Application of synthetic photostable retinoids induces novel limb and facial phenotypes during chick embryogenesis \textit{in vivo}

R. E. Lopez-Real,¹ J. J. R. Budge,¹ T. B. Marder,² A. Whiting,³ P. N. Hunt¹ and S. A. Przyborski¹,⁴

¹School of Biological and Biomedical Sciences, Durham University, Durham, UK
²Institut für Anorganische Chemie, Würzburg, Germany
³Department of Chemistry, Durham University, Durham, UK
⁴Reinnervate Limited, NETpark Incubator, Sedgefield, UK

Abstract

We have recently developed a range of synthetic retinoid analogues which include the compounds EC23 and EC19. They are stable on exposure to light and are predicted to be resistant to the normal metabolic processes involved in the inactivation of retinoids \textit{in vivo}. Based on the position of the terminal carboxylic acid groups in the compounds we suggest that EC23 is a structural analogue of \textit{all-trans} retinoic acid (ATRA), and EC19 is an analogue of \textit{13-cis} retinoic acid. Their effects on the differentiation of pluripotent stem cells has been previously described \textit{in vitro} and are consistent with this hypothesis. We present herein the first description of the effects of these molecules \textit{in vivo}. Retinoids were applied to the anterior limb buds of chicken embryos \textit{in ovo} via ion-exchange beads. We found that retinoid EC23 produces effects on the wing digits similar to ATRA, but does so at two orders of magnitude lower concentration. When larger quantities of EC23 are applied, a novel phenotype is obtained involving production of multiple digit 1s on the anterior limb. This corresponds to differential effects of ATRA and EC23 on \textit{sonic hedgehog} (\textit{shh}) expression in the developing limb bud. With EC23 application we also find digit 1 phenotypes similar to thumb duplications described in the clinical literature. EC23 and ATRA are shown to have effects on the entire proximal–distal axis of the limb, including hitherto undescribed effects on the scapula. This includes suppression of expression of the scapula marker \textit{Pax1}. EC23 also produces effects similar to those of ATRA on the developing face, producing reductions of the upper beak at concentrations two orders of magnitude lower than ATRA. In contrast, EC19, which is structurally very similar to EC23, has novel, less severe effects on the face and rarely alters limb development. EC19 and ATRA are effective at similar concentrations. These results further demonstrate the ability of retinoids to influence embryonic development. Moreover, EC23 represents a useful new tool to investigate developmental processes and probe the mechanisms underlying congenital abnormalities in vertebrates including man.

Key words: chick; craniofacial; development; limb; \textit{Pax1}; retinoic acid; scapula; \textit{sonic hedgehog}; synthetic retinoid.

Introduction

Retinoids are small lipophilic molecules that are essential for a wide range of developmental events in vertebrates (Ross et al. 2000; Duester, 2008). Retinoid levels in tissues are thought to result from the balance between localised enzymatic conversion from non-biologically active retinoid precursors and inactivation by enzymes of the Cyp26 family which are also restricted during embryonic development (Swindell et al. 1999; Ross et al. 2000; Mic et al. 2004; Reijntjes et al. 2004, 2005b; Chambers et al. 2007). The restricted expression of such enzymes corresponds to embryonic regions known to be affected when retinoid levels are raised or lowered experimentally (Soprano & Soprano, 1995; Ross et al. 2000; Yashiro et al. 2004). This suggests that retinoids are required for selected embryonic processes, and that correct retinoid concentration and localisation are necessary.

A major part of retinoid effects are mediated by the binding of \textit{all-trans} retinoic acid (ATRA) to retinoic acid receptors (RARs), which results in modulation in the
transcriptional activity of a range of target genes (Mark et al. 2006). However, there is evidence that retinoids other than ATRA can also exert biological effects. It is known that two isomers of ATRA, 9-cis and 13-cis retinoic acid, can be found in vivo. When a teratogenic dose of ATRA was administered to e10.5 mouse embryos, a considerable increase in the levels of 13-cis retinoic acid was detected in the spinal cord, one of the embryonic regions subject to retinoid teratogenesis (Horton & Maden, 1995). The isomeric 9-cis retinoic acid can be produced by photo-conversion of ATRA and is approximately 25 times more potent than ATRA at inducing digit duplications in chick limbs (Thaller et al. 1993). Oxidation of retinoids by the CYP26 enzymes is generally thought to result in their inactivation (White et al. 1996, 2000; Fujii et al. 1997). However, the abnormalities in vitamin A-deficient quail embryos can be rescued to a large extent by administration of metabolites 4-oxo retinoic acid, 5,6-epoxy retinoic acid or 4-hydroxy retinoic acid (Reijntjes et al. 2005a). Thus, it is possible that some effects of retinoids may be mediated by ATRA metabolites.

There is extensive literature concerning the ability of retinoids to influence a number of systems in vivo. One of the best studied retinoid sensitive systems is the developing forelimb bud, where early work demonstrated that retinoids are able to influence patterning along the anterior–posterior axis (Tickle et al. 1982). In this system retinoids can be applied topically to the anterior limb bud using ion-exchange beads soaked in retinoid-containing solutions. It was found that additional posterior digits on the anterior side of the limb were produced, and the number and identity of the digits developing were proportional to the concentration of retinoid supplied to the limb (Tickle et al. 1985). Thus, naturally occurring retinoids are able to produce a dose-dependent duplication and posteriorisation of digit pattern.

The three digits in the avian wing have been described as corresponding to digits 2, 3 and 4 (Burke & Feduccia, 1997). However, fossil evidence would suggest that digits of the bird wing correspond to digits 1, 2 and 3 in other tetrapods (Burke & Feduccia, 1997; Towers et al. 2011). The recent fate mapping of the contribution of the polarising region to the digit skeleton (Towers et al. 2011) and the transcriptional profiling of digit precursors in chick forelimbs and hindlimbs (Wang et al. 2011) have added further evidence supporting this concept. The nomenclature used throughout this work when referring to wing digits of different identities will reflect these findings.

Endogenous retinoids are required for the establishment of the proximal part of the forelimb field (Stratford et al. 1996; Niederreither et al. 2002; Mic et al. 2004; Yashiro et al. 2004; Zhao et al. 2009; Cunningham et al. 2013). The ability of topical retinoid application to induce duplication and posteriorisation of digits is thought to result from their ability to induce Hand2 and shh expression (Riddle et al. 1993; Fernandez-Teran et al. 2000) and does not reflect a direct endogenous role for retinoids in antero-posterior patterning of the digits. Nevertheless, the graded nature of the response of limb buds to retinoids means that the limb continues to represent a sensitive system for the comparison of the biological effects of retinoids in vivo (Tickle et al. 1985).

There is considerable interest in using retinoids to investigate developmental mechanisms and to control cell differentiation in vitro, e.g. neurogenic differentiation of embryonal carcinoma stem cells (Przyborski et al. 2000; Horrocks et al. 2003; Stewart et al. 2003). However, a limitation in the use of naturally occurring retinoids is that they can give rise to a range of different products upon exposure to light (Christie et al. 2008). This will deplete the quantity of defined retinoid present and generate a mixture of compounds that are also biologically active but with potentially different properties to ATRA (Thaller et al. 1993; Murayama et al. 1997). From the perspective of experimental reproducibility, a compound with similar properties to ATRA but without its instability to light, or to other environmental conditions, would offer considerable advantages.

We have recently developed a range of retinoid analogues which have been demonstrated to be stable on exposure to light (Christie et al. 2008). In addition, they would be predicted to be largely resistant to oxidation by cyp26 enzymes on the basis of their structure (see Discussion herein). Two of these compounds have been demonstrated to promote differentiation of TERA2.cl.SP12 embryonal carcinoma stem cell lines in vitro (Christie et al. 2008) and are illustrated in Fig. 1. The two synthetic compounds differ only in the position of the terminal carboxylic acid group with respect to the tetramethyl-tetrahydronaphthalene unit that replaces the trimethylcyclohexenyl ring and part of the polyene chain found in naturally occurring retinoids (see boxes in Fig. 1 and the structure numbering, highlighting which carbon atoms map onto which, on each structure). The position of the terminal carboxylic acid suggests that EC23 would be a very close analogue of ATRA, as reinforced by the fact that the two structures are essentially superimposable. In contrast, EC19 would be a closer analogue of 13-cis retinoic acid. Consequently, EC23 was able to promote neurogenic differentiation in culture efficiently, like ATRA, although EC23 was significantly more potent. In contrast, EC19 produced epithelioid differentiation characterised by enhanced cytokeratin expression (Christie et al. 2008).

This shows that one synthetic retinoid can give rise to similar effects as those resulting from ATRA exposure. To characterise further these compounds we have explored their properties in vivo. As discussed above, the effects of retinoids on limb digit identity are dose-dependent. In light of the in vitro evidence that they are more potent, we have chosen to use the chick embryonic limb bud system to evaluate the effects of these novel retinoids directly on vertebrate embryogenesis.
Methods

Fertile white leghorn eggs were obtained from PD Hook Hatcheries (Thirsk, North Yorkshire). They were kept at 10 °C and then incubated at 38.4 °C in 50% humidity. All-trans retinoic acid (ATRA; Sigma) was dissolved in dimethyl sulphoxide (DMSO; Sigma). EC23 and EC19 were obtained from Reinnervate Ltd and dissolved in DMSO. Aliquots of ATRA, EC23 and EC19 were stored at -20 °C.

In ovo microsurgery

Eggs were incubated for 4 days and staged according to Hamburger and Hamilton (Hamburger & Hamilton 1951) and Fisher et al. (2008). Eggs staged at HH20–21 were then treated with retinoid or DMSO. AG1-92 beads (diameter 50–150 or 150–300 µm; BioRad) were used as formate derivatives, prepared from chloride or hydroxide forms according to manufacturer’s instructions. Beads were soaked in retinoid or DMSO for 30 min, rinsed in Tyrode’s saline (Tickle et al. 1985) and then inserted into a slit in the anterior limb bud using fine forceps and tungsten needles. Eggs were re-incubated for 7 days for phenotypic screens of the retinoids, 24 h for RNA analysis, and 30 or 48 h for in situ hybridisation.

