Induction and prepatternning of the zebrafish pectoral fin bud requires axial retinoic acid signaling

Yann Gibert, Alexandra Gajewski, Axel Meyer and Gerrit Begemann*

Vertebrate forelimbs arise as bilateral appendages from the lateral plate mesoderm (LPM). Mutants in aldh1a2 (raldh2), an embryonically expressed gene encoding a retinoic acid (RA)-synthesizing enzyme, have been used to show that limb development and patterning of the limb bud are crucially dependent on RA signaling. However, the timing and cellular origin of RA signaling in these processes have remained poorly resolved. We have used genetics and chemical modulators of RA signaling to resolve these issues in the zebrafish. By rescuing pectoral fin induction in the aldh1a2/neckless mutant with exogenous RA and by blocking RA signaling in wild-type embryos, we find that RA acts as a permissive signal that is required during the six- to eight-somite stages for pectoral fin induction. Cell-transplantation experiments show that RA production is not only crucially required from flanking somites, but is sufficient to permit fin bud initiation when the trunk mesoderm is genetically ablated. Under the latter condition, intermediate mesoderm alone cannot induce the pectoral fin field in the LPM. We further show that induction of the fin field is directly followed by a continued requirement for somite-derived RA signaling to establish a prepattern of anteroposterior fates in the condensing fin mesenchyme. This process is mediated by the maintained expression of the transcription factor hand2, through which the fin field is continuously posteriorized, and lasts up to several hours prior to limb-budding. Thus, RA signaling from flanking somites plays a dual early role in the condensing limb bud mesenchyme.

KEY WORDS: Retinoic acid, Raldh2, Limb, Pectoral fin, Somites, Spadetail, No tail, Neckless, Zebrafish

INTRODUCTION

Vertebrate limbs arise bilaterally from the lateral plate mesoderm (LPM) of the embryo. Within the trunk LPM, interactions between the limb bud mesenchyme and the overlying ectoderm result in the initiation of local patterning and distal outgrowth that shape the fore and hindlimbs buds at the appropriate levels along the anteroposterior (AP) axis (reviewed by Capdevila and Izpisúa Belmonte, 2001; Logan, 2003; Tickle, 2003). Fibroblast growth factors (FGFs) have been shown to play an important role in limb induction. When beads are soaked in a range of fibroblast growth factors and are placed in the flank of chick embryos, they induce the formation of ectopic limb buds (Cohn et al., 1995; Ohuchi et al., 1995). Competence of the LPM to respond to these signals is initially distributed along most of the AP axis of the LPM in chick and mouse embryos (Tanaka et al., 2000).

The search for the axial sources of FGF expression in the chick embryo lead to the observation that the intermediate mesoderm (IM), situated between the somites and the LPM, is required for cell proliferation in the adjoining limb bud mesenchyme (Geduspan and Solursh, 1992; Smith et al., 1996); furthermore, FGF8 is produced in the intermediate mesoderm at the time of limb induction and, when applied to the chick flank, induces the development of additional limbs (Crossley et al., 1996; Vogel et al., 1996). On the other hand, when the induction of mesonephros, a component of IM, is blocked rostral to the future limb field, limb buds nevertheless form and develop normally (Fernandez-Teran et al., 1997). In addition, elimination of FGF8 from the IM does not affect limb initiation or outgrowth (Boulet et al., 2004). In addition, genes of the Wnt family are differentially expressed in the chick IM and LPM and are capable of inducing limb outgrowth (Kawakami et al., 2001). Specifically, forelimb induction requires Wnt2b expression in the IM and/or LPM of the chick, while its source in zebrafish appears to be the somites flanking the pectoral fin field (Ng et al., 2002). Thus, the identity of axial signals required for limb development as well as their source(s) remain to be determined.

Retinoic acid (RA) is required for a variety of processes during vertebrate embryonic development. Its effect is transmitted by retinoic acid receptors (RARs) at the level of regulating the expression of target genes (reviewed by Morriss-Kay and Ward, 1999). Three enzymes, Aldh1a1–3 (previously designated Raldh1–3), catalyze the final oxidative step by which vitamin A (retinol) is converted to RA, but only Aldh1a2 has been shown to be responsible for RA synthesis during early stages of embryogenesis. During gastrulation, aldh1a2 is expressed in embryonic mesoderm and is later found in mesodermal derivatives, such as somites, IM and LPM (Niederreither et al., 1997; Berggren et al., 1999; Swindell et al., 1999; Begemann et al., 2001; Chen et al., 2001; Grandel et al., 2002).

Mouse and zebrafish mutants in aldh1a2 have been used to show that the somitic mesoderm acts a source of RA that patterns adjacent axial tissues, such as the hindbrain and pancreas; importantly, aldh1a2 mutants also lack forelimb buds (Begemann et al., 2001; Grandel et al., 2002; Niederreither et al., 2002; Linville et al., 2004; Molotchov et al., 2005; Stafford et al., 2006). The absence of forelimb buds in aldh1a2 mutants is associated with the loss of tbx5 expression, the earliest known marker of the developing forelimb field (Gibson-Brown et al., 1996; Tamura et al., 1999; Begemann and Ingham, 2000). In the mouse, Tbx5 first serves to establish forelimb bud outgrowth by initiating the expression of regulatory loops of Wnt and Fgf proteins (reviewed by Capdevila and Izpisúa Belmonte, 2001; Logan, 2003); while in the zebrafish, tbx5 is required for the migration of mesenchymal LPM cells to the pectoral fin bud. As a result, mutants in both vertebrates do not form forelimb buds (Ahn et al., 2002; Garrity et al., 2002; Agarwal et al., 2003;
The failure to induce \textit{tbx5} expression in the absence of early RA signaling therefore indicates an essential early role for RA during the establishment of the limb/fin field.

