**Int. J. Dev. Biol. 54**: 125-134 (2010)
doi: 10.1387/ijdb.082783sr

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

# Retinoic acid is both necessary for and inhibits myogenic commitment and differentiation in the chick limb

SUSAN REIJNTJES[1], PHILIPPA FRANCIS-WEST[2] and BALJINDER S. MANKOO*[,1]

*[1]Randall Division of Cell and Molecular Biophysics, New Hunt's House and [2]Department of Craniofacial Development, Dental Institute, King's College London, Guy's Campus, London, UK*

**ABSTRACT Retinoic acid (RA) plays an essential role in the development of many embryonic tissues, including the developing tetrapod limb bud. At early stages of limb development, RA levels are highest proximally and regulate the migration of myoblasts into the limb. As the premyogenic progenitor cells migrate into the limb and accumulate in premuscle masses, they express *Pax3* and *Meox2*. Myogenic differentiation is initiated by expression of *Myf5* and *MyoD*, and both *Pax3* and *Meox2* are required for normal *Myf5* expression. We show by loss of function using the inhibitor citral, that RA signalling within the limb bud is required to maintain *Pax3* and *Meox2* in the progenitor and *Myf5* and *MyoD* in the differentiating myoblasts. Treatment with excess RA showed a differential effect: *Meox2* and *Pax3* showed localised down-regulation of expression in the limb. In contrast, there was a dramatic down-regulation of expression of *MyoD*, *Myf5* and *Meox1*. The down-regulation of myogenic gene expression in response to inhibition of RA signalling, and differential response to application of excess RA, in the absence of changes to cell proliferation and apoptosis, indicate that myogenic specification and differentiation in the developing limb possess a complex sensitivity to RA concentrations.**

KEY WORDS: *retinoic acid, myogenesis, tendon, limb bud, chick*

## Introduction

In the early developing vertebrate embryo, signalling molecules are required to control individual cells and to instruct them as to when and where to differentiate into their distinctive tissue types. The limb bud is an excellent model system to investigate the differentiation and patterning of muscle. The cells of the hypaxial dermomyotome of somites adjacent to the presumptive limb bud undergo an epithelial-to-mesenchymal transition in response to signals from the neighbouring lateral plate mesoderm (Chevallier *et al*., 1977; Christ *et al*., 1977; Jacob *et al*., 1978; Solursh *et al*., 1987; Hayashi & Ozawa, 1995) and then delaminate from the somites and migrate into the limb bud. The migratory myogenic precursors initially express the transcription factor Meox2 (Mankoo *et al*., 1999) and CXCR4, Pax3 and Lbx1, the latter three being essential for migration (Vasyutina *et al*., 2005; Williams & Ordahl, 1994; Gross *et al*., 2000). Pax3 is important also for specification and proliferation of limb muscle precursors (Bober *et al*., 1994; Goulding *et al*., 1994). Once the muscle progenitors reach the developing limb, they continue to proliferate and coalesce in the dorsal and ventral pre-muscle masses, and initiate differentiation by expressing the myogenic regulatory transcription factors (MRFs): Myf5, MyoD, MRF4 and Myogenin (reviewed by Pownall *et al*. 2002).

The *Meox* genes have been shown to play an overlapping and essential role in somitogenesis in the mouse (Mankoo *et al*., 2003). In addition to a failure of the axial skeleton to develop, compound mutant embryos (*Meox1*$^{-/-}$;*Meox2*$^{-/-}$) exhibit a dramatic muscle phenotype where most skeletal muscles are absent or reduced in size concomitant with reduced or absent expression of *Pax3*, *Pax7* and *Myf5* in somites (Mankoo *et al*., 2003). Both *Meox1* and *Meox2* are expressed in the developing limb buds and are conserved between chick and mouse (Reijntjes *et al*., 2007a), suggesting a role for both in limb muscle development. The *Meox1* expression domain overlaps that of *MyoD*, while the *Meox2* domain of expression overlaps with *Pax3* and not *MyoD* indicating a differential role of the two *Meox* genes (Reijntjes *et al*., 2007a). During mouse myogenesis, it has been demonstrated

*Abbreviations used in this paper:* MRF, myogenic regulatory factor; RA, retinoic acid.

***Address correspondence to:** Dr. Baljinder S. Mankoo. King's College London, Randall Division of Cell and Molecular Biophysics, New Hunt's House, Guy's Campus, London SE1 1UL, U.K. Fax: +44-207-848-6435. e-mail: baljinder.mankoo@kcl.ac.uk

*Accepted:* 25 November 2008. *Final author corrected PDF published online:* 14 August 2009. *Edited by:* Christine Mummery.