Whole-mount Alcian Blue staining and photography

Embryos recovered after 7 days of incubation with retinoid were then stained with Alcian Blue and Alizarin Red to determine their skeletal phenotype. Briefly, embryos were fixed and stained in ethanolic acetic Alcian Blue for at least 3 days, followed by three washes in 100% ethanol over 3 days. Embryos were imaged for limb and beak malformations using a Spot Idea camera mounted on a Zeiss SV11 dissecting microscope. Embryos were imaged dry or surrounded by 100% ethanol on 3% agar plates. To allow for comparisons of beak length, the left eye was removed to allow the heads to lie flat. Embryos were then stained with Alizarin Red in 1% aqueous potassium hydroxide for 45 min to stain for bone. Embryos were cleared in 1% potassium hydroxide (KOH). Embryos were then further cleared in 1% KOH, 25% glycerol and stored in 1% KOH, 50% glycerol or 80% glycerol. Embryos were then imaged using transmitted light.

Assignment of digit identity

To establish a set of criteria which could be used to identify digits we measured their length in images of 19 control limbs using the line segment function of NIH IMAGEJ. From these measurements we calculated the following length ratios with respect to digit 2:

1 digit 1 – 0.42, 0.05 S.D.
2 digit 2 – 1.00
3 digit 3 – 0.58, 0.05 S.D.

The crucial distinction to be made for our purposes was between digit 1 and digit 2. Based on the above measurements we interpreted a duplicated digit as digit 1 if the length ratio was < 0.5.

Reverse transcription

Chick RNA was reverse-transcribed using the High Capacity DNA Synthesis kit (Applied Biosystems, Paisley, UK) as per the instructions provided. Briefly, 2 μg RNA in 10 μL was added to 1 μL 10× buffer, 0.4 μL dNTP, 1 μL random primers, 1 μL reverse transcriptase and nuclease-free water (Promega, Southampton, UK). This was then placed into a thermocycler (Biometra) with the following programme: 25 °C 10 min, 2 h at 37 °C and 85 °C 10 s.

Polymerase chain reaction

To generate polymerase chain reaction (PCR) products which could in turn be transcribed into labelled RNA probes, a T7 RNA polymerase binding site was added to the 5’ of each reverse primer. Primers were designed using sequences from the National Centre for Biotechnology Information (NCBI) and are shown below. Before designing primers which would generate specific probes, the sequence of interest was BLAST-searched to determine regions of
homology and specificity. The probes which would be generated for each gene were then BLAST-searched to ensure specificity. For primer sequences see Table 1 below. PCR was carried out using GoTaq Flexi DNA Polymerase kit (Promega) according to the manufacturer’s instructions.

Probe synthesis

Digoxigenin-labelled (DIG-labelled) RNA probes were generated from total RNA from HH20 embryos. RNA was extracted and reverse-transcribed as described earlier. The complementary DNA (cDNA) was used as a template for PCR to amplify the region of DNA specific to each probe. The PCR product was then used as a template for in vitro transcription of DIG-labelled RNA probes for whole-mount in situ hybridisation. The PCR fragments were transcribed into DIG-labelled RNA. A 1 µl template was added to 2 µl buffer, 0.2 mM dithiothreitol, 2 µl nucleotides containing DIG-uracil triphosphate (Roche Applied Science; Burgess Hill, UK), 0.5 µl recombinant ribonuclease inhibitor (Rnasin) and 1 µl T7 RNA polymerase (Promega) in 20 µl reaction volume. This was incubated at 37-45 °C for 2 h and analysed on 2% agarose tris-borate EDTA (TBE; Sigma) gel. The size of the product was checked against a low range DNA ladder (Fermentas). The probe was then ethanol-precipitated and washed twice with 80% ethanol before being air-dried to allow it to dry in the air. The probe was then re-suspended in TE for quantification on the NanoDrop spectrophotometer (ND1000).

Prehybridisation, post-hybridisation and visualisation

Whole-mount in situ hybridisation was carried out according to Acloque et al. (2008) with some modifications. Embryos were incubated to the desired stage. They were dissected into ice-cold phosphate-buffered saline (PBS) and fixed overnight in 4% paraformaldehyde (Sigma) prepared in diethylpyrocarbonate (DEPC)-treated PBS at 4 °C. After washing three times in DEPC-PBS containing 0.1% Tween20 (Sigma; PBT), membranes were dissected off before dehydrating to 100% methanol (AnalaR VWR; Lutterworth, UK). Batches were stored in 100% methanol at −20 °C. They were rehydrated to PBT and then bleached in 6% hydrogen peroxide (Sigma). Proteinase K was applied to bleached PBS at 4°C for 1 h. Approximately 1 µg DIG-labelled RNA probe was then dissolved into 1 mL prehybridisation buffer in which embryos were agitated at 70 °C for 16 h.

Embryos were then washed with solution 1 at 70 °C (50% formamide, 5× SSC, 1% sodium dodecyl sulphate, SDS) and then with solution 3 (50% formamide, 2× SSC, 1% SDS) at 70 °C. This was followed by TBST washes [1× TBST, 1% Tween20, 2 mM levamisole (Sigma)] and then blocking with 10% sheep serum (Sigma) : TBST. Anti-DIG antibody 1 µl (Roche Applied Science) was purified using embryo powder for 1 h in 1% sheep serum TBST at 4 °C. The powder was then centrifuged to remove purified antibody in the supernatant and was applied to the embryos at 1 : 3000 dilution. This was rocked overnight at 4 °C.

Antibody was removed and followed with eight washes of TBST and left overnight at 4 °C. The embryos were then placed in NTMT (100 µM sodium chloride, 100 µM TrisHCl pH 9.5, 50 mM magnesium chloride, 0.1% Tween20, 2 mM levamisole). The DIG-antibody was conjugated to alkaline phosphatase, which will catalyse a reaction between nitro-blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolylphosphate toluidine salt (BCIP) to produce a purple precipitate. NBT 4.5 µl ml⁻¹ and BCIP 3.5 µl ml⁻¹ (Promega) were applied with NTMT and agitated in the dark for 20 min and left to develop. Once the colour reaction had developed to the desired extent, embryos were washed three times with PBS and then fixed in 4% paraformaldehyde. Embryos were then imaged using the Idea (Spot) camera on the Zeiss SV11 dissection microscope as described previously. Embryos were imaged on 3% agar plates so that they could be flat and pinned in place.

Embryo dissection, RNA isolation and qPCR

Embryos were exposed to retinoid or DMSO as described above by in ovo microscopy. Twenty-four hours after operating, embryos were dissected into ice-cold DEPC-PBS. Embryos whose limbs still had a bead attached and had developed as expected were included in the pool for RNA isolation. Torso of embryos were dissected and pinned to 3% agar : Tyrode plates to facilitate accurate dissection. The bead was removed and the anterior third of the limb bud was dissected using tungsten needles. The anterior limb portion was transferred into an RNase free tube (Starlabs) in DEPC-PBS. After dissection, DEPC-PBS was removed and RLT (lysis buffer) was then placed on the limb portions. The limb portions were lysed and homogenised. Limb portion lysates were stored at −80 °C until 16 limb portions were collected per repeat. They were then thawed on ice and RNA was isolated using the Qiagen RNeasy Kit (Dorking, UK) as per the manufacturer’s instructions. RNA was quantified using the nanodrop ND1000.

Reverse transcription was carried out using cDNA generated using the high capacity cDNA synthesis kit described earlier. qPCR was carried out using the Applied Biosystems Real Time PCR 7500 System as per the manufacturer’s kits and instructions. The genes

| Table 1 | Sequences used to generate primers for PCR and subsequent whole-mount in situ hybridisation. |
|---------|-----------------------------------------------------------------------------------------------|
| U22046.1 shh (Riddle et al. 1993) | TGGAGCAGACGGGTGGGTA |
| NM_204821.1 Pax1 (Barnes et al. 1996) | ATGAAGAGAACACGGGAGCTGACA TCTCTCGCGGCTTTGTC |
| T7 polymerase | AAGGGATCCCGTCGACATCGATAATACGACTCTACATAAGGGA |

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analysed and the assay IDs (Taqman Gene Expression Assays; 
Applied BioSystems) were the following:

cyp26A1 Gg03345448_g1
Radh2 Gg03348020_m1
Meis2 Gg03338704_m1

Briefly, 20 ng of DNA was used per qPCR reaction, which was 
carried out as per the manufacturer’s instructions.

In all cases no expression was detected in the blank reaction. 
Change in gene expression was calculated as fold change by the 
ΔΔCt method using Gapdh expression as the endogenous control. 
The relative quantification (RQ) was then calculated using the for-
mula: RQ = 2−ΔΔCt, and standard deviations were then calculated 
for these values. The significance of the fold change in response to 
retinoid with respect to DMSO was calculated using an unpaired 
Student’s t-test.

Results

The response of embryos to synthetic retinoids

Our initial experiments compared the toxicity of synthetic 
retinoids EC23 and EC19, and naturally occurring ATRA. 
AG1-X2 resin beads of 200–400 mesh size, diameter 
50–150 μm (classed as small beads), were soaked in retinoid-
containing solutions of different concentration and 
implanted into anterior limb buds of chick embryos at 
Hamburger and Hamilton stage 20 (HH20) (Hamburger & 
Hamilton, 1951). Embryos were incubated until HH35, and 
the number surviving to this stage scored. The results are 
shown in Table 2. It can be seen that EC23 is the most toxic, 
with no embryos surviving applications of beads soaked in a 
centration of retinoid higher than 0.01 mg mL−1. In con-
trast, the toxicity of both EC19 and ATRA was similar to that 
described in the previous literature for ATRA (Tamarin et al. 
1984; Tickle et al. 1985), with embryos surviving applications 
of beads soaked in concentrations up to 10 mg mL−1 ATRA. 
EC23 is thus toxic at three orders of magnitude lower con-
centration than ATRA. The synthetic retinoid EC19 exhibited 
the unusual characteristic of showing increased toxicity com-
pared with the naturally occurring ATRA at 0.01 mg mL−1, 
but less toxicity than ATRA at 1 and 3 mg mL−1.