In zebrafish, \textit{aldh1a2} is expressed in the posterior mesenchyme of the developing pectoral fin and in the LPM (Grandel et al., 2002; Emoto et al., 2005). RA has been detected in the limb bud mesenchyme (Thaller and Eichele, 1987; Niederreither et al., 2002); however, RA production in the cells of the polarizing region of the limb bud has not been demonstrated. Expression analyses in zebrafish and mouse embryos mutant for \textit{Aldh1a2} have shown that RA is required to induce sonic hedgehog (Shh), the protein responsible for the polarizing activity (reviewed by Tickle, 2003), during forelimb outgrowth (Begemann et al., 2001; Grandel et al., 2002; Niederreither et al., 2002), and suggest that RA signaling may be involved in the establishment of AP polarity in the limb. Indeed, maternal RA supplementation of \textit{Aldh1a2} mutant mice rescues forelimb development, but does not restore normal AP patterning of the limb (Niederreither et al., 2002). Rescued limbs are characterized by a lack or abnormal distal distribution of Shh. More recent studies, however, have proposed a different role for RA in limb patterning. Using mice mutant for \textit{Aldh1a2}, Mic et al. (Mic et al., 2004) have shown that RA signaling is required first to initiate forelimb development and later to expand the apical ectodermal ridge (AER) along the distal ectoderm. A study by Yashiro et al. (Yashiro et al., 2004) similarly suggested that a gradient of RA signaling is required to determine proximodistal identity in the developing mouse forelimb.

In this study, we determine the crucial time period for limb induction and the source of RA. By analyzing pectoral fin development in the absence of RA, we uncover that prepatternning by RA occurs several hours prior to the budding of the pectoral fin to establish a functional zone of polarizing activity. Our findings point to an essential role for somite-derived RA in fin field induction, apical fold formation and in establishing posterior cell fates within the condensing fin mesenchyme.

**MATERIALS AND METHODS**

**Zebrafish husbandry**

Zebrafish strains of Konstanz wild type, \textit{nlsl26} (Begemann et al., 2001), \textit{mp48001} (Odenthal et al., 1996) and \textit{spit100} (Kimmel et al., 1989) were reared and staged at 28.5°C according to Kimmel et al. (Kimmel et al., 1995).

**Pharmacological treatments**

Embryos were incubated in the dark at 28.5°C in 10⁻⁸ M all-trans retinoic acid (Sigma), diluted in embryo medium, from a 10⁻² M stock solution in DMSO. The pan-retinoic acid receptor antagonist BMS493 (a kind gift of Bristol Myers Squibb) was diluted to 10⁻⁶ M and 5⁻⁶ M from a 10⁻² M stock solution in ethanol. DEAB (4-diethylaminobenzaldehyde) (Fluka) was applied at a concentration of 10⁻⁵ M from a 10⁻² M stock in DMSO, without shielding from daylight. As controls, wild-type embryos were treated with equivalent concentrations of DMSO. Genotyping to detect homozygosity of the \textit{nlsl} allele was performed as previously described (Begemann et al., 2001).

**In situ hybridization**

Whole-mount in situ hybridization was performed as previously described for \textit{aldh1a2} (Begemann et al., 2001), using the following additional probes: \textit{dlx2a} (Akimenko et al., 1994), \textit{hand2} (Yelon et al., 2000), \textit{hoxc6a} (Molven et al., 1990), \textit{hoxd11a}, \textit{hoxd12a} (Sordino et al., 1995), \textit{msx2} (Ekkert et al., 1992), \textit{myod} (Weinberg et al., 1996), \textit{shh} (Krauss et al., 1993), \textit{tbx5} (Begemann and Ingham, 2000), \textit{tpm1} (Zebrafish Information Network) and \textit{pax2a} (Krauss et al., 1991).

**Mosaic analysis**

Donor embryos were injected at the one-cell stage with 2.5% lysine fixable tetramethyl-rhodamin-dextran and 3.0% lysine fixable biotin-dextran (M, 100,000)(Molecular Probes) dissolved in 0.2 M KCl. At late blastula stages, groups of 10-30 donor cells were transplanted into unlabelled host embryos of the same stage, derived from pair-matings of \textit{nlsl} heterozygotes, and placed along the margins of the blastoderm, which gives rise to the mesendoderm (Kimmel et al., 1990). Transplants were carried out blindly, and host genotypes determined at 24 hpf. Transplanted cells were examined at 48 hpf for fluorescence in all \textit{nlsl} hosts prior to or after fixation and in situ hybridization.

**RESULTS**

**Induction of the pectoral fin field requires RA signaling during early somitogenesis stages**

RA signaling during gastrulation has been shown to regulate the development of the neuroectoderm, endoderm and cardiac mesoderm, respectively (Grandel et al., 2002; Kudoh et al., 2002; Stafford and Prince, 2002; Keegan et al., 2005). In order to determine in more detail the developmental stages during which RA signaling is required to induce the pectoral fin field, we employed two complementary strategies: rescuing \textit{neckless (nlsl)} through exogenous RA; and inhibiting fin field formation by blocking RA signaling in wild-type zebrafish.