ISSN: Online 1696-3547, Print 0214-6282

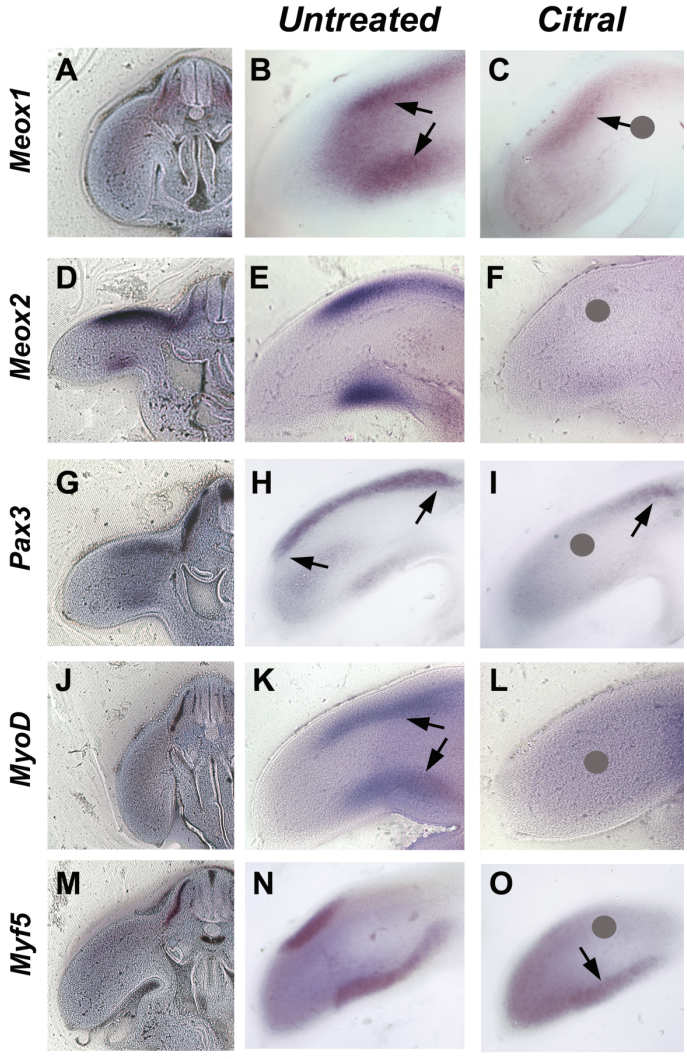



**Fig. 1. Retinoic acid (RA) signalling is required at early stages of limb myogenesis.** *Views of transverse vibratome sections of stage 22 chick embryos before application of a bead of citral to the limb (A,D,G,J,M) and at stage 25 after bead application and incubation (B,C,E,F,H,I,K,L,N,O) to examine the effect on expression of* Meox1 **(A-C)**, Meox2 **(D-F)**, Pax3 **(G-I)**, MyoD **(J-L)** *and* Myf5 **(M-O)**. *The citral treated limbs have been inverted for comparison with the untreated, contralateral limbs. The location of beads is identified by a grey circle.* **(B)** *Section of the untreated limb shows the normal expression of* Meox1 *in the dorsal limb (upper black arrow) and the ventral limb (lower black arrow).* **(C)** *Treatment with citral has resulted in a down regulation of* Meox1 *expression in the dorsal domain of the limb (black arrow) and a complete absence of expression in the ventral domain.* **(E)** *Normal* Meox2 *expression in the untreated contralateral limb bud.* Meox2 *expression is absent after treatment with citral* **(F)**. **(H)** *Section of the untreated limb shows the normal expression of* Pax3 *in the dorsal pre-muscle mass (black arrows) and the ventral pre-muscle mass.* **(I)** *Treatment with citral has completely ablated* Pax3 *expression in the limb apart from a small domain in the dorsal pre-muscle mass (black arrow).* **(K)** *Normal* MyoD *expression in the dorsal and ventral pre-muscle masses (black arrows); after treatment with citral,* MyoD *expression is undetectable* **(L). (N)** *Expression of* Myf5 *in the untreated contralateral limb is in the dorsal and ventral pre-muscle masses.* **(O)** Myf5 *expression in the dorsal pre-muscle mass is absent after treatment with citral and expression in the ventral pre-muscle mass is reduced (black arrow) compared to the untreated control limb bud (N).*

that *Meox2* and *Pax3* are co-expressed in migrating myoblasts as they de-epithelialize from the dermomyotome, migrate into the limb bud mesenchyme and accumulate in the premuscle masses. Mice homozygous for a null mutation of *Meox2* have reduced muscle mass and an absence of specific muscles, and reduced *Pax3* expression in limb muscle progenitors (Mankoo *et al*., 1999).

Retinoic acid (RA) is a low molecular weight (300 Da), signalling molecule that is known to affect pattern formation in vertebrate embryos including limb outgrowth and axial patterning. (reviewed by Maden, 2001). Many tissues such as the eye, hindbrain and spinal cord depend on a temporally and spatially regulated supply of RA. Without this supply of RA, many congenital defects occur. Paradoxically, exposure to this carefully regulated system by administering excess RA results in many defects similar to those generated by a lack of RA. Accumulated evidence has shown that inadequate levels (excess or deficiency) of RA cause a wide range of malformations in developing tissues. Deficiency of RA inhibits longitudinal bone growth (Wolbach *et al*., 1947) and excess RA also inhibits longitudinal bone growth by causing premature fusion of the epiphyseal growth plate (Pease, 1962, Standeven *et al*., 1996). In the hindbrain, retinoid excess targets predominantly the anterior rhombomeres (r1-4) whereby the anterior hindbrain was altered towards a more posterior segmental phenotype (Durston *et al*., 1989; Morriss-Kay *et al*., 1991; Conlon and Rossant, 1992; Marshall *et al*., 1992; Sundin and Eichele, 1992; Wood *et al*., 1994; Hill *et al*., 1995) and retinoid deficiency targets the posterior rhombomeres (r4-8) whereby they never differentiate (Gale *et al*., 1999; White *et al*., 2000). In the developing eye RA treated zebrafish and mouse embryos exhibited anophthalmia (Reijntjes *et al*., 2007b; Sulik *et al*., 1995) a condition also observed in RA deficiency (Wilson *et al*., 1953).

Endogenous all-trans-RA was first identified in the chick limb bud using HPLC (Thaller and Eichele, 1987) and components of the retinoid signalling pathway such as CRABP-II, RARα, RARβ are expressed in domains of the limb associated with muscle formation (Momoi, *et al*., 1992). In the chick limb bud, RALDH2, a RA generating enzyme, is expressed in the region of the presumptive muscle masses, vasculature and motor axons (Berggren *et al*., 2001); and in the mouse limb the establishment of pre-muscle masses, directed by HGF, has been proposed to be under the control of RALDH2 (Mic and Duester, 2003). RA receptors are expressed in muscle cells, RA receptors and MyoD up-regulate each other's transcriptional activity, these proteins interact physically and their transcriptional co-activation requires a RA receptor-MyoD complex that binds to MyoD DNA binding sites in muscle cells (Froeschlé *et al*., 1998). In chick, RARa is expressed in all limb bud cells at stage 20-22, just prior to or at the onset of myogenic commitment (Momoi *et al*., 1992).

Previous studies using cell lines have demonstrated that RA induces myogenic differentiation of a rat rhabomyosarcoma-derived cell line, C2 myoblast cells and primary adult chick satellite cells, the cells that contribute to post-natal growth of muscle (Arnold *et al*., 1992; Albagli-Curiel *et al*., 1993; Halevy and Lerman, 1993). However, RA has also been shown to inhibit myosin heavy chain protein accumulation in mouse embryonic primary muscle cultures (Xiao *et al*., 1995) and depending on its concentration activate or repress myogenesis in the craniofacial mesenchyme (Langille *et al*., 1989). The physiological role of

retinoids during limb myogenesis remains ambiguous as most studies have focused at late stages of differentiation and morphogenesis (Robson *et al*. 1994) or have used cultures of dissociated muscle: different concentrations of RA have been reported to increase or decrease MyoD expression in chick limb buds (Momoi *et al*., 1992). The subdivision of muscle masses and the development of individual tendons require interactions between muscle and tendon (Kardon, 1998; Rodriguez-Guzman *et al*., 2006) and components of the RA signalling pathways have been detected in the developing muscles and tendons of the mouse and chick limb (Abu-Abed *et al*., 2002; Berggren *et al*., 2001; Rodriguez-Guzman *et al*., 2006).

We have focused our attention on a possible role for RA in the early stages of myogenic commitment and differentiation. Firstly, we examined whether there is a requirement for RA signalling in myogenic commitment and/or differentiation, by use of an inhibitor of RA synthesis, citral, on beads applied to the developing chick limb where these genes are expressed. Secondly, we analysed the expression profiles of genes expressed in limb muscle progenitors (*Meox2, Pax3, Myf5* and *MyoD*) in response to excess all-*trans*-RA (tRA) treatment. The absence of RA resulted in downregulation of expression of *Meox2*, *Pax3, MyoD, Myf5* and *Meox1* and indicates a requirement for RA in the induction of myogenesis. We observed a differential sensitivity to RA dependent on the differentiation status of the cells: with a localised down regulation of *Meox2* and *Pax3* expression in progenitors and a dramatic down-regulation in the expression of muscle differentiation genes *MyoD* and *Myf*5 and also of *Meox1*. Furthermore, both citral and RA treatments resulted in a decrease in myogenic differentiation. As myogenic differentiation takes place in conjunction with tendon differentiation we also investigated whether tendon progenitors in the limb respond in a similar fashion to RA as the myogenic precursors at stages when their development is occurring independently of each other. Taken together, these results suggest a complex sensitivity to RA concentrations during myogenesis and tendon development in the limb.

