptional programme of the anterior heart field, including *Tbx1*, *Mef2cAHF* enhancer and *Fgf10* enhancer. At E9.5, this establishes a boundary between expression of *Tbx1* in the anterior heart field and that of *Tbx5* in the posterior heart field [123]. At this later stage, this segregates progenitor cell populations of the arterial and venous poles. The *RARE* regulatory sequences of *Tbx5* are not functional [54], and *Tbx5* is directly activated by the RA target NR2F2 [46]. This suggests indirect activation of *Tbx5* by RA, unless NR2F2 is part of the WHHERE complex, together with the RA receptor RAR, as shown in the paraxial mesoderm. This remains to be demonstrated in cardiac progenitor cells. Inactivation of *Nr2f2*, which is expressed in the posterior heart field, leads to hypoplastic atria and sinus venosus at the venous pole [124]. Later inactivation in conditional *Nr2f2* mutants switches the identity of atrial cardiomyocytes into ventricular, which is reminiscent of the inverted switch, ventricular to atrial, by FGF deficiency in the fish [37].

In the mouse cardiac mesoderm, patterning by the RA/FGF signalling antagonism regulates sequentially the size of the heart field, its antero-posterior subdivision and cardiomyocyte chamber identity. After E10.5, RA is required for the septation of the outflow tract [50,99] and is required in the liver to induce erythropoietin (EPO), which activates growth factor production in the epicardium, ultimately affecting the transmural growth of the ventricular myocardium and the formation of coronary vessels [36,41].

6. Conclusion and perspectives

With advances in genetic engineering, the roles and redundancy of different components of endogenous RA signalling have now been largely dissected *in vivo*. A comparison between animal models and between tissues has accelerated research. A common point between the different mesodermal tissues analysed here is the alternative expression of *Cyp26/Fgf/Aldh1a2* (figures 1 and 2), which reflects molecular cross-regulations and antagonistic signalling between RA and FGF. The lateralization may differ, as exemplified by CYP26 being anterior relative to the cardiac mesoderm, but posterior relative to the paraxial mesoderm, or by FGF8 being a right determinant for chick but not mouse somitogenesis. Another common characteristic is the feedback mechanisms, amplifying RA signalling by induced expression of receptors and potentially of synthesizing enzymes, or restricting RA signalling by induced expression of degrading enzymes. The dose of RA, determined by the integrated output of different components of the pathway, appears as a key factor determining the phenotype. However, there does not seem to be a linear relationship between dose and phenotype, when deficiency and excess lead to similar phenotypes (table 1), which is similar to other morphogen gradients [2]. Recent developments of more quantitative measures of RA signalling [17], three-dimensional imaging of signalling [104] and

mathematical frameworks of spatial patterning [16] open up novel perspectives to model how positional information is encoded by RA/FGF signalling. Although RA signalling is a widespread factor of antero-posterior patterning in mesoderm, endoderm and neural ectoderm, conserved between chordates [42], it displays spatio-temporal dynamics, associated with differential response. It will be important to identify more of the tissue-specific cofactors and effectors of RA signalling to further understand how it induces, for example, cell apoptosis between digits, cell differentiation in the heart field or cell identity in cardiac chambers. The role of RA in left–right patterning appears divergent, to buffer intrinsic asymmetries and sustain symmetric morphogenesis of the forelimbs and somites, whereas it is upstream of Bmp signalling for the asymmetric morphogenesis of the fish heart. Opposed gradients of morphogens is a general principle of spatial patterning [2]. They initiate transcription factor networks, defining cell compartment boundaries and identity reflecting position. This is the case

for RA/FGF upstream of TBX5 and HOXA1/B1. However, RA signalling also directly regulates growth factors (e.g. FGF8, EPO), thus modulating cell behaviour to shape organs. Dissection of the direct roles of RA in specific cellular contexts remains a challenge, relevant to its teratogenicity but also its use in tissue engineering and cancer therapies.

Data accessibility. This article has no additional data.

Authors' contributions. S.B. and S.M.M.: conceptualization, writing and editing the manuscript.

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