Initiation of forelimb bud formation is characterized by the expression of \textit{tbx5} in the anterior lateral plate mesoderm and precedes the emergence of visible forelimb buds (Ruvinsky et al., 2000; Ahn et al., 2002; Garrity et al., 2002; Ng et al., 2002). Pectoral fin development is characterized by the appearance of an apical fold that expresses \textit{dlx2a} (Akimenko et al., 1994). We treated wild-type embryos with 10⁻⁶ M BMS493, which inhibits the activation of all three RA-receptor (RAR) subforms in amniotes by reinforcing co-repressor binding to RAR (Wendling et al., 2000; Dupé and Lumsden, 2001) from 10 hpf onwards, and confirmed that fin buds do develop and express both \textit{tbx5} and \textit{dlx2a} (Grandel et al., 2002) (Fig. 1B,F). However, when treated with 5×10⁻⁶ M BMS493 or with 10⁻⁵ M diethylaminobenzaldehyde (DEAB), a competitive reversible inhibitor of retinaldehyde dehydrogenases (Begemann et al., 2004) from 10 hpf onwards, no fin buds emerged and none of the genes were expressed (Fig. 1C,D,G,H). We next refined these tests of RA requirement by incubations in 10⁻³ M DEAB or in a range of 10⁻⁵ to 5×10⁻⁶ M BMS493. We observed that blocking RAR activation after 13 hpf, or inhibiting endogenous RA synthesis after 12 hpf, has no effect on the onset of \textit{tbx5} expression in the pectoral fin, while treatments commencing earlier disrupt fin field induction (Table 1A). These results suggest that RA signaling is required for fin development up to 13 hpf.

We next supplied embryos derived from parents that were heterozygous for \textit{nlsl} with an exogenous supply of 10⁻⁸ M RA, commencing treatment from successively later stages onwards, and monitored expression of \textit{tbx5} at 24 hpf, when the mesenchymal core of the pectoral fin bud has formed (Tamura et al., 1999; Begemann and Ingham, 2000) (Fig. 1Q). \textit{nlsl} mutants were identified either through expansion of \textit{krox20} (egr2b – Zebrafish Information Network) expression in the hindbrain when treated after 10 hpf (not shown), or by genotyping when treated prior to 10 hpf. In agreement with a requirement for RA after 10 hpf, treatments initiated as late as 12 hpf are sufficient to rescue \textit{tbx5} expression to approximately wild-type levels, while treatment at 13 hpf or later is not sufficient (Table 1B).

To confirm the hypothesis that RA signaling after gastrulation is required for fin induction, RA synthesis was inhibited with DEAB in wild-type embryos starting from 5 hpf onwards, and at 11 hpf was supplanted with exogenous RA; fin bud induction in these fish proceeded indistinguishably from wild-type development (Fig. 1LJ). Together, these experiments suggest that pectoral fin field
development requires RA signaling after the end of gastrulation and prior to the 12 hpf (six-somite stage). The sufficiency of this short period of treatment indicates that RA is required for induction, rather than maintenance of the pectoral fin field.

To test if development in RA-rescued fins in nls proceeds normally, we analyzed the expression of further genes involved in pectoral fin development. fgf10 acts genetically downstream of fgf24 (Fischer et al., 2003) and is expressed in the fin mesenchyme at 24 hpf (Ng et al., 2002). In RA-rescued fins of nls mutants, fgf10 is expressed strongly (Fig. 1K,L), suggesting that its upstream component fgf24 must also be active. tbx15 is strongly expressed throughout the pectoral fin mesenchyme starting from 30 hpf (Begemann et al., 2002) and is detected in RA-rescued nls fins (Fig. 1M,N). We conclude that genes downstream of tbx5 in the fin mesenchyme are activated following RA-treatment in nls. By contrast, expression of wnt2b, which is required for tbx5 expression and at 22 hpf is found in the ventral part of somites flanking the pectoral fin bud (Ng et al., 2002), is not abolished in wild type fish treated with DEAB or a pan-antagonist of all RA-receptors from 11-22 hpf (Fig. 10,P and not shown), which includes the time of fin induction and early patterning. Thus, RA is dispensable for somitic wnt2b expression. Like aldha1a2, wnt2b acts upstream of tbx5 during fin induction (Ng et al., 2002), so that both genes might operate in parallel pathways. Alternatively, earlier wnt2b expression outside the somites may be required to regulate tbx5.

**Somitic mesoderm is required for pectoral fin induction**

During segmentation stages, aldha1a2 is expressed in the somitic and intermediate mesoderm (Begemann et al., 2001; Grandel et al., 2002). As both these embryonic structures are in close proximity to the future pectoral fin field, we wanted to distinguish whether the source of RA that triggers pectoral fin induction is the intermediate or somitic aldha1a2 expression domain. To this end, we genetically ablated the somitic mesoderm in embryos double mutant for spadetail (spr) and no tail (ntl) (Amacher et al., 2002; Goering et al., 2003), taking advantage of the fact that double-mutants for the allelic combination of spr<sup>+/−</sup> and ntl<sup=+/−</sup> lack somitic mesoderm, but retain a reduced intermediate mesoderm (IM), as marked by the expression of pax2a, a marker of the pronephric duct and the glomerulus (Majumdar et al., 2000) (Fig. 2A-D). Similarly, somitic aldha1a2 expression is reduced in spr and is absent in spr<sup>+/−</sup>ntl<sup>−/−</sup> (Fig. 2E-H'). Because a faint aldha1a2 staining remains in double mutants, we wanted to identify unambiguously double-mutant embryos by simultaneously monitoring both myod and aldha1a2 expression. At 13 hpf, when RA signaling is required for pectoral fin field induction, aldha1a2 is expressed in the wild-type IM, and can be clearly detected at reduced levels in the IM of spr<sup>+/−</sup>ntl<sup>−/−</sup> (Fig. 2J,J'). These results establish that in spr<sup>+/−</sup>ntl<sup>−/−</sup> the IM is likely to produce RA.

spr<sup>+/−</sup>ntl<sup>−/−</sup> double mutants do not develop any morphological signs of pectoral fin development and tbx5 expression in the pectoral fin field is indistinguishable from wild type in ntl (Fig. 3B). In
agreement with the reduction of aldha1 expression in trunk mesoderm, tbx5 expression is slightly reduced in spt (Fig. 3C). By contrast, double-mutant embryos lack tbx5 expression (Fig. 3D), suggesting that fin field induction requires RA signaling from somites rather than from IM.