## Results

### *RA signalling is necessary for myogenic commitment and differentiation*

RA is known to produce diverse, dosage-dependent, effects on muscle differentiation, but it is not known whether myogenic differentiation requires RA signalling. We exposed chick forelimb buds to the RA synthesis inhibitor citral (3,7-dimethyl-2,6-octadienal), a reactive and volatile β-unsaturated aldehyde. Citral has been shown by HPLC of treated chick limb buds to inhibit RA synthesis (Tanaka *et al*., 1996) and in zebrafish, a RA reporter was down-regulated by citral (Perz-Edwards, *et al*., 2001). SM2 beads were incubated in 2.9 x $10^{-3}$M citral in medium and were locally applied to the forelimb of a stage 22 chick embryo, when

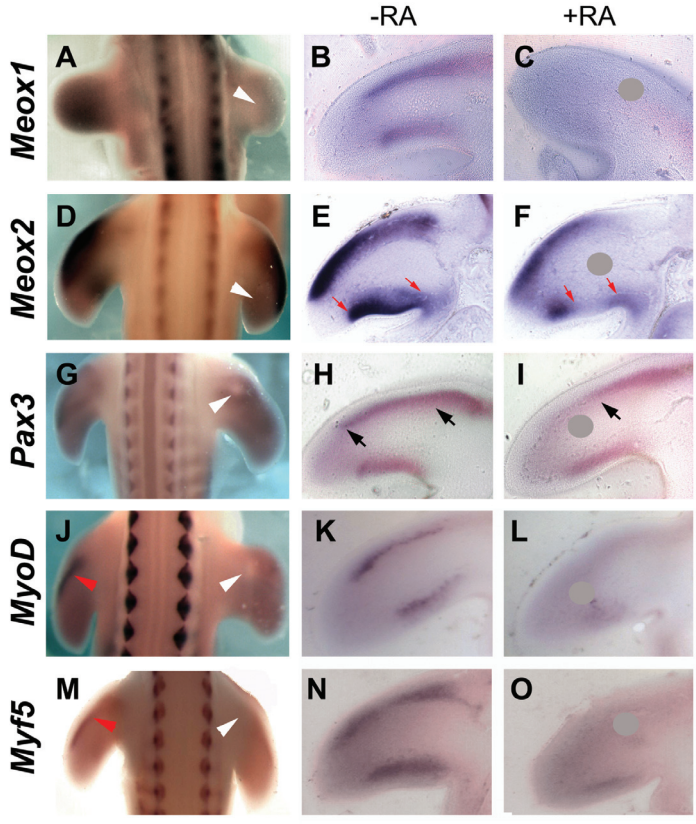



**Fig. 2. Retinoic acid differentially inhibits myogenic gene expression.** *Views of whole mount and transverse vibratome sections of stage 25 chick embryos after application of a bead of all-*trans*-RA to the limb to examine the effect on expression of* Meox1 **(A-C)**, Meox2 **(D-F)**, Pax3 **(G-I)**, MyoD **(J-L)** *and* Myf5 **(M-O)**. *White arrowheads mark the position of the beads (A,D,G,J,M). The RA treated limb sections have been inverted for comparison with the untreated, contralateral limbs. The location of beads is identified by grey circles.* **(A)** *A dorsal view of an embryo which received a bead of all-*trans*-RA in the right limb bud.* Meox1 *expression appears down-regulated around the position of the bead compared to the untreated contralateral limb bud, left side.* **(B)** *Section through a tRA treated embryo shows the normal expression of* Meox1 *in the untreated control limb bud.* **(C)** *Section of the RA treated limb bud reveals that* Meox1 *expression is down-regulated throughout the limb.* **(D)** *Whole mount embryo revealing that the expression of* Meox2 *has been down-regulated around the RA bead in the ventral region of the limb (compare to untreated limb).* **(E)** *A section through the embryo in D showing the normal expression of* Meox2 *in an untreated limb. Red arrows mark the ventral expression domain.* **(F)** *Treatment with a bead of RA results in a less dramatic effect on the expression of* Meox2 *than the expression of* Meox1 *in (C). This section shows localised down-regulation of* Meox2 *in the ventral domain corresponding to the position of the bead (compare red arrows in F to red arrows in E). The affect of RA on the expression of* Pax3 *is not immediately apparent in a whole mount embryo* **(G)** *(right limb bud, white arrowhead).* **(H)** *A section through the embryo in (G) shows expression of* Pax3 *in the untreated limb bud. Black arrows indicate the normal expression in the dorsal pre-muscle mass.* **(I)** *Sectional analysis of RA treated limbs shows a localised effect on* Pax3 *in the proximity of the bead with expression down-regulated distally in the dorsal pre-muscle mass domain (black arrow, compare with H).* **(J)** *Treatment with a bead of RA appears to completely ablate the expression of* MyoD *in the limb (compare expression in treated limb bud, white arrowhead, with untreated left side, red arrowhead),* **(K,L)** *Sections confirm that RA treatment causes a substantial down-regulation of* MyoD *compared to normal limb expression. RA treatment also has a dramatic effect on the expression of* Myf5 **(M-O).** *No expression can be detected in the treated limb bud (white arrowhead, right side; and section in O) compared to the untreated contralateral limb bud (red arrowhead, left side, and section in N).*

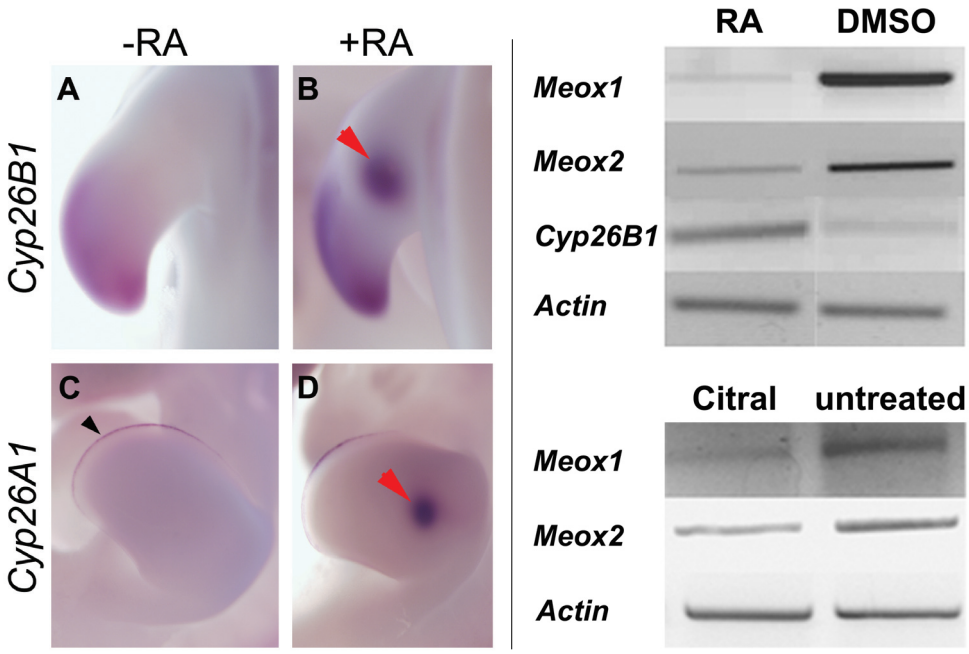



**Fig. 3 (Left). Retinoic acid treatment induces ectopic *Cyp26* expression.** *Views of the limb buds of stage 25 chick embryos to examine the effect of all-*trans*-RA on Cyp26 expression. Red arrowheads mark the position of the bead.* **(A)** *Dorsal view of the untreated contralateral limb bud showing normal* Cyp26B1 *expression in the progress zone.* **(B)** *In addition to the endogenous expression in the progress zone, a bead placed in the limb bud has up-regulated* Cyp26B1 *expression in the limb mesenchyme, right side.* **(C)** *Lateral view of an untreated limb showing normal* Cyp26A1 *expression in the apical ectodermal ridge.* **(D)** *RA treated, contralateral limb from (C) showing up-regulation of* Cyp26A1 *expression around the bead in the limb mesenchyme.*