The naturally occurring retinoid ATRA is known to pro-
duce digit duplications (Tickle et al. 1982). In addition to 
this, topical application of ATRA to the limb has been 
shown to produce selective reduction in the formation of 
the upper beak (Tamarin et al. 1984). This may be either 
because of proximity of the limb bud to the facial primor-
dial in ovo or because the retinoid levels in the entire 
embryo are systemically raised after topical application to 
the limb (Tickle et al. 1985). The limb bud and its vicinity 
are already extensively vascularised at the time when beads 
are applied (Vargesson, 2003; Therapontos et al. 2009). This 
vasculature could potentially act as a route by which other 
parts of the embryo, including the face, could be exposed 
retinoid. The two synthetic retinoids differed in their 
ability to induce either of these biological effects. The 
relative frequencies of different limb and facial phenotypes 
over a range of different retinoid concentrations are shown 
in Fig. 2. Overall EC23 was similar to ATRA in the spectrum 
of digit duplications it produced, but was able to do so at a 
10–100-fold lower concentration. EC23 produced a full spec-
trum of digit duplications at 0.001 and 0.01 mg mL−1, 
whereas ATRA could only produce the mildest form of digit 
duplication, a duplicated digit 1, at 0.01 mg mL−1. Figure 3 
(A,B) shows duplications of digit 1 obtained with 0.001 and 
0.01 mg mL−1 EC23, respectively. Figure 3(C,D) shows duplica-
tions of digits 3, 2 and 1 with 0.01 mg mL−1 EC23. Higher 
doses of ATRA could produce the full spectrum of digit 
duplications previously described (Tickle et al. 1982). In 
inaddition, EC23 was able to produce a novel type of digit 
duplication not previously described after ATRA treatment. 
This was obtained after treatment with 0.01 mg mL−1 
EC23, and involves multiple duplications of digit 1, in the 
absence of the formation of any other types of duplicated 
digit. Figure 3(C,D) shows this digit duplication phenotype, 
which is labelled as 1I123 in Fig. 2. EC19 produced duplica-
tions of a single digit 1 at low frequency. It is notable that 
many higher doses of EC19 were required to obtain this 
phenotype compared with EC23, even though both reti-
noids have been shown to be stable. This is consistent with 
evidence of their potency in vitro (Christie et al. 2008). Thus, 
we have found that these synthetic retinoids can have simi-
lar effects to naturally occurring retinoids on the develop-
ing limb. However, there are some differences in their 
toxicity and effective dose compared with naturally occur-
ring retinoids. In addition, we have found that the most

Table 2. Embryo survival rate through to stage HH35 (Hamburger & Hamilton, 1951) after receiving implants to the anterior limb bud of 50–150-μm diameter resin beads soaked in different concentrations of retinoid. The data show the number of operations carried out at the retinoid concentrations used and the percentage survival rate.

| Concentration (mg mL−1 in DMSO) | Number of operations conducted | Percentage (number) of embryos surviving up to stage 35 |
|----------------------------------|-------------------------------|------------------------------------------------------|
| EC23                             |                               |                                                      |
| 0.0001                           | 32                            | 75 (24)                                              |
| 0.001                            | 70                            | 36 (25)                                              |
| 0.01                             | 57                            | 53 (30)                                              |
| 0.1                              | 31                            | 0 (0)                                                |
| EC19                             |                               |                                                      |
| 0.01                             | 26                            | 23 (6)                                               |
| 0.1                              | 19                            | 47 (9)                                               |
| 3                                | 25                            | 80 (20)                                              |
| 1                                | 31                            | 68 (21)                                              |
| ATRA                             |                               |                                                      |
| 0.01                             | 135                           | 31 (42)                                              |
| 0.1                              | 47                            | 30 (14)                                              |
| 3                                | 24                            | 38 (9)                                               |
| 10                               | 22                            | 50 (11)                                              |

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potent novel retinoid, EC23, produces a novel phenotype of multiple digit 1 duplications that has not been previously described after ATRA treatment.

The response of embryos to larger quantities of synthetic retinoids

Our toxicity data indicate that increasing the concentration of EC23 to 0.1 mg mL\(^{-1}\) prevented any embryos surviving to HH35. We wished to investigate the phenomenon of the multiple digit 1 duplications further. We therefore investigated the consequences of applying a larger quantity of retinoid to limb buds at the same maximum concentration found to be compatible with embryo survival. To do this we used ion exchange beads of 50–100 mesh size, with diameters of 150–300 µm (classed as large beads). As a consequence of their size, these beads have four times the surface area of the smaller beads used in our initial experiments.

The survival rate of embryos to HH35 when treated with the larger beads is shown in Table 3. The outcomes are similar to those observed with smaller beads soaked in similar concentrations of retinoid shown in Table 2. Survival rates with EC23 at 0.01 mg mL\(^{-1}\) are similar to those seen with smaller beads, and the increased survival of embryos treated with 0.1 mg mL\(^{-1}\) EC19 compared with 0.01 mg mL\(^{-1}\) EC19 is confirmed. This is a relatively low survival rate despite administration of retinoid to the limb, a structure that is not required for embryonic survival. This may reflect that the limb is sufficiently well vascularised at this stage for retinoids to be transferred to other, more vital structures, such as the heart. The phenotypes resulting from treatment with this quantity of retinoid are shown in Table 4. With an increased quantity of EC23, it is notable that embryos either have no alterations to the digit pattern or exhibit an additional or multiple digit 1s. Duplications of digits 2 and 3 are rarely seen with this quantity of EC23. In contrast, increasing the quantity of 1 mg mL\(^{-1}\) ATRA delivered still resulted in duplication of posterior digits in addition to digit 1, as shown in Table 4. This indicates that the change in bead size alone is unlikely to be causing the formation of multiple digit 1s. ATRA application is only able to result in the duplication of one additional digit 1, whereas multiple digit 1 duplications are the outcome specifically caused by the novel stable retinoid EC23, as shown in Fig. 4.

What is also notable is that we found no cases after EC23 treatment in which more than three additional digit 1s were formed. In Fig. 4(E) the central additional digit has a
length ratio of 0.59, putting it outside the range of digit 1. This may represent a unique example of an additional digit intermediate in identity between digit 1 and digit 2. Delivery of an increased quantity of EC19 did not result in any observed digit duplications \((n = 16)\) even at 0.1 mg mL\(^{-1}\) \((n = 10)\), which produced digit duplications with small beads (Figure 2, data not shown).

**Table 3** Toxicity of the synthetic retinoids EC23 and EC19 compared with ATRA when applied on beads of 150–300 μm diameter. This shows the number of operations carried out at the retinoid concentrations used. This is used to calculate the frequency/% survival 7 days after operation. % survival is presented as a percentage (number of embryos).

| Retinoid concentration (mg mL\(^{-1}\) in DMSO) | Number of operations | % survival (number) surviving to 7 days |
|-----------------------------------------------|----------------------|----------------------------------------|
| ATRA  | EC23 | EC19 | ATRA  | EC23 | EC19 |
| 0.01  | 0    | 58   | 20    | –    | 50 (29) | 30 (6) |
| 0.1   | 0    | 0    | 24    | –    | –     | 42 (6) |
| 1     | 14   | 0    | 43 (6) | –    | –     | –     |

**Limb buds are responding to added retinoid at the transcriptional level**

Retinoids are known to exert their effects by a range of cellular mechanisms. Most effects in developing systems are assumed to be mediated by modulation of transcription, and a large number of genes have been described as regulatory targets of retinoids (Balmer & Blomhoff, 2002). Two genes known to be retinoid-regulated are Cyp26a1 (Reijntjes et al. 2005a) and Raldh2, an enzyme that converts retinaldehyde to the biologically active retinoic acid (Niederreither et al. 1999). Cyp26a1 is retinoid-inducible, whereas Raldh2 expression is suppressed by retinoids. A third gene, Meis2, is known to be a proximal limb marker and is upregulated by ATRA (Mercader et al. 2000). We used qPCR to investigate whether EC23 was likely to be exerting its effects via transcription. We compared the expression of these three known retinoid target genes in limb buds 24 h after treatment with DMSO and retinoids. Figure 5 shows that expression of Cyp26a1 is induced 60-fold by ATRA and 35-fold by EC23. In contrast expression of Raldh2 is reduced 2.5-fold by ATRA and 3.25-fold by EC23. This is consistent with the previously described response of these genes to...
retinoids, and suggests that the effects of EC23 are mediated transcriptionally. It also suggests that the system is exhibiting feedback inhibition in response to excess retinoid. Increased Cyp26 levels would increase the rate of breakdown of retinoids susceptible to metabolism, whereas reduced levels of Raldh2 would reduce levels of ATRA synthesis. Levels of the marker of proximal wings, Meis2, are induced 2-fold and 2.5-fold by ATRA and EC23, respectively. This is consistent with earlier work showing this gene is retinoid-inducible, and that cells are being proximalised as a result of retinoid treatment (Mercader et al. 2000).