**RA is sufficient to rescue fins in the absence of somites**

As the lack of tbx5 expression in nls/spt could either be due to the lack of somite-derived signals distinct from RA or because the LPM may not be competent to form fins, we asked if exogenous application of RA after gastrulation would rescue pectoral fin field induction. Interestingly, application of $10^{-8}$ M RA, beginning at 10 hpf, efficiently rescues tbx5 expression in sptb104/ntltc41, suggesting that the LPM is competent to respond to a somite-derived signal to form fins (Fig. 3I,K). As RA application after gastrulation does not reconstitute somite development in double mutants (Fig. 3J-M), RA is identified as the signal emanating from somites that induces the pectoral fin field. Moreover, these findings further suggest that somite-derived signals other than RA are not required to initiate tbx5 expression.

**RA from anterior somites induces pectoral fin field formation**

In order to determine more directly the source of RA, we next investigated whether somitic cells of wild-type origin were able to induce tbx5 expression in the lateral plate mesoderm of nls mutants. We transplanted lineage-labeled wild-type cells at blastula stages into the lateral marginal zone of nls mutant hosts, a region fated to give rise to trunk somites (Melby et al., 1996). nls hosts were identified by their hindbrain phenotype at 22-24 hpf and scored for the number and distribution of transplanted cells (Table 2). In such an experiment, we not only identified the nls hosts with rescued fins, but the presence of wild-type cells in somites without rescue is equally informative. None of the nls hosts had donor cells in

### Table 1. The temporal requirement of RA for pectoral fin field induction

#### A Inhibition of pectoral fin development

<table>
<thead>
<tr>
<th>Beginning of antagonist treatment*</th>
<th>Sample size (n)</th>
<th>Antagonist (concentration)</th>
<th>Number of embryos lacking tbx5a expression in pectoral fins (% of sample)</th>
<th>Loss of tbx5a expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.3 hpf</td>
<td>60</td>
<td>BMS ($10^{-5}$ M)</td>
<td>60 (100%)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>39</td>
<td>BMS ($5 \times 10^{-6}$ M)</td>
<td>39 (100%)</td>
<td>+</td>
</tr>
<tr>
<td>8 hpf</td>
<td>64</td>
<td>BMS ($10^{-5}$ M)</td>
<td>64 (100%)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>41</td>
<td>BMS ($5 \times 10^{-6}$ M)</td>
<td>41 (100%)</td>
<td>+</td>
</tr>
<tr>
<td>10 hpf</td>
<td>102</td>
<td>BMS ($10^{-5}$ M)</td>
<td>102 (100%)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>41</td>
<td>BMS ($5 \times 10^{-6}$ M)</td>
<td>41 (100%)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>DEAB ($10^{-5}$ M)</td>
<td>30 (100%)</td>
<td>+</td>
</tr>
<tr>
<td>11 hpf (3 s)</td>
<td>40</td>
<td>BMS ($10^{-5}$ M)</td>
<td>40 (100%)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>DEAB ($10^{-5}$ M)</td>
<td>30 (100%)</td>
<td>+</td>
</tr>
<tr>
<td>12 hpf (6 s)</td>
<td>40</td>
<td>BMS ($10^{-5}$ M)</td>
<td>40 (100%)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>DEAB ($10^{-5}$ M)</td>
<td>26 (87%)</td>
<td>+</td>
</tr>
<tr>
<td>13 hpf (8 s)</td>
<td>40</td>
<td>BMS ($10^{-5}$ M)</td>
<td>18 (45%)</td>
<td>+/-</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>DEAB ($10^{-5}$ M)</td>
<td>0 (0%)</td>
<td>+</td>
</tr>
<tr>
<td>14 hpf (10 s)</td>
<td>40</td>
<td>BMS ($10^{-5}$ M)</td>
<td>0 (0%)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>DEAB ($10^{-5}$ M)</td>
<td>0 (0%)</td>
<td>+</td>
</tr>
<tr>
<td>14.5 hpf (11 s)</td>
<td>40</td>
<td>BMS ($10^{-5}$ M)</td>
<td>0 (0%)</td>
<td>+</td>
</tr>
<tr>
<td>15.5 hpf (13 s)</td>
<td>40</td>
<td>BMS ($10^{-5}$ M)</td>
<td>0 (0%)</td>
<td>+</td>
</tr>
</tbody>
</table>