**Fig. 4 (Right). Semi-quantitative gene expression analysis following citral and tRA treatment.** *Semiquantitative RT-PCR experiments on stage 25 chick forelimbs treated with beads of citral (lower left panel), control untreated contralateral limb (lower right panel), all-*trans*-RA (upper left panel) and control DMSO (upper right panel). The upper panel shows the results of* Meox1 *with a decrease in expression after the addition of citral compared to the untreated control limb; a decrease in* Meox2 *expression after citral treatment and no change in the expression of* Actin *after treatment with citral, compared to the untreated control. The upper panel shows the results for* Meox1 *with a decrease in expression after the addition of all-*trans*-RA; a similar decrease in* Meox2 *after addition of all-*trans*-RA; an increase in expression of* Cyp26B1 *after the addition of all-*trans*-RA. There is no change in the expression of* Actin *with all-*trans*-RA.*

myogenic differentiation has started, and examined by *in situ* hybridisation after overnight incubation. *In vivo* implantation of citral-beads arrested limb growth resulting in a decreased limb size compared to the untreated contralateral limb.

In citral treated limbs *Meox1* expression is present in the dorsal domain but is absent in the ventral domain local to the citral bead (Fig. 1 B,C, 2/2 embryos), while a reduced domain of dorsal expression persists. In contrast, inhibition of RA synthesis by citral has a dramatic affect on *Meox2* expression affecting both dorsal and ventral domains resulting in a small region of greatly reduced expression remaining in the ventral part of the treated limb (Fig. 1 E,F, 2/2 embryos).

Compared to normal *Pax3* expression in the pre-muscle masses, only a small domain of *Pax3* remains in the dorsal pre-muscle mass after citral treatment (Fig. 1 H,I). Citral treatment down-regulates expression of *MyoD* in the dorsal and ventral pre-muscle masses (Fig. 1 K,L, 2/2 treated embryos). Citral treatment also results in a down-regulation of expression of *Myf5*; however, in contrast to *MyoD,* the effect on *Myf5* expression is much less dramatic, with a localised reduction of expression (Fig.1 N,O. 3/3 embryos).

### *RA has a differential effect on gene expression in limb myogenic progenitors*

We investigated whether myogenic commitment and differentiation are sensitive to RA by administration of excess tRA, to the developing chick limb. For these experiments, we used local administration of AG 1-X2 beads soaked in 1.33 x $10^{-2}$M tRA dissolved in DMS0. This dose has been found to duplicate the chick limb bud (Tickle *et al*. 1982). A tRA soaked bead was locally applied to the forelimb of a stage 22 chick embryo when myoblasts have migrated into the limb bud and the limbs were examined for gene expression by *in situ* hybridisation after overnight incubation. In all cases control beads which had been soaked in DMSO had no effect on the embryos either in terms of morphology or gene expression.

*Meox1* and *Meox2* have overlapping and non-overlapping domains of expression in the limb bud (Reijntjes *et al*. 2007). tRA soaked beads caused a dramatic decrease in the limb expression pattern of *Meox1* (Fig. 2 A-C, 9/11 treated embryos). Vibratome sectioning through the treated limb bud reveals that *Meox1* expression is decreased throughout the limb in both dorsal and ventral muscle domains (Fig. 2C). In contrast, down-regulation of *Meox2* expression after treatment with tRA is predominantly localised to the proximity of the bead (Fig. 2 D-F). *Meox2* expression is down-regulated only in the ventral domain of the right forelimb bud in the region of the bead compared to the untreated control side (Fig. 2F, 9/9 embryos).

On initial observations, it appears that *Pax3* expression around the location of the bead is not affected by tRA treatment (Fig. 2G) but sectioning of this embryo reveals that compared to the normal expression in the dorsal and ventral pre-muscle masses, there is down-regulation of this gene in the dorsal pre-muscle mass in the proximity of the bead, compared to the control limb (Fig. 2I, 5/5 embryos), while more distant dorsal and the entire ventral expression domains remain unchanged.

*MyoD* expression is induced in the premuscle masses at the onset of terminal differentiation. Expression of MyoD is significantly down-regulated throughout the limb bud by tRA treatment (Fig.2 J-L, 3/3 embryos). Sections through a tRA treated embryo demonstrate a small domain of reduced expression of *MyoD* remaining in the ventral pre-muscle mass (compare Fig. 2J with

2L). A similar effect is observed with *Myf5* which is strongly down-regulated by tRA beads when placed in the forelimb (Fig. 2 M-0, 4/4 embryos). A small domain of reduced expression remains in the ventral pre-muscle mass after tRA treatment (Fig. 20).

These results demonstrate that myogenic progenitors in the limb have a differential response to RA, which reflects the differentiation status of the cells: *Meox2* and *Pax3* expression in undifferentiated progenitors shows only local down-regulation in regions of highest RA concentration, while both *MyoD* and *Myf5* expression in differentiating myoblasts was reduced throughout the limb bud.

### *Local concentrations of RA up-regulate expression of RA responsive genes in the limb*

To confirm that the concentration of tRA used in these experiments has a specific effect on genes involved in limb myogenesis and not a generalized effect on gene expression, *in situ* hybridisation was performed using the *Cyp26A1* and *Cyp26B1* anti-sense RNA probes after tRA treatment. Cyp26A1 and Cyp26B1 enzymes are members of the cytochrome P450 superfamily which catabolise RA to products such as 4-oxo-RA, 4-OH-RA and 18- OH-RA (Fujii *et al*., 1997; White *et al*., 1997, 2000). Both genes are expressed in the developing chick limb, *Cyp26A1* in the apical ectodermal ridge (AER) (Swindell *et al*., 1999) and *Cyp26B1* in the distal mesenchyme of the progress zone (Reijntjes *et al*., 2003); and both are known to be inducible by RA (Abu-Abed *et al*., 1998; Fujii *et al*., 1997; Ray *et al*., 1997, Reijntjes *et al*., 2005; White *et al*., 1996; White *et al*., 1997). *Cyp26B1* expression is up-regulated around the tRA bead in the forelimb mesenchyme, proximal to the progress zone, in 2/2 embryos (Fig. 3B). Similarly, but to a lesser degree, a bead of tRA induces *Cyp26A1* in the limb mesenchyme in 2/2 embryos (Fig. 3D). This confirms the effects observed on the myogenic genes are not due to a non-specific down regulation of gene expression.

To again confirm the observations by *in situ* hybridisation, limb buds of stage 22 embryos were treated separately with beads soaked with $2.9 \times 10^{-3}$M citral, and $1.33 \times 10^{-2}$M tRA or DMSO and incubated for 24 hours before collection and RNA extraction. Semi-quantitative RT-PCR was performed to analyse expression levels relative to those for actin. *Meox1* and *Meox2* expression are down-regulated after treatment with citral compared to the untreated limb (Fig. 4, lower panel). Similarly, RT-PCR confirms that *Meox1* and *Meox2* RNA levels are down-regulated by tRA compared to DMSO and *Cyp26B1* expression is up-regulated compared to DMSO control (Fig. 4, upper panel).