The effect of retinoids on Shh expression
Previous work has shown that retinoid-induced digit duplications are associated with the induction of ectopic, anterior domains of shh expression after 24–30 h of retinoid treatment (Riddle et al. 1993). Furthermore, ectopic shh expression alone is able to induce digit duplications (Riddle et al. 1993; Chang et al. 1994). The expression of shh after retinoid treatment was therefore investigated by whole-mount in situ hybridisation. Figure 6(A,C) shows that after 24 h treatment with either ATRA or EC23 no ectopic shh

Table 4 Frequency and nature of limb phenotypes generated with ATRA and EC23 applied on beads with diameter of 150–300 μm. Normal limb development is referred to as 123. Extra digits are denoted by numbers before this and refer to the digit identity assigned by length of ectopic digits.

| Retinoid | Concentration (mg mL⁻¹) | Frequency (number) of limb phenotypes seen in embryos surviving to 7 days |
|----------|--------------------------|-----------------------------------------------------------------------|
|          |                          | 123  | 1123 | 11 | 123 | 111 | 123 | 2123 | 21 | 123 | 1223 | 32 | 123 |
| EC23     | 0.01                     | 35 (10) | 24 (7) | 17 (5) | 3 (1) | 0 | 0 | 0 | 0 | 0 |
| ATRA     | 1                        | 0 (0) | 33 (2) | 0 | 0 | 17(1) | 17 (1) | 17 (1) | 17 (1) |
Domain is observed on the anterior wing bud after retinoid treatment. However, as seen in Fig. 6(B), ATRA was able to induce shh expression in the anterior wing bud at a similar frequency to its ability to produce severe digit duplications \((n = 2/6)\) after 30 h of retinoid treatment. EC23 was never observed to induce shh expression in the anterior wing \((n = 11)\) as shown in Fig. 6D. This difference in shh induction suggests that digit duplication and/or limb development is inhibited to a greater extent in EC23-treated wings.

**Synthetic retinoids are able to affect proximal limb elements**

Application of ATRA to the posterior proximal wing can affect zeugopod and autopod elements (Tickle et al. 1985), and element length has been affected in RAR\(\alpha/\gamma\) and cyp26 null mice (Lohnes et al. 1994; Yashiro et al. 2004; Dranse et al. 2011). Effects on the proximal wing were also noted with EC23 and ATRA after application of large beads. The frequencies of alterations to more proximal limb elements after application of ATRA and EC23 to anterior limb buds are shown in Tables 5 and 6. Example phenotypes are illustrated in Fig. 7. It can be seen from Table 5 that EC23 and ATRA cause malformation to proximal regions of the wing. The frequency of digit duplication was 65% in EC23-treated wings and 100% in ATRA-treated wings, which was similar to the frequency of effects on zeugopod element size: 69% in EC23-treated wings and 100% in ATRA-treated wings. Both EC23 and ATRA also affected the humerus with a similar frequency. However, in keeping with their differential effects on the digits, EC23 and ATRA showed differential effects on development of the humerus, radius and ulna.

Application of EC23 at HH20 was able to cause a significant decrease in the humerus length : width ratio, which is
Table 5  Effect of EC23 and ATRA on development of humerus, radius and ulna. This shows the frequency of phenotypes and number of examples with a smaller length : width ratio of radius or ulna. Note that embryos exhibiting smaller radius or ulna are both included in the column designated RU change. Some embryos show changes to one or both elements.

| Retinoid | Concentration (mg mL\(^{-1}\)) | % of embryos (number) surviving after 7 days’ treatment with malformations to humerus, radius or ulna cartilages |
|----------|---------------------------------|------------------------------------------------------------------------------------------------------------------|
|          |                                 | Radius/ulna change | Small radius | Small ulna | Humerus                                  |
| EC23     | 0.01                            | 69 (20)           | 48 (14)      | 48 (14)    | 48 (14)                                  |
| ATRA     | 1                               | 100 (6)           | 100 (6)      | 17 (1)     | 83 (14)                                  |

Table 6  Effect of EC23 and ATRA on size of radius, ulna and humerus. Average length : width ratio of the cartilage elements is shown (± S.D.). Significance was tested using an unpaired Student’s t-test to compare retinoid-treated with DMSO-treated cartilage elements.

| Retinoid | Concentration (mg mL\(^{-1}\)) | Average LW ratio of radius, ulna and humerus 7 days after treatment |
|----------|---------------------------------|---------------------------------------------------------------------|
|          |                                 | Radius | Ulna | Humerus          |
| DMSO/untreated | N/A                           | 12.6 (±2.5) | 13.2 (±2.2) | 11.9 (±1.0) |
| EC23     | 0.01                            | 8.6 (±2.5)** | 9.2 (±3.1)** | 9.4 (±2.3)** |
| ATRA     | 1                               | 7.4 (±1.1)** | 10.8 (±2.1) * | 12.4 (±1.8) NS |

*P < 0.05, **P < 0.001. NS, not significant.

characterised phenotypically by a thickened and shorter humerus. This can be seen by comparison of Fig. 7(A) with Fig. 7(D-F), and Table 6. It can also be seen from Table 5 that ATRA causes alterations to the humerus length : width ratio compared with control embryos at twice the frequency of EC23. However, as shown in Table 6, EC23 causes a statistically significant decrease in humerus length : width ratio compared to controls, whereas ATRA causes a slight increase in this ratio, although this is not statistically significant.

Table 5 shows that 69% of embryos treated with 0.01 mg mL\(^{-1}\) EC23 exhibit a change in the length : width ratio of the radius and/or the ulna, whereas the frequency of change to these elements with ATRA is 100%. As can be seen from Fig. 7, ATRA and EC23 can affect the length of both zeugopod elements equally (not shown, Fig. 7E) or one element more than the other (Fig. 7B,D,F). Both retinoids are able to produce a significant reduction in length of both elements, as shown in Table 6. Treatment with 1 mg mL\(^{-1}\) ATRA causes a change to the radius (Fig. 7B) or both elements (not shown) in every embryo analysed. There is a notable difference in the frequencies with which EC23 and ATRA affect the different elements. EC23 effects the development of the radius and ulna at the same frequency, whereas ATRA shortened the length of the more anterior radius with a higher frequency than the more posterior ulna (Table 5). In addition, the two retinoids differed in the magnitude of the reductions they produced. ATRA caused a greater shortening of the radius than EC23, whereas EC23 caused a greater shortening of the ulna (Table 6). Effects on the elbow joint were also seen, with fusions between the humerus, radius and ulna, as shown in Fig. 7(C,E).

These data suggest that EC23 and ATRA both affect the entire proximal-distal axis. ATRA has been suggested to affect proximal areas previously (Tickle et al. 1985; Tickle & Crawley, 1988). In the present study this has been shown after application to the anterior wing bud at HH20 rather than the posterior wing bud. There is evidence for a difference in the effects produced by the stable retinoid EC23 compared with ATRA on the zeugopod and stylopod elements. These differences are in the frequency with which changes are seen, the nature and the magnitude of the changes. These proximal effects are consistent with our findings on the induction of the proximal marker Meis2, shown in Fig. 5(C).

Both EC23 and ATRA affect elbow development and cause digit fusions

Both EC23 and ATRA can be seen to generate fusions to the elbow joint or between digits during wing development. Table 7 shows the frequencies of elbow phenotypes seen after treatment with EC23 and ATRA. Figure 8 shows representative examples of fusions between different cartilage elements. EC23 has not been observed to cause fusion of the humerus-ulna and ATRA has not been observed to
cause fusion of the humerus–radius elements, as shown in Table 7. However, EC23 can fuse humerus–radius, as shown in Fig. 8(B) and ATRA can fuse humerus–ulna, as shown in Fig. 8F. Both EC23 and ATRA can generate fusion of all three elements at varying severity. EC23 0.01 mg mL$^{-1}$ causes fusion of humerus, radius and ulna with varying degrees of severity (cf. Fig. 8C,D). As seen from Table 7, EC23 and ATRA cause fusions to developing digits at similar frequencies. Figures 3(D,E), 4(B,E), 7(E) and 8(D) show examples of digit fusions in response to 0.01 mg mL$^{-1}$ EC23, and Fig. 8(E,F) shows fusions after treatment with 1 mg mL$^{-1}$ ATRA. These fusions are largely between cartilage elements of digit 1s, and are similar to radial polydactyly abnormalities described in the clinical literature (Manske & Oberg, 2009).

Because we have observed fusion of elements throughout the proximal–distal axis of the limb we favour this as an explanation of our observations with respect to the digits. An interesting alternative interpretation which we cannot exclude or support without further experiments is that the digit phenotypes listed in the above paragraph arise from bifurcation rather than fusion of elements.

### Synthetic retinoids truncate the limb girdle

Previous research has shown that retinoids can affect the shoulder girdle if applied at HH18-20 in chick embryos and has shown that retinoids caused production of ectopic cartilage or duplication of the coracoid (Oliver et al. 1990). A high concentration of ATRA (10 mg mL$^{-1}$) was required.

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**Table 7** Frequency and nature of cartilage element fusion in wing development after treatment with EC23 and ATRA. Note that some examples with digit fusions also exhibited more proximal fusions, and are included in the HR, HU and HRU frequencies.

| Retinoid | Concentration (mg mL$^{-1}$) | % of embryos (number) surviving after 7 days treatment with fusions |
|----------|-------------------------------|---------------------------------------------------------------|
|          |                               | HR   | HU   | HRU  |Digit    |
| EC23     | 0.01                          | 17 (5) | 0   | 21 (6) | 10 (3) |
| ATRA     | 1                             | 0    | 17 (1) | 17 (1) | 33 (2) |

HR, humerus-radius; HU, humerus-ulna; HRU, humerus-radius-ulna.
to produce an ectopic cartilaginous spur on the scapula when applied at HH20. We found that naturally occurring and synthetic retinoids were able to affect the scapula when using large beads, but in ways that differed from those which had been described previously (Oliver et al. 1990).

Malformation to the scapula occurs at a similar frequency to digit duplication and alterations of stylopod and zeugopod elements, as shown in Table 8. All embryos treated with 1 mg mL\(^{-1}\) ATRA that survived to HH35 exhibited a scapula malformation, whereas 0.01 mg mL\(^{-1}\) EC23 caused scapula malformations at a lower frequency. In all cases, scapula phenotypes were seen with digit duplications in response to 1 mg mL\(^{-1}\) ATRA. Scapula phenotypes were not always concurrent with digit duplication in response to 0.01 mg mL\(^{-1}\) EC23: 24% scapula malformations seen had normal digit development. However, in most cases of EC23 treatment if there was a digit phenotype, there was also a malformation of the scapula: 78% limb phenotypes had a malformed scapula and 67% scapula malformations were seen with digit duplication. In all, 67% scapula phenotypes

Table 8 The effect of EC23 and ATRA on scapula development. The third column shows the frequency of embryos (number) surviving to HH35 with normal scapula on the operated side. The remaining columns show the frequency (number) of embryos with various scapula malformations: reductions, foramen, bending, ectopic cartilage or absent head structure. Note that some examples exhibited more than one of these phenotypes.

| Retinoid | Concentration (mg mL\(^{-1}\)) | % normal at HH35 (number) | % embryos at HH35 (number) with one or more scapula malformations |
|----------|--------------------------------|--------------------------|------------------------------------------------------------------|
|          |                                |                          | Reduced | Foramen | Bend | Ectopic cartilage | Absent head |
| EC23     | 0.01                           | 24 (7)                   | 59 (17) | 0       | 7 (2) | 14 (4)            | 48 (14)     |
| ATRA     | 1                               | 0                        | 83 (5)  | 17 (1)  | 0    | 17 (1)            | 67 (4)      |

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were seen concomitant with a change in the size of the radius and/or ulna and 52% of scapula malformations were seen with a change in the size of the humerus.