#### B Rescue of pectoral fin development

<table>
<thead>
<tr>
<th>Beginning of RA treatment</th>
<th>Sample size (n)</th>
<th>Number of nls mutants¹ (expected number of nls mutants)</th>
<th>Number of nls mutants expressing tbx5a in pectoral fins (% of rescued nls mutants)</th>
<th>Rescue of tbx5a expression in nls</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO control 5.3 hpf</td>
<td>51</td>
<td>6 (13)</td>
<td>6 (100%)</td>
<td>–</td>
</tr>
<tr>
<td>5.3 hpf</td>
<td>55</td>
<td>Not detectable (18)</td>
<td>Not detectable (100%)</td>
<td>+</td>
</tr>
<tr>
<td>8 hpf</td>
<td>80</td>
<td>Not detectable (20)</td>
<td>Not detectable (100%)</td>
<td>+</td>
</tr>
<tr>
<td>9 hpf</td>
<td>80</td>
<td>Not detectable (20)</td>
<td>Not detectable (100%)</td>
<td>+</td>
</tr>
<tr>
<td>10 hpf</td>
<td>33</td>
<td>6 (8)</td>
<td>6 (100%)</td>
<td>+</td>
</tr>
<tr>
<td>11 hpf (3s)</td>
<td>49</td>
<td>9 (12)</td>
<td>9 (100%)</td>
<td>+</td>
</tr>
<tr>
<td>12 hpf (6s)</td>
<td>117</td>
<td>35 (29)</td>
<td>33 (94%)</td>
<td>+</td>
</tr>
<tr>
<td>13 hpf (8s)</td>
<td>134</td>
<td>36 (33,5)</td>
<td>0 (0%)</td>
<td>–</td>
</tr>
<tr>
<td>16 hpf (14s)</td>
<td>43</td>
<td>10 (11)</td>
<td>0 (0%)</td>
<td>–</td>
</tr>
<tr>
<td>19 hpf (20s)</td>
<td>50</td>
<td>10 (12,5)</td>
<td>0 (0%)</td>
<td>–</td>
</tr>
<tr>
<td>22 hpf (26s)</td>
<td>38</td>
<td>12 (9,5)</td>
<td>0 (0%)</td>
<td>–</td>
</tr>
</tbody>
</table>

*Incubations lasted until 24 hpf, at which stage tbx5 expression was assayed. s, number of somites.

¹As identified by expansion of krox20 expression in the hindbrain and reduction of hoxb4a expression in the anterior spinal cord, or by genotyping.
derivatives of the intermediate mesoderm. In one host with rescued fins \((n=2)\), the majority of muscle fibers in somites 5-7 were donor derived, with only a few wild-type cells in somite 3 and 4 (Fig. 4A-C). In another successful rescue, the majority of muscle fibers in somites 3-6 were wild-type derived (Fig. 4D-F). In hosts with only a few wild-type cells occupying somites 4-7 \((n=6)\), or where donor cells gave rise to part or all of the muscle fibers in somites posterior to somite 8 \((n=6)\), no rescue was observed (Table 2; Fig. 4H).

Interestingly, one transplanted embryo showed very strong contribution of wild-type cells to the dorsal halves of somites 1-8, yet did not lead to pectoral fin development (Fig. 4G).

These transplantation experiments show that RA signaling from somites 3-7 is sufficient for pectoral fin development; moreover, it appears that signaling originates from cells in the ventral myotome.

**Sources and requirement of RA in the developing pectoral fins**

To resolve the role of RA in pectoral fin bud development and during the outgrowth phase, we first determined the expression pattern of *aldh1a2* in more detail and in later stages of fin development than previously shown. *aldh1a2* is first re-expressed at 28 hpf in the posterior-most part of the pectoral fin bud and in the adjacent LPM (Fig. 5A,B). Between 32 and 48 hpf, *aldh1a2* is expressed in the entire pectoral fin mesenchyme (Fig. 5C,D) and becomes restricted proximally at later stages (Fig. 5E,H). Thus, during initiation of fin outgrowth, RA is produced from a posterior source of *aldh1a2* expression, is synthesized uniformly in the entire fin at later stages, and becomes gradually restricted to the proximal anterior fin mesenchyme by day 5.

We next investigated the roles of RA signaling during later stages of pectoral fin development. Wild-type embryos treated with either BMS493 (not shown) or DEAB from 16 hpf onwards develop pectoral fin buds, although they are smaller than in wild type (Fig. 6). This suggests that the initiation of bud outgrowth is independent of RA, although normal cell proliferation within the pectoral fin bud may require full RA signaling.

To determine to what extent fin patterning is affected in the absence of RA, we examined gene expression in the fin bud mesenchyme and apical fold. The bHLH transcription factor *hand2* is required for the expansion of the pectoral fin-forming
Development 133 (14)

RA signaling is abolished, anteriorly as the fin bud grows (Molven et al., 1990) (Fig. 6I). When anterior half of the fin mesenchyme at 28 hpf and is restricted more findings indicate that early AP patterning of the developing fin bud
when the anterior half of the mesenchyme at 38 hpf (Fig. 6J). These

When RA synthesis is suppressed, expression of hoxd12a

immediate lack of RA, or may be a consequence of the loss of

expression of which does not depend on shh

of wild-type fin buds at 40 hpf (Sordino et al., 1995) (Fig. 6E,G), the

expression. In wild type, shh is strongly downregulated (Fig. 6C,D). Both

Expression of which does not depend on shh

expression may be due to the immediate lack of RA, or may be a consequence of the loss of hand2.

hoxd11a and hoxd12a are markers of the posterior mesenchyme of wild-type fin buds at 40 hpf (Sordino et al., 1995) (Fig. 6E,G), the

expression. When RA synthesis is suppressed, expression of hoxd11a

and hoxd12a is downregulated (Fig. 6E-H). hoxc6a is marker of the

anterior half of the fin mesenchyme at 28 hpf and is restricted more

anteriorly as the fin bud grows (Molven et al., 1990) (Fig. 6I). When

RA signaling is abolished, hoxc6a is expanded posteriorly to cover

the anterior half of the mesenchyme at 38 hpf (Fig. 6J). These

findings indicate that early AP patterning of the developing fin bud

is compromised in the absence of RA, leading to an expansion of the

anterior marker hoxc6a and the downregulation of markers of the

posterior fin bud mesenchyme.