### *Tendon differentiation is sensitive to RA signalling*

The development of muscle requires a coordinated differentiation of both muscle and tendons, although each tissue derives from different progenitors: the limb muscle from immigrant myoblasts originating from adjacent somites, and tendons from limb mesenchyme. We investigated whether tendon progenitors respond to RA in a similar manner as myogenic precursors, by analyzing expression of the transcription factor *Scleraxis,* which is expressed in all tendon progenitors in the limb (Schweitzer *et al*. 2001).

*Scleraxis* expression shows reduced expression in response to tRA (Fig. 5 A-C, in 3/3 embryos). Sections confirm that *Scleraxis* expression is reduced proximally in both the ventral and dorsal regions of the limb bud in the region of the bead (Fig. 5C). However, the reduction observed for *Scleraxis* expression is much less than seen for the myogenic genes. Citral inhibition of RA signalling resulted in an absence of *Scleraxis* expression in the dorsal domain of the limb and substantially reduced expression in the ventral domain (Fig. 5E, 5/5 embryos).

### *RA treatment does not result in detectable changes in cell proliferation or cell death*

We next asked whether these treatments caused a decrease in cell proliferation, as RA can induce growth arrest in myogenic cells (Albagli-Curiel *et al*., 1993). The limbs of stage 22 embryos were treated with a bead of tRA, citral and DMSO at the same concentrations used for the *in situ* analyses and incubated for 24 hours before collection. At stage 25 dividing cells were detected using anti-phosphohistone H3 antibody staining on sections and were present in all treatments in similar numbers and distribution as the untreated, contralateral control limb, indicating that neither treatment with excess RA nor inhibition of RA signalling causes detectable changes in cell proliferation (data not shown).

RA administration has been shown to cause mouse limb defects partly by inducing massive cell death in developing limbs

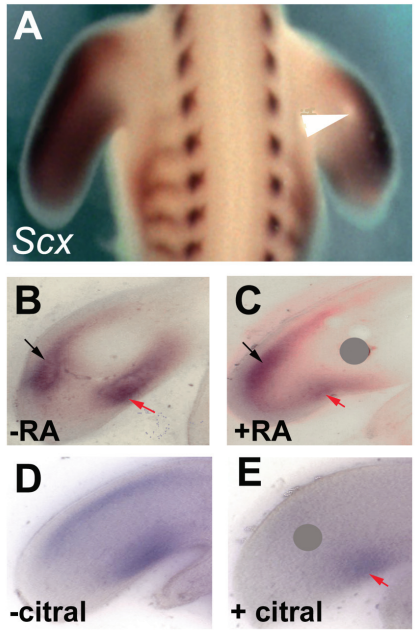



**Fig. 5. Tendon development is responsive to retinoic acid signalling.** *The effect of RA* **(A-C)** *and citral* **(D-E)** *treatments on the expression of* Scleraxis *(Scx). The location of beads is identified with grey circles in* **(C,E)**. **(A)** *A whole mount* in situ *hybridisation with* Scleraxis *reveals that compared with the untreated limb bud, there is localised down-regulation in the proximal limb bud around the position of the bead after RA treatment (white arrowhead).* **(B)** *A section through the embryo in A, shows the untreated limb bud with normal* Scleraxis *expression in the dorsal limb bud (black arrow) and the ventral limb bud (red arrow).* **(C)** *A section of the RA treated limb bud reveals that* Scleraxis *expression is reduced proximally both dorsally (compare to black arrow in B) and ventrally (compare to red arrow in* B*).* Scleraxis *expression is down-regulated after treatment with citral compared with normal expression in the untreated limb* **(D)***, resulting in a residual small domain of expression in the ventral limb bud* **(E)** *(red arrow).*

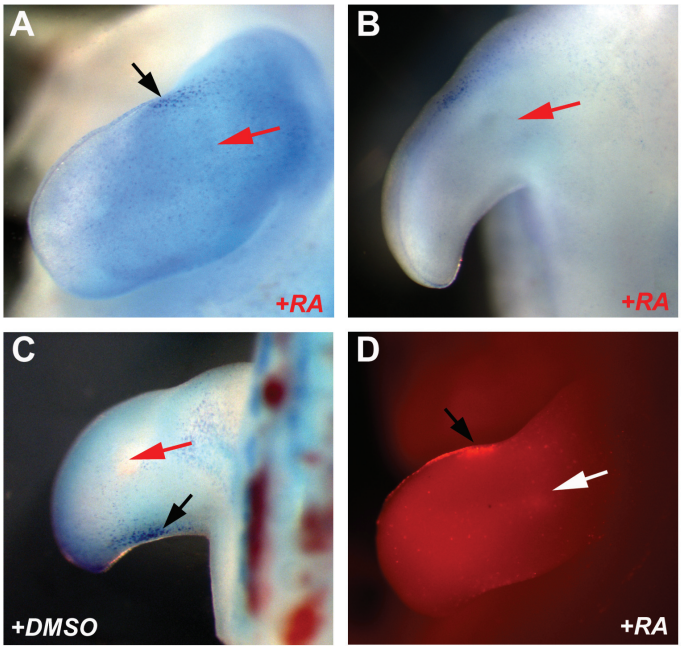



**Fig. 6. Programmed cell death is not increased by application of tRA.** *Cell death was assayed by Nile blue sulphate* **(A-C)** *and LysoTracker Red* **(D)** *staining of stage 25 chick embryos right forelimbs.* **(A,D)** *Dorsal views of limbs to which a RA bead has been applied to the forelimb (white arrows) demonstrates that there is no cell death detected around the bead.* **(B)** *A ventral view of the same limb in (A) (limb has been inverted for ease of analysis) where no cell death can be detected around the location of the bead (white arrow). A DMSO control bead indicated by a red arrow* **(C)** *does not result in increased cell death. Cell death associated with sculpting of the limb can be detected in (A,C,D) (black arrow).*

(Zakeri and Ahuja, 1994). In particular muscle morphogenesis in the distal limb is regulated by RA-induced apoptosis (Rodriguez-Guzman *et al*., 2006). Therefore, we performed cell death assays on the limbs of stage 25 embryos treated with beads of tRA and DMSO at stage 22. Nile blue sulphate staining reveals the characteristic pattern of apoptosis in the anterior necrotic zone and the ectoderm, sculpting the shape of the limb during limb development (Fig. 6A). A bead of tRA, used at the same concentration as used for the *in situ* analyses described above, does not induce apoptosis in the limb dorsally (Fig. 6A) or ventrally (Fig. 6B). LysoTracker Red staining after tRA treatment also reveals that administering tRA at this concentration does not result in increased apoptosis dorsally (Fig. 6D) or ventrally (not shown) compared to the control limbs.

### *Muscle cell differentiation in micromass cultures is decreased by citral and RA treatments*

The effect of excess RA or inhibition of RA on terminal myogenic differentiation was examined by treating micromass cultures of stage 22 chick forelimbs with 1.75 x $10^{-5}$ M citral,1 x $10^{-5}$ M RA and DMSO. We determined the concentration of citral by using the lowest amount that did not result in cell death. The concentration of RA used was based on that used by others to study the response of myogenic differentiation to RA (Xiao *et al*., 1995). Fresh medium and factor were added every 48 hrs and the cultures were examined on day 4. Four cultures from each treatment group were pooled for analysis and the number of myosin HC positive cells as a proportion of the total number of cells was counted following citral and RA and treatments. (Cell counts per culture: DMSO, mean = 429/668, S.D. = 71; citral, mean = 230/571, S.D. = 24; RA, mean = 245/705, S.D. = 88). Therefore, compared to controls, citral and RA treatment result in myogenic inhibition by 47% and 43% respectively.