As can be seen from Table 8, the scapula malformations generated ranged in severity. The most frequent scapula malformation seen with either EC23 or ATRA was a shortening of the scapula blade, as shown in Fig. 9. This was often concurrent with absence of the scapula head in EC23 and ATRA-treated embryos, as shown in Fig. 9(C–E) and Fig. 9(G–H). There was a range in the severity of this phenotype in both EC23- or ATRA-treated embryos. For example, compare Fig. 9(C′) with Fig. 9(E′) for EC23 and Fig. 9(F′) with Fig. 9(H′) for ATRA.

The least severe phenotypes are those of ectopic cartilage, bending and formation of a foramen, as shown in Figs 9(C,C′ and F,F′). Both EC23 and ATRA can generate ectopic cartilage around the scapula as seen in Fig. 9(G,G′), consistent with previous literature (Oliver et al. 1990). In contrast, duplication of the coracoid was never seen with EC23 and ATRA in our hands. Only EC23 was seen to have any effect on the coracoid, causing one embryo to develop with a thickened coracoid (not shown). Similarly, significant bending of the scapula blade was only seen in response to treatment with 0.01 mg mL\(^{-1}\) EC23, as shown in Fig. 9(C). The development of a foramen in the scapula was only seen once, in response to 1 mg mL\(^{-1}\) ATRA, as shown in Fig. 9(F′). Thus, EC23 and ATRA are shown to affect the limb girdle as well as the entire proximo-distal axis of the wing.

Both ATRA and EC23 inhibit expression of the scapula precursor marker \textit{Pax1}

The mechanism behind this shortening of the scapula was investigated by analysing the effect of retinoids on \textit{Pax1} expression. \textit{Pax1} has been implicated in scapula development, as the \textit{Pax1} mutant mouse (\textit{undulated}) shows a malformed acromion (head) and spina scapula (Timmons et al. 1994). Its expression pattern has also been documented in mouse and chick development (Timmons et al. 1994; LeClair et al. 1999; Huang et al. 2000). \textit{Pax1} expression in chick has been shown to precede the condensation of scapular cartilage and can be regarded as a marker for scapula development (Huang et al. 2000). Therefore, the mechanism behind the scapula reductions in EC23 and ATRA treated embryos was explored by investigating the effect of these retinoids on \textit{Pax1} expression.

Figure 10 shows whole-mount \textit{in situ} hybridisation for \textit{Pax1} expression in the wing after 48 h of retinoid treatment with large beads. In untreated wing buds, \textit{Pax1} is seen to be expressed in a domain at the antero–proximal wing.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig9}
\caption{Effect of retinoids on scapula development. Whole-mount skeletal preparations of retinoid-treated torsos and isolated scapulae are shown. (A) DMSO control embryo. (B–E′) Embryos treated with 0.01 mg mL\(^{-1}\) EC23 ranging from least to most severely affected. (F–H′) Embryos treated with 1 mg mL\(^{-1}\) ATRA, ranging from least to most severe affected. (A–H) Dorsal views of the torso with the operated wing on the right and anterior to the top. (C′–H′) Flat views of both scapulae after dissection: right is from operated side. (C–E) Examples of bending, ectopic cartilage and truncation, respectively. Arrow in (C) indicates the scapular head on the un-operated wing. Arrowheads in (C′–H′) indicate absence of the scapular head on the operated side. Panels F and F′ shows that scapula can develop a foramen (arrow in F′) in the presence of ATRA. (G,G′,H,H′) Reduction to the scapula blade in the presence of ATRA. c, coracoid; s, scapula; vc, vertebral column. Scale bars: 1 mm.}
\end{figure}
bud (Huang et al. 2000) at HH24–26. Both EC23 and ATRA down-regulate Pax1 expression in wings treated at HH20 with retinoid-soaked beads. This down-regulation can be complete, as shown in Fig. 10 (n = 2/6 ATRA or n = 3/5 EC23), or there can be a reduction in Pax1 levels (n = 3/6 ATRA or n = 1/5 EC23). These data are consistent with the idea that retinoids cause the scapula phenotypes observed via a down-regulation of Pax1.

Effect of EC23 and ATRA on upper beak outgrowth

Retinoids are known to affect the development of the upper beak when applied to the anterior chick wing at HH20 (Tamarin et al. 1984). The whole embryo, including the face, can become exposed to retinoid when it is applied topically to the limb (Thaller & Eichele, 1988). The levels of additional retinoid in the treated limb are found to be 10-fold higher than the rest of the embryo. No overall reduction in embryo size was observed after retinoid treatment, indicating that the facial effects observed reflect a particular sensitivity of these structures to retinoids. The effect of EC23, EC19 and ATRA on upper beak development was compared after application of retinoid to the limb via the large 150–300-μm beads. Table 9 shows the frequencies of facial phenotypes at concentrations chosen for EC23 and ATRA and two concentrations of EC19. EC23 and EC19 generated a range of facial phenotypes (Fig. 11). ATRA generated a mild reduction of upper beak outgrowth in 50% of embryos and the more severe truncated upper beak in 33% of embryos, as previously described (Tamarin et al. 1984).

Exposure to 0.01 mg mL$^{-1}$ EC23 resulted in asymmetric upper beak outgrowth (Fig. 11B,B$'$) and the production of an overbite (Fig. 11C,C$'$) at low frequencies. At 0.01 mg mL$^{-1}$, EC23 also caused both reduced and truncated upper beak outgrowth in 38 and 14% of embryos, respectively (Table 9, Fig. 11D,D$'$,E,E$'$).

Retinoid EC19 caused mild effects on upper beak outgrowth. At 0.01 mg mL$^{-1}$ EC19, only 17% of embryos exhibited reduced upper beak outgrowth (Table 9 and data not shown). However, at 0.1 mg mL$^{-1}$, EC19 reduced upper beak outgrowth in a similar frequency to EC23 (Fig. 11H,H$'$,I,I$'$). Application of 0.1 mg mL$^{-1}$ EC19 also generated a high frequency (60%) of asymmetric upper beak outgrowth (Fig. 11F,F$'$,G,G$'$). The frequency of this phenotype is higher than the frequency of the equivalent seen with 0.01 mg mL$^{-1}$ EC23. It is also notable that although retinoid was always applied to the right wing, the asymmetry can be either towards or away from the source of retinoid (cf. Fig. 11F with Fig. 11G$'$). In the most severe phenotype generated by 0.1 mg mL$^{-1}$ EC19, there is both reduced and asymmetric upper beak outgrowth (Fig. 11I,I$'$). These data indicate that EC23 is more potent than EC19 in its ability to affect upper beak development. EC23 is able to generate more severe phenotypes at a lower concentration than EC19. This is consistent with our findings on limb phenotypes (Fig. 2) and previous reports (Christie et al. 2008).

Discussion

After applying novel stable synthetic retinoids in vivo to chick embryo limb buds, we find that one compound, EC23, has very similar biological effects to all-trans retinoic acid. Within the appropriate concentration range, EC23 can be regarded as equivalent to ATRA but with the added benefit of being more stable, both to light and on the basis of its
structure, to enzymatic degradation. We found that EC23 is 100–1000-fold more effective than ATRA in vivo. Thus, synthetic retinoid EC23 offers the potential to be used as a more consistent experimental tool in vivo as well as in vitro (Christie et al. 2008). In addition, when higher doses of EC23 are applied, we find that novel phenotypes are produced compared with ATRA; in particular, a novel type of digit duplication was observed. We have also seen effects on the entire proximo-distal axis of the limb with both EC23 and ATRA. These include a novel effect on the pectoral girdle. Differences in outcomes compared with ATRA may be a result of the stability of EC23, or differential receptor activation. A second synthetic retinoid, EC19, differs from EC23 only in the position of the terminal carboxylic acid group. This structure is consistent with the idea that EC19 may be a stable analogue of 13-cis retinoic acid. We find that EC19 shows a markedly different spectrum of phenotypic effects in vivo compared with EC23, as it did in vitro (Christie et al. 2008). 13-cis Retinoic acid has been shown to be present at low concentrations in vivo, like 9-cis retinoic acid, and is increased after the application of excess amounts of ATRA (Horton & Maden, 1995). However 13-cis retinoic acid has been shown to be less potent than ATRA (Kistler, 1987) and its effects have been attributed to its inter-conversion with ATRA by isomerases (Chen & Juchau, 1998; Ruhl et al. 2001). Thus, EC19 may represent an experi-

Table 9 The effect of ATRA, EC23 and EC19 on upper beak development in embryos surviving to HH35. Shows the frequency of embryos which survived to HH35 with either normal, asymmetric, reduced, truncated or overbite facial phenotypes. Reduction is characterised as decreased upper beak outgrowth whereas truncation is complete absence of the upper beak (see Figure 11 D, D’, E, E’, H, H’). Numbers are presented as percentages of number surviving to HH35 (number).