To test whether loss of RA signaling affects the development of the apical fold, a structure required for fin outgrowth, we examined dlx2a expression. As in wild type, dlx2a is expressed along the AP axis of the apical fold during fin budding in the absence of RA synthesis (Akimenko et al., 1994) (Fig. 6K,L). In a subset of embryos treated with DEAB from 14 hpf, rather than 16 hpf, onwards, slight variations in the AP extent of dlx2a expression was observed, such as downregulation in the anterior and posterior apical fold (not shown). This suggests that full RA signaling is required to establish a complete apical fold.

**Axial RA signaling patterns the fin field prior to budding**

**aldh1a2** is widely expressed in the pectoral fin bud (Fig. 5), whereas in the mouse it is restricted to the lateral plate mesoderm (Mic and Duester, 2003; Mic et al., 2004; Yashiro et al., 2004). We therefore determined if pectoral fin patterning is mediated by RA synthesized within the fin bud mesenchyme, by blocking signaling after initiation, but prior to aldh1a2 expression in the fin bud. Wild type were

region of the LPM and is expressed in the pectoral fin-bud mesenchyme, with the exception of its anteriormost aspect (Yelion et al., 2000). In the absence of RA, hand2 is not expressed (Fig. 6A,B). Because mutants in hand2 fail to express sonic hedgehog (shh), we next analyzed shh expression. In wild type, shh is expressed in the posterior fin bud mesenchyme at 28 hpf, colocalizing with the zone of polarizing activity (ZPA). In the absence of RA, shh is strongly downregulated (Fig. 6C,D). Both

shh and hand2 remain downregulated at least up to 48 hpf (not shown). Thus, downregulation of shh expression may be due to the immediate lack of RA, or may be a consequence of the loss of hand2.

**Alkaline phosphatase staining**

Alkaline phosphatase staining was used to visualize the developing fin field and to identify the anterior somites that were transplanted to the nls host embryos. The staining reaction was carried out according to the protocol of Ikeda and Kuroiwa (2000). After antibody staining, sections were counterstained with hematoxylin.

### Table 2. Transplanted wild-type cells in anterior somites rescue pectoral fin induction in nls

| Pectoral fin present | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
|---------------------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|
| Yes                 | – | + | ++| ++| ++| ++| – | – | – | –  | –  | –  | –  | –  | –  | –  | –  |
| Yes                 | – | – | – | + | ++| ++| ++| ++| + | + | + | + | + | + | + | + | + |
| No†                | ++| ++| ++| ++| ++| ++| ++| ++| + | + | + | + | + | + | + | + | + |
| No                 | + | + | – | – | – | – | – | + | + | – | – | – | – | – | – | – | – |
| No                 | – | – | – | + | ++| ++| ++| ++| + | + | + | + | + | + | + | + | + |
| No                 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| No                 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| No                 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| No                 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| No                 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| No                 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |

Rescue of pectoral fin development in nls embryos.

*nls* hosts were identified by their hindbrain phenotype (otic placode abuts the first somite) at 22-24 hpf; nls hosts were scored for the number and distribution of transplanted cells, and presence of a pectoral fin bud, at 48-50 hpf.

*Shown are transplants located in the first 16 somites; donor-derived muscle fibres were counted in somites (vertical columns), indicated as absence (–), or fewer (+) or more (+++) than ten fibers per somite.

†In this nls fish, all transplanted cells were located dorsal to the horizontal myoseptum.

Fig. 4. Retinoic acid signaling from anterior somites rescues pectoral fin development in nls. Anterior is towards the left: lateral (A,B,D,E,G,H) and dorsal views (C,F). Transmitted light (A,C,D,F) and fluorescent (B,E,G,H) images taken at 48 hpf. (A-C) Wild-type donor cells (red fluorescence in B) occupying somites (s) 2-7 in a nls host are able to rescue pectoral fin (pec) development and tbx5 expression on the transplanted side (dark area in pectoral fin in A,B; arrow in C); chevron-shaped lines mark somite boundaries. The embryo in B was processed for in situ hybridization prior to photography, resulting in apparent enhancement of unspecific autofluorescence within the yolk. (D-F) Wild-type donor cells occupying most of somites 3-6 in a nls host rescue pectoral fin development (arrow in D) and tbx5 expression (arrow in F). (G,H) Donor cells transplanted to the dorsal aspect of somites 1-7 (G) or to somites posterior to somite 6 (H) are unable to rescue pectoral fin development (not shown). Scale bar: 100 μm.
incubated in 10 μM DEAB from 14, 18 and 22 hpf onwards, and assayed for shh expression at 32 hpf. Inhibiting RA signaling from 14 hpf results in reduced fin buds devoid of detectable shh expression and inhibition from 18 hpf onwards lead to a reduction of most shh expression (Fig. 6O,P). By contrast, blocking RA signaling after 22 hpf had little effect on shh expression (Fig. 6M,N). This suggests that AP patterning within the pectoral fin field occurs throughout early to mid-somitogenesis and prior to the formation of the limb bud. As aldha1a2 is expressed in the fin bud after its AP pattern has been established, somite-derived RA is required to pattern the developing field along with the condensation of the fin mesenchyme.

Effects of RA depletion during pectoral fin outgrowth

To examine the function of aldha1a2 expression within the developing fin bud, we compared the effects of blocking RA production by incubation in DEAB in wild type between 11 and 17 hpf, and during budding and outgrowth of the fin after 28 hpf. Larvae (n=80) resulting from the former treatment lack pectoral fins. At 6 dpf, only the exoskeletal component of the shoulder girdle, the cleithrum, is present, while all endoskeletal derivatives, which develop from within the fin bud mesenchyme, are absent. Only in a few cases (n=5/80) a much reduced scapulocoracoid had formed (Fig. 7B). Larvae (n=80) incubated in DEAB from 28 hpf onwards exhibit defects in the development of proximal skeletal elements of the shoulder girdle (n=80) (Fig. 7C). More distal regions, including the endoskeletal disc, do not show any defects that are detectable at the molecular or morphological level (Fig. 7D-G). We conclude that RA is required for the proper differentiation of the cartilaginous elements of the shoulder girdle along their dorsoventral extent (relative to the animal’s axis).