## Discussion

RA signalling is important in the morphogenesis of many embryological systems including the hindbrain, the heart and the eye and but its role during early stages of myogenic specification and differentiation in the limb is unknown. We analysed the effect of both inhibiting RA synthesis and applying excess RA on the limb myogenic progenitors, post migration and at the onset of differentiation.

In particular we are interested in deciphering which signals that are involved in differentiation and morphogenesis of limb muscles are mediated via Meox homedomain transcription factors. Previously we have reported on the role of the *Meox* homeobox gene family in limb muscle development (Mankoo *et al*., 2003), and we have demonstrated that *Meox2* is a component of the genetic cascade that regulates skeletal myogenesis in the limb (Mankoo *et al*., 1999); and in the chick limb these genes are differentially expressed: *Meox2* is expressed in a broader domain in the dorsal and ventral limb overlapping *Pax3*, whereas *Meox1* expression is deeper within the limb and overlaps with *MyoD*, and there is a narrow region of overlap of *Meox1* and *Meox2* (Reijntjes *et al*., 2007a). In the work described here, we have studied whether *Meox* expression is responsive to RA signalling in the developing chick limb.

Several studies have investigated the effect of RA on myogenic differentiation in the limb. Most studies have focused at late stages of differentiation and morphogenesis (Robson *et al*. 1994) or have used cultures of dissociated muscle: different concentrations of RA have been reported to increase or decrease MyoD expression in chick limb buds (Momoi *et al*., 1992). Both citral and RA treatments altered development of distal limb muscle bellies, demonstrating that muscle-tendon interactions are mediated in part by apoptosis regulated by RA signalling (Rodriguez-Guzman *et al.,* 2006). Conditionally rescued RALDH2 mutant mouse embryos showed abnormal migration of limb muscle precursors in the forelimb, but expressed *MyoD* at E11.5, which could be interpreted as evidence for there being no requirement for RA for myogenic differentiation (Mic and Duester, 2004). However, in that study there was rescue by application of RA to pregnant mothers, which leads to the possibility that RA-dependent gene expression had been initiated before the RA was cleared. Moreover, the rescued limb was small and the MyoD expression occupied a much smaller domain than control limbs. Here we show that loss of RA signalling within the limb does affect differentiation of myogenic progenitors. Treatment with the RA inhibitor citral resulted in down-regulation of expression of myogenic genes in both progenitor cells (*Pax3* and *Meox2, Meox2* down-regulation was greater than that of *Pax3*) and myoblasts committed to differentiation (*Myf5* and *MyoD, MyoD* down-regulation was greater than that of *Myf5*). These results are consistent with the down-regulation in expression of neural *Pax3* and the

myogenic differentiation gene *Myogenin* in somites in the absence of RA, as reported in the vitamin A deficient quail (Wilson *et al*., 2004; Maden *et al*., 2000). Additionally, *in vivo* studies in zebrafish embryos demonstrated that RA signalling is required for onset of myogenic differentiation in somites (Hamade *et al*., 2006). The decreased expression of *MyoD* may be direct, as MyoD expressing cells respond to RA and physically interact with RXRs to form DNA binding complexes (Froeschle *et al*. 1998), or may be a consequence of down regulation of *Pax3* and *Myf5*, although *MyoD* induction in Meox2 mutant limbs occurs normally when *Pax3* and *Myf5* expression is reduced (Mankoo *et al.,* 1999). In summary, these data demonstrate that RA is required for myogenic commitment and differentiation, but the differential response of the myogenic genes indicates the requirement for RA signalling is complex.

Analysis of tRA treated forelimb buds indicates that *Meox1* expression is strongly down-regulated throughout the limb bud whereas *Meox2* expression was less affected, with down-regulation only occurring around the location of the tRA soaked bead. This supports overlapping and unique functions for each gene within their expression domains, with *Meox2* being expressed in the progenitor cells and *Meox1* in deeper layers of differentiated cells. Interestingly, *Meox1* and *Meox2* expression is also altered differentially in explanted neural crest in response to RA, *Meox1* expression is up-regulated and *Meox2* expression is down-regulated (Williams *et al*., 2004), indicating that response of *Meox* genes to RA is also context dependent, resulting in unique functions for these closely related genes.

Application of excess tRA induced localised down-regulation of *Pax3* in the chick limb in a manner similar to *Meox2*. The co-expression of *Meox2* and *Pax3* in the chick limb bud (Reijntjes *et al*., 2007a) and in migrating limb myoblasts in the mouse, and the down-regulation of *Pax3* expression in these cells in the absence of *Meox2* (Mankoo *et al*. 1999); strongly implies a role for Meox2, along with Pax3, in muscle cell specification. In a manner similar to its affect on *Meox1*, excess tRA induced strong down-regulation of *Myf5* and *MyoD* expression in the developing limb bud. The strong effect of both citral and tRA resulting in a broad down-regulation of gene expression indicates a greater sensitivity to the concentration of citral and tRA, as it is assumed there is a decreasing concentration of chemical away from the bead. Localised down-regulation, conversely, indicates that relatively high concentrations are required to produce an effect. Both citral and RA treatment resulted in a severe decrease in the formation of myosin positive myotubes. The differential sensitivity to RA shown by *Pax3* expressing progenitors and *Myf5* expressing myoblasts is noteworthy, as Pax3 is known to directly regulate *Myf5* expression in the hypaxial somite and limb muscle progenitors (Bajard *et al*., 2006); and indicates a rapid change in the RA response as cells switch from a progenitor state to differentiation. The expression of RA receptors in muscle progenitors (Froeschle *et al*., 1998) suggests the effects of RA on myogenesis we observed are direct

The negative effect of RA on *Myf5* and *MyoD* expression has been observed following RA treatment on primary muscle cultures prepared from dissociated neonatal mouse hind legs, *MyoD* transcripts were reduced and *Myf5* and *MRF4* transcripts were undetectable (Xiao *et al*., 1995). Using primary dissociated cultures of 13.5 dpc mouse limb buds and neonatal hind leg muscles treated with $10^{-5}$ M RA, these authors also demonstrated RA treatment resulted in a dramatic loss of MyHC expression. Similarly, in chick, micromass cultures of cells prepared from stage 20 forelimb buds, treated with RA resulted in fewer myogenic cells differentiating (Robson *et al*., 1994). In the work described here, we show that RA treatment resulted in a 43% decrease in MyHC positive cells in chick micromass cultures. These data strongly suggest that differentiating limb myoblasts have a greater sensitivity and therefore a different response to RA signalling than progenitors. Similarly myogenic differentiation showed a similar response in the somites of vitamin A deficient quail embryos, in which RA is absent, *Myogenin* expression was down-regulated but *Myf5* expression was not (Maden *et al.,* 2000). This is also supported by the observation that the myogenic response of rhabdomyosarcoma cells to RA is dependent on their differentiation status (Ricaud *et al*., 2005), and a relationship between differentiation state and sensitivity to RA has also been reported in ovarian cancer cells (Caliaro *et al*., 1994).

RA receptors bind their cognate binding sites via heterodimerisation with RXR proteins. We analysed the number of RXR binding sites in 200 bp of sequence upstream and 100 bp downstream of the transcription start site of each mouse homologue of the myogenic genes using Genomatix MatInspector software (Cartharius *et al*., 2005). Interestingly, the number of RXR sites shows a positive correlation with sensitivity to citral treatment (*MyoD*>*Meox2*>*Pax3*> *Meox1*>*Myf5*), however, this correlation does not extend to the response to tRA treatment; indicating that RA function at early stages of myogenesis is complex.