| Retinoid | Concentration (mg mL⁻¹) | Frequency of embryos surviving to HH35 with facial phenotypes% (number) |
|----------|--------------------------|---------------------------------------------------------------------|
|          |                          | Normal | Assymetric | Reduced | Truncated | Overbite |
| EC23     | 0.01                     | 41 (12)| 3 (1)     | 38 (11)| 14 (4)    | 3 (1)    |
| ATRA     | 1                        | 17 (1)| 0         | 2 (3)  | 33 (2)    | 3 (1)    |
| EC19     | 0.01                     | 83 (5)| 0         | 17 (1) | 0         | 3 (1)    |
| EC19     | 0.1                      | 10 (1)| 60 (6)    | 40 (4) | 0         | 0        |

![EC23](image1) ![ATRA](image2)

Fig. 11 Effects of retinoids on upper beak outgrowth. (A-I) Lateral views of embryos stained with Alcian Blue and (A’-I’) show frontal views of the same embryo. (A,A’) Effect of DMSO on upper beak outgrowth. (B-E’) Phenotypes produced with 0.01 mg mL⁻¹ EC23 with increasing severity. (F-I’) Effects of 0.1 mg mL⁻¹ EC19 with increasing severity. (B,B’) Asymmetric upper beak outgrowth. (C,C’) Production of an overbite. (D,D’) Reduction in upper beak outgrowth. (E,E’) Show complete truncation of the upper beak. (F,F’) Asymmetric upper beak outgrowth with the upper beak skewed to the unoperated side. (G,G’) Asymmetric upper beak outgrowth with the upper beak skewed towards the operated side. (H,H’) Reduction in upper beak outgrowth. (I,I’) Both reduced and asymmetric upper beak outgrowth. e, eye; l, lower beak; u, upper beak. Scale bars: 1 mm.
mentally useful compound for the study of the effects of 13-cis retinoic acid in the absence of its conversion to ATRA.

Potential mechanisms for the differential effects of EC23

Two possibilities may explain the differences in the activities of EC23, ATRA and EC19: differential receptor activation and decreased metabolism in vivo. Considering the increased stability and potency of EC23, it is likely that a decrease in its metabolism in vivo may explain its alternative action compared with ATRA. The structure of EC23 is similar to that of another synthetic retinoid, TTNPB. TTNPB has been shown to be three orders of magnitude more potent than ATRA (Eichele et al. 1985), which is similar to the potency we find with EC23. TTNPB potency has also been shown to be due to its resistance to metabolism (Pignatello et al. 2002) rather than to changes in binding affinity with retinoid receptors or CRABP (Pignatello et al. 1999). When considering the structure of EC23, it may be predicted that its metabolism by the cyp26 enzymes should be blocked by the replacement of trimethylcyclohexenyl unit of ATRA with a tetramethyl-tetrahydroanaphthalene unit, which blocks oxidation at the carbon atoms numbered as 4, 5 and 6 on the structures in Fig. 1 (position 4 in ATRA is the centre which becomes oxidised to give the so-called 4-oxo derivative). There remains the question, however, as to whether the other equivalent positions in EC23 and EC19 could be oxidised, as they are in the proposed oxidative metabolites of ATRA (Topletz et al. 2012), i.e. positions 3, 16 and 18. Assuming that the 4-position is the more readily oxidised in these systems, this would cause ATRA to be inactivated much more efficiently than EC23 by cyp26, as EC23 would be unable to undergo the same oxidation at position 4 due to its geminal-dimethyl substitution at that centre. Also, because EC19 shares a similar structure to EC23, differing only in the position of the terminal carboxylic acid group, it would be just as stable as EC23. Hence, the fact that EC19 shows differential effects compared with both ATRA and EC23 suggests that differential receptor activation is the underlying mechanism for the effects of EC19.

Retinoids and digit development

ATRA and EC23 produce digit duplications after application to the anterior wing bud at HH20. ATRA has been shown previously to generate duplications (Tickle et al. 1985), as has the synthetic retinoid TTNPB (Eichele et al. 1985), which has some structural similarities to EC23. These early experiments on the limb indicated that retinoids were able to produce changes in the anterior-posterior patterning of the digits. These findings were extended by studies showing that after retinoid treatment there is up-regulation of genes involved in setting up antero–posterior polarity, for example hoxb8 after 30 min (Lu et al. 1997; Stratford et al. 1997) and Hand2 after 20 h (Fernandez-Teran et al. 2000). ATRA application was also shown to up-regulate shh after 24 h (Riddle et al. 1993). Shh is known to be the protein secreted by the Zone of Polarising Activity (ZPA) (Riddle et al. 1993) and to be required for patterning of the AP axis of the limb after the stylopod/zeugopod transition (Chiang et al. 2001; Ros et al. 2003). It has, therefore, been proposed that retinoids cause digit duplication through induction of a second ZPA in the anterior mesenchyme. When EC23 is applied via large beads, we have observed the development of multiple digit 1s without duplication of other digits. This phenotype is not observed in response to ATRA (Tickle et al. 1985; Budge, 2010) or TTNPB (Eichele et al. 1985), despite evidence that TTNPB is also resistant to metabolism (Pignatello et al. 2002).

When a fixed quantity of additional retinoid is applied via an ion-exchange bead, the effects of the additional retinoid will eventually be attenuated by metabolism of the added retinoid and growth of the embryo, both of which will reduce retinoid concentrations in tissues. If a retinoid is resistant to metabolism, only the increase in size of the embryo would attenuate retinoid effects. Thus, expression of immediate retinoid targets would be expected to persist for longer in a limb bud treated with a stable retinoid (Pignatello et al. 1999). After applying retinoids via large beads we observed induction of shh expression in anterior limb buds after 30 h with ATRA but not EC23. This would mean that shh induction occurs later and/or less shh is produced with EC23. Lower levels of shh would then be produced in the anterior limb, compared with ATRA treatment, which would lead to the duplication of only anterior digits (Towers et al. 2008). An example of 11123 pattern digit duplications has been previously described in the literature at low frequency, in an experiment where shh at low concentration was applied to the limb bud via a bead for an extended period (Yang et al. 1997). This would suggest that the phenotype described here with EC23 is in some way shh-dependent and results from a long duration of low level shh activity. In the clinical literature, mutations affecting shh regulation have been implicated in radial polydactyly (Lettice et al. 2003; Gurnett et al. 2007). However, the clinical phenotypes involve single duplicated thumbs, not the multiple duplications described here in response to EC23.

In some contexts, retinoids are known to exhibit proliferative effects. Retinoic acid in keratinocytes is known to be able to promote proliferation via an alternative receptor pathway involving PPARβ/δ and FABP5 (Shaw et al. 2003; Schug et al. 2007). Potentially, this enhanced proliferation could also be occurring in the limb after EC23 treatment. As a result, multiple additional digits are formed, but they all have digit 1 identity associated with low shh levels. In this sense, our anterior digit 1 duplications are occurring under similar conditions to those producing abnormalities in talpid3 mutants, where shh transduction is blocked but cell proliferation can still occur (Davey et al. 2006; Bangs et al. 2011).
An alternative, non-exclusive possibility relates to the fact that digit 1 is the last to form, as indicated by the initiation of Sox9 expression in its condensation (Welten et al. 2005). If the presence of EC23 suppresses a number of aspects of limb development, then establishment of digits with identities of 2 and 3 may not be possible in the EC23-affected region of the limb bud, even in the presence of shh. By the time EC23 concentrations fall to levels compatible with digit initiation, it is only possible to form condensations of digit 1 morphology before digits can no longer be initiated at all. In the context of promotional models of digit specification (Towers & Tickle, 2009; Towers et al. 2011), our data would be consistent with there being insufficient time for any promotion of duplicated digits occurring at all after EC23 treatment.

Retinoids and regulation of cartilage element size

All-trans retinoic acid and EC23 have been observed to reduce the length of the humerus, radius and ulna during limb development. They have also been seen to affect these elements differentially: ATRA has less effect on the humerus and ulna than EC23 (Fig. 6, Tables 5 and 6). We have also observed synostosis of different components of the elbow. These findings are consistent with the malformations described in mice, zebrafish and man in situations in which cyp26b1 function is impaired (Yashiro et al. 2004; Laue et al. 2008, 2011; Spoorendonk et al. 2008). Cyp26b1 inactivation would be predicted to give rise to increases in endogenous retinoid levels.

Previously, retinoids have been shown to down-regulate fgfs and distally expressed genes in limb development, causing cell identity to become proximalised (Mercader et al. 2000). The effect of both EC23 and ATRA on cartilage element length may be due to transient down-regulation of the AER and decreased proliferation of progenitors. The effects on the ulna are consistent with a deficiency of shh during its development (Chiang et al. 2001; Ros et al. 2003), which would be consistent with our observations of a delay in shh induction.

The shortened and thickened zeugopod elements we have seen are similar to those produced by over-expression of Meis1 (Mercader et al. 1999, 2009). This would be consistent with up-regulation of the proximal marker, Meis2, seen in treated limb buds, suggesting that retinoids are proximalising limbs via induction of Meis1 and Meis2. We have unpublished data from a microarray analysis (data not shown) of retinoid-treated limbs indicating that, in addition to Meis2, there is also up-regulation of Meis1 in ATRA- and EC23-treated limbs compared with DMSO. Meis1 up-regulation is consistent across all retinoid-treated samples, although the level of variation between replicate samples means that the observation does not pass filtering criteria for significantly altered target genes. The enhanced ability of EC23 to induce proximal markers compared with ATRA may mean that it represents a useful tool for exploring the role of retinoids in the establishment of proximal identity in limb buds.

Effect of retinoids on scapula development

EC23 and ATRA cause shortening to the scapula blade, and absence of the scapula head is seen at high frequency. Both ATRA and EC23 down-regulated Pax1 expression. Pax1 has been proposed to be a marker of scapula-forming cells and is expressed during the extension of the scapula blade (Huang et al. 2000). Pax1 null mice (undulated) have been shown to exhibit malformations in the shoulder girdle (Timmons et al. 1994). Increased BMP signalling has been implicated in scapula malformation and inhibition of Pax1 expression between HH19 and 25 (Hofmann et al. 1998). Injection of Noggin-expressing cells at varying somite levels between HH20 and 22 caused lack of scapula blade development in the corresponding somite level (Wang et al. 2005). ATRA and EC23 may truncate scapula development via perturbation of BMP signalling. Pbx1 has also been implicated in the control of scapula development, given its expression pattern and knockout mice exhibiting reduction of the scapula blade (Selleri et al. 2001; Capellini et al. 2010). Interestingly, Pbx1 has also been documented as a retinoid target during limb development (Qin et al. 2002). Alteration in the expression of these two transcription factors may be a mechanism by which ATRA and EC23 affect scapula development.