DISCUSSION

Somites provide RA for pectoral fin field induction

The contribution of RA signaling to the establishment of the forelimb field is poorly understood and the source for RA signaling establishing the fin/limb field has remained elusive. We have investigated this RA requirement in more detail and show that RA induces pectoral fin field formation after gastrulation and prior to the formation of somites 6-8. Thus, RA signaling between 12 and 13 hpf is sufficient for pectoral fin field induction as marked by tbx5 expression. We show that RA signaling originates from expression of aldha1a2 in somites 3-7, which appear during this time (Fig. 4). RA synthesis from anterior somites thus fulfills several important developmental roles: induction of the fin field, as well as neural patterning and motoneuron differentiation in the anterior spinal cord (Begemann et al., 2004; Linville et al., 2004). Our findings also explain the results of a previous study, in which treatment of zebrafish embryos with citral, an unspecific inhibitor of retinaldehyde dehydrogenases, from 80% epiboly to the two-somite stage leads to morphologically normal fish that lack the pectoral fins (Vandersea et al., 1998).

We observed a notable correspondence of temporal RA requirement with the development of the somites flanking the prospective forelimb field. In zebrafish, the pectoral fin develops...
lateral to the second and third somite (Grandel and Schulte-Merker, 1998), and RA is required while these somites are formed. Similarly, in the mouse embryo the forelimbs develop adjacent to somites 8-12, and limited maternal RA treatment that includes the development of the first 4-8 somites in Aldh1a2-mutant mice rescues initiation of the forelimb field (Mic et al., 2004). Considering the variation inherent to rescue studies in the mouse in general, and that most RA may not be cleared immediately, limb field induction in mice can be viewed as taking place concurrently with the emergence of flanking somites. This observation holds particularly true for the chick embryo, where forelimb induction has occurred by the 20-25 somite stage, and the wing bud develops adjacent to somites 15-20 (reviewed by Martin, 1998). We therefore propose a general mechanism by which somite formation and forelimb field induction are coupled through a window of RA responsiveness.

In zebradish, the pelvic fins develop more than 2 weeks after completion of somitogenesis, in a position ventrolateral to the ninth and tenth myotome (Grandel and Schulte-Merker, 1998). If RA is equally required to induce pelvic fin development, pelvic fin induction may either occur shortly after the formation of the corresponding somites, or may be concomitant with the formation of the mesenchymal layer present in the prospective pelvic area that is present during the third week postfertilization (Grandel and Schulte-Merker, 1998). In the latter case, a heterochronic shift in signaling from RA alone or in conjunction with another signal may have enabled the delay in pelvic fin development in zebrafish. Alternatively, RA-mediated induction may not be in place for the hindlimbs. Appropriate RA rescue and inhibition assays will have to be performed to test the extent of conservation in the molecular mechanisms governing fore- and hindlimb induction.

RA is a permissive factor for fin development

Unlocalized exogenous RA rescues tbx5-expressing fin buds in nls or RA-depleted embryos to an extent indistinguishable from wild type (Fig. 1). RA thus cannot be an instructive signal, but has to act as a permissive factor whose ectopic presence does not interfere with fin formation.

As RA is sufficient to rescue fin induction in the absence of somitic mesoderm, other somite-derived signals are not required to allow fin field induction. This finding appears to be in disagreement with a previously proposed role of wnt2b, which acts genetically upstream of tbx5. wnt2b is expressed in the ventral part of somites flanking the pectoral fin bud at 22 hpf (Ng et al., 2002), several hours after fin induction. Morpholino-induced inactivation of wnt2b leads to a failure of expression of tbx5, as well as of fgf10 and fgf24, in the fin bud mesenchyme, which can be rescued by injection of tbx5 mRNA (Ng et al., 2002; Fischer et al., 2003). Our findings therefore imply that wnt2b may be expressed outside the somites during pectoral fin field induction, in LPM or IM. Unfortunately, wnt2b is only weakly expressed, hampering detection of expression outside the somites or during the early segmentation period (not shown).

RA acts over a short range during pectoral fin induction

The somitic mesoderm and LPM are in close proximity during pectoral fin field induction, separated by the IM. Diffusion of RA from forming somites must therefore travel through IM or the ectoderm to the LPM. The occurrence of one transplant with considerable contribution of wild-type cells to the dorsal myotome that fails to rescue fin induction suggests that RA may diffuse over a short distance only (Fig. 8). In agreement with the temporal requirement of RA, aldhl1a2-expressing cells posterior to the eighth somite either emerge too late or are located too far from the fin field for induction. Alternatively, the LPM may have lost the competence to respond to RA once the embryo has passed the eight-somite stage.

In the absence of somites, the molecular targets of RA are likely to be found in the LPM. Of the possible target genes that RA may act on, tbx5 is the earliest marker expressed in cells of the LPM that contribute to the fin bud. Expression of tbx5 starts at the seventhsomite stage, in two paraxial domains that flank the anterior head and trunk of the embryo (Tamura et al., 1999; Begemann and Ingham, 2000; Ruvinsky et al., 2000). The temporal requirement for RA signaling coincides with the onset of tbx5 expression in the LPM, therefore RA may directly induce tbx5 expression in the presumptive fin field. However, we have been unable to identify sequences of putative binding sites for RA receptors (RA-responsive elements) in the putative promoter region of human Tbx5 (the genomic sequence of the zebrafish tbx5 region is currently incomplete) that could mediate direct regulation of tbx5. Agarwal et al. (Agarwal et al., 2003) have proposed that in the mouse an axial signal may exist that serves to support Tbx5 expression, or that might confer competence to the LPM to induce forelimb bud outgrowth by Tbx5. Our experiments in zebrafish suggest that RA is synthesized at the right time and place to qualify as such a signal.