Loss of gene expression was not due to non-specific effects on cell death as shown by cell death analyses, ability to induce Cyp26 expression and the differential response to excess RA. Moreover, the tRA induced ectopic expression of Cyp26A1 and Cyp26B1 in the limb bud mesenchyme is novel as it had been previously thought that excess tRA could only induce Cyp26 expression in other Cyp26 expression domains, but not in regions where the Cyp26s are never expressed, for example, ectopic Cyp26A1 expression can be induced in the developing vasculature, a site of Cyp26B1 expression (Reijntjes *et al*., 2005).

The development of muscle requires closely integrated differentiation of both myogenic and muscle-associated connective tissue precursors. RALDH2, the major RA generating enzyme is expressed in the connective tissue precursors, motor neurons and vasculature in the chick limb, although not within myoblasts (Berggren *et al*. 2001). RA signalling components have been detected in the developing tendons and muscle in mouse (Abu-Abed *et al*., 2002) and chick limbs (Rodriguez-Guzman *et al.,* 2006) demonstrating RA plays a role at later stages of tendon development. RA has been implicated in regulating morphogenesis of distal muscle and tendons by an apoptosis-mediated mechanism (Rodriguez-Guzman *et al.,* 2006). However, the role of RA on tendon progenitors is unknown. We observed down-regulation of expression of the tendon-specific gene *Scleraxis* in response to both excess RA and inhibition of RA signalling. Significantly, this loss of *Scleraxis* expression is independent of the effects of RA on muscle as myogenic and tendon progenitors initially develop independently (Kardon, 1997), suggesting a mechanism that coordinates the differentiation of these tissues.

To conclude, we have shown that RA signalling is essential for

maintenance of premyogenic cells and myogenic commitment within the limb bud. Excess RA signalling inhibits myogenic differentiation suggesting that a gradient of RA activity controls the rate of myogenic commitment. We show that RA signalling is essential for the early stages of myogenesis and tendon development, RA could also modulate the rate of myogenic differentiation by interactions with tendons and the vascular system at later stages of morphogenesis. We have shown that both inhibition and excess RA can negatively effect myogenic differentiation emphasizing the importance of an exquisitely controlled local concentration of RA. Moreover we have shown the response to RA is dependent on the state of differentiation of limb muscle precursors.

## Materials and Methods

### *Embryos*

Fertilised hens eggs (mixed flock, Henry Stewart and Co. Ltd., Louth, Lincolnshire) were incubated in a humidified atmosphere at 37°C. Embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951).

### *Experimental manipulation of the forelimb*

All-*trans*-RA (Sigma) was dissolved in dimethyl sulfoxide (DMSO), (Sigma-Aldrich, USA) to a concentration of 1.33 x 10-2M and incubated in analytical grade anion exchange resin beads AG1-X2, 100-200 Mesh Formate Form (Bio-RA). To visualise the beads 1ml of Dulbecco's MOD eagle Medium (Gibco) was added. SM2 beads (BioRad) were incubated in 2.9 x 10-3M Citral (Fluka) in M16 medium (Sigma). Eggs were fenestrated at 3.5 days after incubation and beads were implanted in the developing right forelimb of stage 22 embryos. The embryos were then incubated for 24 h at 37°C and then samples were processed for *in situ hybridisation*, or RNA extraction or immunostaining. The all-*trans*-RA experiments were controlled by parallel experiments implanting DMSO soaked beads.

### *Whole-mount* in situ *hybridization and sectioning*

Digoxigenin (Roche) labelled antisense RNA probes for *Meox1*, *Meox2* and *Scleraxis* were prepared as described (Reijntjes *et al*., 2007). *Pax3, MyoD* and *Myf5* antisense-probes were prepared by Philippa Francis-West and *Cyp26A1* and *Cyp26B1* probes were prepared as previously described (Reijntjes *et al*., 2005). Whole-mount *in situ* hybridisation was carried out using standard procedures, prehybridisation and hybridisation was carried out at 70°C with a probe concentration of 1 μg/ml and visualised with digoxigenin antibodies conjugated to alkaline phosphatase reacted with NBT/BCIP purple (Roche). Embryos were embedded in 3.6ml vibratome embedding mix (gelatine type A, egg albumin, Sigma, sucrose, BDH), incubated for 2 h at room temperature and then 400 μl glutaraldehyde (Sigma) was added). Sections were cut at 80 μm on a vibratome (Leica VT 1000S), placed on glass microscope slides and mounted in glycerol.

### *Semiquantitative reverse transcription-PCR analysis*

RNA was isolated from all-trans-RA, DMSO and Citral treated and untreated forelimbs by the guanidinium thiocyanate method of Chomczynski and Sacchi (1987). For all semiquantitative RT-PCR reactions, single-stranded cDNA was synthesised from 1 μg of forelimb RNA. The cDNA synthesis was achieved by using cloned AMV 1st strand cDNA synthesis kit (Invitrogen). One microgram of total RNA and 1 μl Oligo(dT)20 primer was heated for 5 min at 65°C and then reverse transcribed at 50°C for 1 h using 4 μl 5x cDNA synthesis buffer, 1 μl DTT (100 nM), 1 μl RNaseOUT (40 U/μl) and 1μl cloned AMV reverse transcriptase (15 units/μl) in a 20 μl reaction. The PCR reaction was carried out using 2 μl of the retrotranscription reaction in a final volume of 50 μl, containing 10x PCR buffer, 200 μM each dNTPs, 0.2 μM each primer and 1.25 units of Taq DNA polymerase (New England Biolabs).

Actin and Cyp26B1 primers were designed as previously described (Reijntjes *et al*., 2005). For Meox1 primers were designed using GENBANK accession no. NM_204765 to generate a 403-bp product, for Meox2 primers were designed from NM_001005427 to generate a 368-bp product. The forward and reverse primers were, respectively:

Meox1: 5'-TGAATTCTCAGCCTCCTGCT-3' and
5'-TCTCCTGAGCCTTGTCAGGT-3';
Meox2: 5'-TCCCTCAGATGTCGTCTCCT-3' and
5'-ATCTCCTCAGCCTGGTCAAA-3'.

The actin cDNA was co-amplified in each experiment to verify equal amounts of cDNA in the samples. The amplification reaction was initiated by incubation of PCR samples at 94°C for 2 min followed by the cyclic program, 94°C for 30 s, 58°C for 30 s and 72°C for 1 min for 35 cycles for *Cyp26B1* amplification, 94°C for 30 s, 50°C for 30 s and 72°C for 1 min for 38 cycles for *Meox1* and 94°C for 30 s, 55°C for 30 s and 72°C for 1 min for 32 cycles for *Meox2*. For each primer set, an increasing number of PCR cycles were performed with otherwise fixed conditions to determine the optimal number of cycles to be used. PCR reactions were performed twice with two separate cDNA pools of treated and untreated forelimb buds and each gene product was verified by sequencing.

### *Cell proliferation and cell death*

Paraffin-wax-embedded tissue sections were processed and mounted on glass slides. To label proliferating cells a rabbit polyclonal antibody to the phosphorylated serine 10 residue of histone H3 (anti-phospho-H3) was used (BD Pharmingen) as a marker of cells entering mitosis (Clayton *et al*., 2000). The antibody was used at a concentration of 1:8000 followed by a Vector ABC Elite kit (Vector Laboratories) to visualise immunoreactivity.