Retinoids and upper beak outgrowth

All-trans retinoic acid and EC23 can be seen to reduce or truncate upper beak outgrowth. These results are consistent with those reported for ATRA (Tamarin et al. 1984). Upper beak outgrowth and dorso-ventral polarity are controlled by a region called the frontonasal ectodermal zone (FEZ), which develops at HH20 in chick embryos. Shh and fgf8 are essential for outgrowth and morphogenesis of the upper beak, as removal of the FEZ or inhibition of fgf8 at HH17 causes truncation of the upper beak (Hu & Marcucio, 2009) and excess shh causes duplications or widening of the upper beak (Hu & Helms, 1999). Inhibition of shh signalling has been documented to cause truncation, but it has been shown that the epithelium can re-specify itself after FEZ excision at HH20 to produce normal upper beak development (Hu & Helms, 1999). Retinoids may cause decreased upper beak outgrowth by interfering with the FEZ. Consistent with this, shh expression is absent from 30 h after ATRA treatment in the developing frontonasal mass (FN M), whereas fgf8 is unaltered (Helms et al. 1997). ATRA is known to have an antagonistic relationship with fgf8 in the limb bud and other areas (Mercader et al. 2000). Excess retinoid may cause incorrect levels of fgf8, which has been shown to result in decreased proliferation and outgrowth.
of the FNMs (Song et al. 2004). To support this, it has been shown that after excess shh is applied to the FNMs, there is an increase in Raldh2 expression and a concurrent decrease in fgf8 expression which also leads to truncation of the upper beak (Hu & Marcucio, 2009). ATRA has been shown to decrease Msx expression in the developing facial processes, which can also lead to decreased outgrowth of the FNMs (Brown et al. 1997; Song et al. 2004).

EC19 can also produce facial phenotypes (Fig. 10, Table 9); however, those observed appear to be distinct from those produced by ATRA or EC23 in that the outgrowth of the FNMs is reduced or asymmetric. This may be a consequence of its potential activity as an equivalent of 13-cis retinoic acid that cannot be converted to ATRA.

Conclusions

We have observed that EC23 and EC19 exhibit differential effects on toxicity, limb and craniofacial development in vivo. EC23 mimics ATRA to an extent in the production of digit duplications, shortening of the cartilage elements and malformation of the scapula but also generates a novel digit duplication in the production of multiple digit 1s. EC19, however, is never seen to generate digit duplications at the higher concentrations tested herein. EC23 mimics the effects of ATRA on upper beak outgrowth, whereas EC19 causes far milder effects: reductions and asymmetrical outgrowth. We have demonstrated that novel stable retinoids are biologically active and can be used to uncover new aspects of retinoid function in vivo. As a result, EC23 and EC19 may represent useful new tools to investigate the mechanisms underlying congenital abnormalities in mammals, including man.

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References

Acloque H, Wilkinson DG, Nieto MA (2008) In situ hybridization analysis of chick embryos in whole-mount and tissue sections. Methods in Cell Biology 87, 169–185.

Balmer JE, Blomhoff R (2002) Gene expression regulation by retinoic acid. J Lipid Res 43, 1773–1808.

Bangs F, Antonio N, Thongnuek P, et al. (2011) Generation of mice with functional inactivation of talpid2, a gene first identified in chicken. Development 138, 3261–3272.

Barnes GL, Hsu CW, Mariani BD, et al. (1996) Chicken Pax-1 gene: Structure and expression during embryonic somite development. Differentiation 61, 13–23.

Brown JM, Robertson KE, Weddell SE, et al. (1997) Alterations in Msx 1 and Msx 2 expression correlate with inhibition of outgrowth of chick facial primordia induced by retinoic acid. Anat Embryol 195, 203–207.

Budge JKR (2010) The biological effects of novel synthetic retinoids. Masters thesis, Durham University.

Burke AC, Feduccia A (1997) Developmental patterns and the identification of homologies in the avian hand. Science 278, 666–668.

Capellini TD, Vaccari G, Ferretti E, et al. (2010) Scapula development is governed by genetic interactions of Pbx1 with its family members and with Emsx2 via their cooperative control of Alx1. Development 137, 2559–2569.

Chambers D, Wilson L, Maden M, et al. (2007) RALDH-independen generation of retinoic acid during vertebrate embryogenesis by CYP1B1. Development 134, 1369–1383.

Chang DT, Lopez A, Vokkessler DP, et al. (1994) Products, genetic-linkage and limb patterning activity of a murine hedgehog gene. Development 120, 3339–3353.

Chen H, Juchau MR (1998) Biotransformation of 13-cis- and 9-cis-retinoic acid to all-trans-retinoic acid in rat conceptal homogenates – evidence for catalysis by a conceptual isomerase. Drug Metab Dispos 26, 222–228.

Chiang C, Litingtung Y, Harris MP, et al. (2001) Manifestation of the limb prepattern: limb development in the absence of Sonic hedgehog function. Dev Biol 236, 421–435.

Christie VB, Barnard JD, Batsanov AS, et al. (2008) Synthesis and evaluation of synthetic retinoid derivatives as inducers of stem cell differentiation. Org Biomol Chem 6, 3497–3507.

Cunningham JM, Zhao X, Sandell LL, et al. (2013) Antagonism between retinoic acid and fibroblast growth factor signaling during limb development. Cell Rep, 3, 1–10.

Davey MG, Paton IR, Yin Y, et al. (2006) The chicken talpid2 gene encodes a novel protein essential for Hedgehog signaling. Genes Dev 20, 1365–1377.

Dranse HJ, Sampaio AV, Petkovich M, et al. (2011) Genetic deletion of Cyp26b1 negatively impacts limb skeletogenesis by inhibiting chondrogenesis. J Cell Sci 124, 2723–2734.

Duester G (2008) Retinoic acid synthesis and signaling during early organogenesis. Cell 134, 921–931.

Eichele G, Tickle C, Alberts BM (1985) Studies on the mechanism of retinoid-induced pattern duplications in the early chick limb bud – temporal and spatial aspects. J Cell Biol 101, 1913–1920.

Fernandez-Teran M, Piedra ME, Kathiriya IS, et al. (2000) Role of dHAND in the anterior-posterior polarization of the limb bud: implications for the Sonic hedgehog pathway. Development 127, 2133–2142.

Fisher ME, Clelland AK, Bain A, et al. (2008) Integrating technologies for comparing 3D gene expression domains in the developing chick limb. Dev Biol 317, 13–23.

Fujii H, Sato T, Kaneko S, et al. (1997) Metabolic inactivation of retinoic acid by a novel P450 differentially expressed in developing mouse embryos. EMBO J 16, 4162–4173.

Gurnett CA, Bowcock AM, Dietz FR, et al. (2007) Two novel point mutations in the long-range SHH enhancer in three families with triphalangeal thumb and preaxial polydactyly. Am J Med Genet A 143A, 27–32.

Hamburger V, Hamilton HL (1951) A series of normal stages in the development of the chick embryo. J Morphol 88, 49–92.

Helms JA, Kim CH, Hu D, et al. (1997) Sonic hedgehog participates in craniofacial morphogenesis and is down-regulated by teratogenic doses of retinoic acid. Dev Biol 187, 25–35.

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Hofmann C, Drossopoulou G, McMahon A, et al. (1998) Inhibitory action of BMPs on Pax1 expression and on shoulder girdle formation during limb development. Dev Dyn 213, 199–206.

Horrocks GA, Lauder L, Stewart R, et al. (2003) Formation of neuroepithelium from human embryonal carcinoma stem cells. Biochem Biophys Res Commun 304, 411–416.

Horton C, Maden M (1995) Endogenous distribution of retinoids during normal development and teratogenesis in the mouse embryo. Dev Dyn 202, 312–323.

Hu D, Helms JA (1999) The role of sonic hedgehog in normal and abnormal craniofacial morphogenesis. Development 126, 4873–4884.

Hu D, Marcucio RS (2009) A SHH-responsive signaling center in the forebrain regulates craniofacial morphogenesis via the facial ectoderm. Development 136, 107–116.

Huang RJ, Zhi QX, Patel K, et al. (2000) Ductal origin and segmental organisation of the avian scapula. Development 127, 3789–3794.

Kistler A (1987) Limb bud cell-cultures for estimating the teratogenic potential of compounds – validation of the test system with retinoids. Arch Toxicol 60, 403–414.

Laue K, Janicke M, Plaster N, et al. (2008) Restriction of retinoic acid activity by Cyp26b1 is required for proper timing and patterning of osteogenesis during zebrafish development. Development 135, 3775–3787.

Laue K, Pogoda HM, Daniel PB, et al. (2011) Craniosynostosis and multiple skeletal anomalies in humans and zebrafish result from a defect in the localized degradation of retinoic acid. Am J Hum Genet 89, 595–606.

LeClair EE, Bonfiglio L, Tuan RS (1999) Expression of the paired-box genes Pax-1 and Pax-9 in limb skeleton development. Dev Dyn 214, 101–115.

Lettice LA, Heaney SJH, Purdie LA, et al. (2003) A long-range Shh enhancer regulates expression in the developing limb and fin and is associated with proximal polydactyly. Hum Mol Genet 12, 1725–1735.

Lohnes D, Mark M, Mendelsohn C, et al. (1994) Function of the retinoic acid receptors (RARs) during development. 1. Craniofacial and skeletal abnormalities in RAR double mutants. Development 120, 2723–2748.

Lu HC, Revelli JP, Goering L, et al. (1997) Retinoid signaling is required for the establishment of a ZPA and for the expression of Hoxb-8, a mediator of ZPA formation. Development 124, 1643–1651.