Limb initiation through axial signals

Axial signals that regulate limb development are likely to be derived from paraxial or intermediate mesoderm (IM). The finding that tbx5 expression, and thus the presumptive fin field, is absent in ntl/spt embryos, identifies somitic aldhl1a2 expression, rather than expression in the IM, as the earliest genetically confirmed source of an axial signal that triggers fin field initiation.

A role of IM in limb induction is still debated. Experiments using foil barriers and ablation of IM tissue in the chick embryo have suggested that the IM provides important signals for limb
development (Stephens and McNulty, 1981; Strecker and Stephens, 1983; Geduspian and Sorulsh, 1992). However, this view has been challenged by the observation that wing buds form in experiments blocking chick mesodermic differentiation, and more specifically IM-expressed FGF8 is dispensable for mouse limb induction (Fernandez-Teran et al., 1997; Boulet et al., 2004); finally, the loss of IM cells does not affect normal development of limbs in Pax2<sup>−/−</sup>/Pax8<sup>−/−</sup> mouse embryos (Bouchard et al., 2002). Although we did not observe transplanted wild-type cells in the IM of nls mutants, the rescue of fin development by somites alone suggests that IM-derived RA signaling is not required for pectoral fin initiation in wild type.

**Continuous RA signaling prior to fin outgrowth is required for AP-pre-patterning and apical fold formation**

Taking advantage of the relative ease with which RA signaling can be manipulated using exogenous agents in the zebrafish, we have uncovered that following RA-mediated induction of the pectoral fin field at 12-13 hpf, RA remains to be required continuously throughout the pre-budding stage to establish posterior cell fates in the fin field. Importantly, this RA dependency occurs several hours prior to the development of a visible mesenchymal condensation and of apical fold formation. This is exemplified by the absence of shh expression in fin buds devoid of RA prior to 22 hpf (Fig. 6M-P). As aldhl<sub>1a</sub>2 is not expressed within the fin field until a visible bud emerges at 26-28 hpf, the strong expression of aldhl<sub>1a</sub>2 in adjacent somites is most likely to provide RA to the developing pectoral fins.

In addition, hoxc<sub>6a</sub>, a marker of the anterior pectoral mesenchyme, is expanded posteriorly in RA-depleted fins. We interpret this as a consequence of loss of shh, which is required to repress the posterior expression of hoxc<sub>6a</sub> (Neumann et al., 1999). By contrast, posterior expression of hoxd<sub>11a</sub> and hoxd<sub>12a</sub> is independent of shh (Neumann et al., 1999), yet both genes are never expressed in fin buds lacking RA. Similarly, hand<sub>2</sub> is not expressed in the absence of RA, and forelimbs in both zebrafish and mice that resemble limbs that develop without RA, in that they never express hoxd<sub>11a</sub> and hoxd<sub>12a</sub> in the fin mesenchyme (Yelon et al., 2000) or Shh in the limb bud (Charité et al., 2000).

hand<sub>2</sub> expression in the LPM has been implicated in regulating AP prepatternning prior to the formation of the ZPA and is required for early expansion of the presumptive fin field (Yelon et al., 2000). Similarly, in Aldh1<sub>1a</sub>2<sup>−/−</sup> mutant mice, Hand<sub>2</sub> is not expressed in forelimbs, but expression can be rescued by RA (Niederreither et al., 2002; Mic et al., 2004). Our results thus suggest that continued RA signaling from somites acts via hand<sub>2</sub> expression to establish a prepattern in the fin field mesenchyme. This process occurs during early somitogenesis and advocates a requirement of somite-derived RA for the maintenance of hand<sub>2</sub> expression in the LPM (Fig. 8).

It has been suggested that Aldh1<sub>1a</sub>2 expression in proximal limb mesoderm of the mouse establishes a proximodistal gradient needed for AER formation in the forelimb buds (Mic et al., 2004). We find that zebrafish embryos blocked immediately after the initiation stage (starting at 14 hpf) infrequently formed incomplete apical folds, while fin buds that developed during a RA block starting at 16 hpf did not exhibit apical fold defects (not shown and Fig. 6K-L). At this early stage, somite-derived RA-signaling is unlikely to form a gradient in the fin mesenchyme, and has been detected throughout the pre-budding mouse forelimb (Mic et al., 2004). This suggests that RA signaling is required between 14-16 hpf, when dlx<sub>2a</sub> is first expressed in the fin field (Akimenko et al., 1994), for full AP extension of dlx<sub>2a</sub> expression in the mature apical fold. Likewise, AER-formation in mammalian forelimbs is more likely to be a consequence of early deficits in full RA signaling, rather than the failure to establish a gradient of RA activity over the outgrowing limb bud. This notion is further supported by the expression of zebrafish aldhl<sub>1a</sub>2 throughout the entire fin bud mesenchyme during the outgrowth phase.

The ability to manipulate endogenous RA levels in zebrafish embryos at any stage of development has made it possible to reveal a link between somite-maturation and pectoral fin field induction, and has shown that the molecular mechanisms leading to apical fold formation and patterning of the posterior limb originate from RA signaling at the earliest stages of fin field formation. In the future, it should be possible to apply this approach successfully to a variety of RA-dependent developmental processes.

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