Manske PR, Oberg KC (2009) Classification and developmental biology of congenital anomalies of the hand and upper extremity. J Bone Joint Surg Am 91A, 3–18.

Mark M, Ghyselinck NB, Chambon P (2006) Function of retinoid nuclear receptors. Lessons from genetic and pharmacological dissections of the retinoic acid signaling pathway during mouse embryogenesis. Annu Rev Pharmacol Toxicol 46, 451–480.

Mercader N, Leonardo E, Azpilzu N, et al. (1999) Conserved regulation of proximodistal limb axis development by Meis1/Hth. Nature 402, 425–429.

Mercader N, Leonardo E, Piedra ME, et al. (2000) Opposing RA and FGF signals control proximodistal vertebrate limb development through regulation of Meis genes. Development 127, 3961–3970.

Mercader N, Selleri L, Criado LM, et al. (2009) Ectopic Meis1 expression in the mouse limb bud alters P-D patterning in a Pbx1-independent manner. Int J Dev Biol 53, 1483–1494.

Mic FA, Sirbu IO, Dueter G (2004) Retinoic acid synthesis controlled by Raldh2 is required early for limb bud initiation and then later as a proximodistal signal during apical ectodermal ridge formation. J Biol Chem 279, 26698–26706.

Murayama A, Suzuki T, Matsuji M (1997) Photosomerization of retinoic acids in ethanol under room light: a warning for cell biological study of geometrical isomers of retinoids. J Nutr Sci Vitaminol 43, 167–176.

Niederreither K, Subbarayan V, Dolle P, et al. (1999) Embryonic retinoic acid synthesis is essential for early mouse post-implantation development. Nat Genet 21, 444–448.

Niederreither K, Vermot J, Schubbaur B, et al. (2002) Embryonic retinoic acid synthesis is required for forelimb growth and anteroposterior patterning in the mouse. Development 129, 3563–3574.

Oliver G, Derobertis EM, Wolpert L, et al. (1990) Expression of a homeobox gene in the chick wing bud following application of retinoic acid and grafts of polarizing region tissue. EMBO J 9, 3093–3099.

Pignatello MA, Kauffman FC, Levin AA (1999) Multiple factors contribute to the toxicity of the aromatic retinoid TTTNPB (Ro 13-7410): interactions with the retinoic acid receptors. Toxicol Appl Pharmacol 159, 109–116.

Pignatello MA, Kauffman FC, Levin AA (2002) Liarozole markedly increases all-trans-retinoic acid toxicity in mouse limb bud cell cultures: a model to explain the potency of the aromatic retinoid (E)-4-(2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)ene)-1-propenyl] benzoic acid. Toxicol Appl Pharmacol 178, 186–194.

Przyborski SA, Morton IE, Wood A, et al. (2000) Developmental regulation of neurogenesis in the pluriotent human embryonal carcinoma cell line NTERA-2. Eur J Neurosci 12, 3521–3528.

Qin P, Cimildoro R, Kochhar DM, et al. (2002) PBX, MEIS, and IGF-I are potential mediators of retinoic acid-induced proximodistal limb reduction defects. Teratology 66, 224–234.

Reijntjes S, Gale E, Maden M (2004) Generating gradients of retinoic acid in the chick embryo: Cyp26c1 expression and a comparative analysis of the Cyp26 enzymes. Dev Dyn 230, 509–517.

Reijntjes S, Bientic A, Gale E, et al. (2005a) The control of morphogen signalling: regulation of the synthesis and catabolism of retinoic acid in the developing embryo. Dev Biol 285, 224–237.

Reijntjes S, Gale E, Maden M (2005b) The role of the retinoic acid catabolising enzymes CYP26A1, CYP26B1 and CYP26C1 in development. Mech Dev 122, 539–540.

Riddle RD, Johnson RL, Lauffer E, et al. (1993) Sonic hedgehog mediates the polarizing activity of the ZPA. Cell 75, 1401–1416.

Rus MA, Dahn RD, Fernandez-Teran M, et al. (2003) This chick oligozeugodactyly (ozd) mutant lacks sonic hedgehog function in the limb. Development 130, 527–537.

Ross SA, McCaffery PJ, Drager UC, et al. (2000) Retinoids in embryonal development. Physiol Rev 80, 1021–1054.

Ruhl R, Plum C, Elmazar MMA, et al. (2001) Embryonic subcellular distribution of 13-cis- and all-trans-retinoic acid indicates differential cytosolic/nuclear localization. Toxicol Sci 63, 82–89.

Schug TT, Berry DC, Shaw NS, et al. (2007) Opposing effects of retinoic acid on cell growth result from alternate activation of two different nuclear receptors. Cell 129, 723–733.

Selleri L, Depew MJ, Jacobs Y, et al. (2001) Requirement for Pbx1 in skeletal patterning and programming chondrocyte proliferation and differentiation. Development 128, 3543–3557.
Shaw N, Elholm M, Noy N (2003) Retinoic acid is a high affinity selective ligand for the peroxisome proliferator-activated receptor beta/delta. J Biol Chem 278, 41589–41592.

Song Y, Hui JN, Fu KK, et al. (2004) Control of retinoic acid synthesis and FGF expression in the nasal pit is required to pattern the craniofacial skeleton. Dev Biol 276, 313–329.

Soprano DR, Soprano KJ (1995) Retinoids as teratogens. Annu Rev Nutr 15, 111–132.

Spoorenendonk KM, Peterson-Maduro J, Renn J, et al. (2008) Retinoic acid and Cyp26b1 are critical regulators of osteogenesis in the axial skeleton. Development 135, 3765–3774.

Stewart R, Christie V, Przyborski SA (2009) Manipulation of retinoids in the craniofacial skeleton. Curr Rev Nutr 1, 214–222.

Stratford T, Horton C, Maden M (1996) Retinoic acid is required for the initiation of outgrowth in the chick limb bud. Curr Biol 6, 1124–1133.

Stratford TH, Kostakopoulou K, Maden M (1997) Hoxb-8 has a role in establishing early anterior-posterior polarity in chick forelimb but not hindlimb. Development 124, 4225–4234.

Swindell EC, Thaller C, Sockanathan S, et al. (1999) Complementary domains of retinoic acid production and degradation in the early chick embryo. Dev Biol 216, 282–296.

Tamarin A, Crawley A, Lee J, et al. (1984) Analysis of upper beak defects in chicken embryos following with retinoic acid. J Embryol Exp Morphol 84, 105–123.

Thaller C, Eichele G (1988) Characterization of retinoid metabolism in the developing chick limb bud. Development 103, 473–483.

Thaller C, Hofmann C, Eichele G (1993) 9-cis-retinoic acid, a potent inducer of digit pattern duplications in the chick wing bud. Development 118, 957–965.

Therapontos C, Erskine L, Gardner ER, et al. (2009) Thalidomide induces limb defects by preventing angiogenic outgrowth during early limb formation. Proc Natl Acad Sci U S A 106, 8573–8578.

Tickle C, Crawley A (1988) The effects of local application of retinoids to different positions along the proximo-distal axis of embryonic chick wings. Wilhelm Roux Arch Dev Biol 197, 27–36.

Tickle C, Alberts B, Wolpert L, et al. (1982) Local application of retinoic acid to the limb bud mimics the action of the polarizing region. Nature 296, 564–566.

Tickle C, Lee J, Eichele G (1985) A quantitative-analysis of the effect of all-trans-retinoic acid on the pattern of chick wing development. Dev Biol 109, 82–95.

Timmons PM, Wallin J, Rigby PWJ, et al. (1994) Expression and function of Pax-1 during development of the pectoral girdle. Development 120, 2773–2785.

Topletz AR, Thatcher JE, Zelter A, et al. (2012) Comparison of the function and expression of CYP26A1 and CYP26B1, the two retinoic acid hydroxylases. Biochem Pharmacol 83, 149–163.

Towers M, Mahood R, Yin Y, et al. (2008) Integration of growth and specification in chick wing digit-patterning. Nature 452, 882–886.

Towers M, Tickle C (2009) Growing models of vertebrate limb development. Development 136, 179–190.

Towers M, Signolet J, Sherman A, et al. (2011) Insights into bird wing evolution and digit specification from polarization region fate maps. Nat Commun 2, 426. doi: 10.1038/ncomms1437.

Vargesson N (2003) Vascularization of the developing chick limb bud: role of the TGFβ signalling pathway. J Anat 202, 93–103.

Wang BG, He LW, Ehehalt F, et al. (2005) The formation of the avian scapula blade takes place in the hypaxial domain of the somites and requires somatopleure-derived BMP signals. Dev Biol 287, 11–18.

Wang Z, Young RL, Xue HL, et al. (2011) Transcriptomic analysis of avian digits reveals conserved and derived digit identities in birds. Nature 477, 583–5835.

Welt en MCM, Verbeek FJ, Meijer AH, et al. (2005) Gene expression and digit homology in the chicken embryo wing. Evol Dev 7, 18–28.

White JA, Guo YD, Baetz K, et al. (1996) Identification of the retinoic acid-inducible all-trans-retinoic acid 4-hydroxylase. J Biol Chem 271, 29922–29927.

White JA, Ramshaw H, Taimi M, et al. (2000) Identification of the human cytochrome P450, P450RAI-2, which is predominately expressed in the adult cerebellum and is responsible for all-trans-retinoic acid metabolism. Proc Natl Acad Sci U S A 97, 6403–6408.

Yang Y, Drossopoulou G, Chuang PT, et al. (1997) Relationship between dose, distance and time in Sonic Hedgehog-mediated regulation of anteroposterior polarity in the chick limb. Development 124, 4393–4404.

Yashiro K, Zhao XL, Uehara M, et al. (2004) Regulation of retinoic acid distribution is required for proximodistal patterning and outgrowth of the developing mouse limb. Dev Cell 6, 411–422.

Zhao XL, Sirbu IO, Mic FA, et al. (2009) Retinoic acid promotes limb induction through effects on body axis extension but is unnecessary for limb patterning. Curr Biol 19, 1050–1057.