Nile blue sulphate (NBS) staining is a vital stain that is sequestered into apoptotic bodies and phagolysosomes found in cells neighbouring apoptotic cells (Allen *et al*., 1997). All-trans-RA and DMSO treated stage 25 embryos were dissected from extraembryonic membranes and transferred to 1:50,000 NBS in lactated Ringer's solution (Kotch *et al*., 1992) and incubated at 37°C for 30 min. After staining embryos were rinsed in cold Ringer's solution and photographed. LysoTracker Red (LTR) staining. LTR (Molecular Probes) concentrates in membrane bound, acidic compartments of cells indicating regions of high phagocytosis and lysosomal activity correlating to apoptosis activity (Zucker *et al*., 1998, 1999). Embryos were dissected in Hanks and transferred to 24-well plates containing 1:100 LTR in fresh Hanks warmed to 37°C. This was removed and replaced with 1:3 LTR in Hanks, plates were covered and incubated at 37°C for 30 min. After staining embryos were rinsed 4x5 min with Hanks and fixed overnight in 4% paraformaldehyde (Sigma) at 4°C. Embryos were rinsed in PBS, dehydrated in 100% MeOH and viewed under fluorescence in MeOH.

### *Micromass culture*

The ectoderm of stage 22 chick forelimbs was removed following treatment with cold trypsin. The mesenchyme was then disaggregated and the cells were suspended in modified F-12 tissue culture medium (Gibco advanced DMEM/F12 + 2 mM L-glutamine + 100 units/ml penicillin, 100 μg/ml streptomycin + 10% foetal calf serum (Gibco). The cells were plated out in 10 μl drops at a final density of 2 $x10^5$ cells per 10 μl. The cultures were then incubated at 37°C with 5% $CO_2$ for 1 hour and then flooded with modified F-12 medium. RA was added to the medium resulting in a final concentration of $10^{-5}$M, DMSO was added at equivalent amounts to RA (1 μl/ml) and citral added in a final concentration of 1.75 x $10^{-5}$ M. Medium, plus reagents, was replaced every 48 hrs. On day 4 after plating, cells were fixed for immunohistochemical staining.

### *Immunohistochemistry*

Cells were washed three times with PBS and then fixed on the plates

with 4% PFA for 10 min. After two washes with PBS, non-specific proteins were blocked by incubation with PBS/5% horse serum for 5 mins. Pan myosin MyHC antibody (a gift from Simon Hughes) was diluted with PBS/2% horse serum and added onto the plates and incubated overnight at 4°C and then washed 3 times with PBS. Subsequent incubation was performed at 4°C overnight with peroxidase labelled anti-mouse IgG (H+L) secondary antibody (Vector laboratories), diluted 1 in 500 in PBS/2% horse serum. Substrate solution made from DAB tablets (Sigma) following manufacturer's instructions was used to stain the plates for 5 min. Staining was recorded with microscopic photography and cell counts performed for each treatment (four separate micromass culture samples each).

*Acknowledgements*

*This work was supported by the BBSRC and MYORES FP6 Network of Excellence. We thank Professor Malcolm Maden for technical assistance and reagents; and also the helpful comments of anonymous reviewers.*

## References

ABU-ABED, S.S., BECKETT, B.R., CHIBA, H., CHITHALEN, J.V., JONES, G., METZGER, D., CHAMBON, P.and PETKOVICH, M. (1998). Mouse P450RAI (CYP26) expression and retinoic acid-inducible retinoic acid metabolism in F9 cells are regulated by retinoic acid receptor gamma and retinoid X receptor alpha. *J Biol Chem* 273: 2409-2145.

ABU-ABED, S.S., MACLEAN, G., FRAULOB, V., CHAMBON, P., PETKOVICH, M. and DOLLE, P. (2002). Differential expression of the retinoic acid-metabolizing enzymes CYP26A1 and CYP26B1 during murine organogenesis. *Mech Dev* 110: 173-177.

ALLEN, R.T., HUNTER, W.J. and AGRAWAL, D.K. (1997). Morphological and biochemical characterization and analysis of apoptosis. *J Pharmacol Toxicol Meth* 37: 215-228.

ARNOLD, H.H., GERHAZ, C.D., GABBERT, H.E. and SAMINEN, A. (1992). Retinoic acid induces myogenin synthesis and myogenic differentiationin the rat rhabdomyosarcoma cell line BA-Han-1C. *J Cell Biol* 118: 877-887.

ALBAGLI-CURIEL, O., CARNAC, G., VANDROMME, M., VINCENT, S., CREPIEUX, P. and BONNIEU, A. (1993). Serum-induced inhibition of myogenesis is differentially relieved by retinoic acid and triodothyronine in C2 murine muscle cells. *Differentiation* 52: 201-210.

BAJARD, L., RELAIX, F., LAGHA, M., ROCANCOURT, D., DAUBAS, P. and BUCKINGHAM, M.E. (2006). A novel genetic hierarchy functions during hypaxial myogenesis: Pax3 directly activates Myf5 in muscle progenitor cells in the limb. *Genes Dev* 20: 2450-2564.

BERGGREN, K., EZERMAN, E.B., MCCAFFERY, P. and FOREHAND, C.J. (2001). Expression and regulation of the retinoic acid synthetic enzyme RALDH-2 in the embryonic chicken wing. *Dev Dyn* 222: 1-16.

BOBER, E., FRANZ, T., ARNOLD, H.H., GRUSS, P. and TREMBLAY, P. (1994). Pax-3 is required for the development of limb muscles: a possible role for the migration of dermomyotomal muscle progenitor cells. *Development* 120: 603-612.

CALIARO, M.J., MARMOUGET, C., GUICHARD, S., MAZARS, P., VALETTE, A., MOISAND, A., BUGAT, R. and JOZAN, S. (1994). Response of four human ovarian carcinoma cell lines to all-trans retinoic acid: relationship with induction of differentiation and retinoic acid receptor expression. *Int J Cancer* 56: 743–748.

CARTHARIUS, K., FRECH, K., GROTE, K., KLOCKE, B., HALTMEIER, M., KLINGENHOFF, A., FRISCH, M., BAYERLEIN, M. and WERNER, T. (2005). MatInspector and beyond: promoter analysis based on transcription factor binding sites. *Bioinformatics* 21: 2933-2942.

CHEVALLIER, A., KIENY, M. and MAUGER A. (1977). Limb-somite relationship: origin of the limb musculature. *J Embryol Exp Morph* 41: 245-258.

CHRIST, B., JACOB, H.J. and JACOB, M. (1977). Experimental findings on muscle development in the limbs of the chick embryo. *Verh Anat Ges* 71: 1231-1237.

CHOMCZYNSKI, P. and SACCHI, N. (1987). Single-step of RNA isolation by acid guanidinium thiocynate-phenol-chloroform extraction. *Anal Biochem* 162: 156-159.

CONLAN, R.A. and ROSSANT, J. (1992). Exogenous retinoic acid rapidly induces anterior ectopic expression of murine Hox-2 genes *in vivo*. *Development* 116: 357-368.

DURSTON, A.J. TIMMERMANS, J.P., HAGE, W.J., HENDRIKS, H.F., DE VRIES, N.J., HEIDEVELD, M. and NIEUWKOOP, P.D. (1989). Retinoic acid causes an anteroposterior transformation in the developing central nervous system. *Nature* 340: 140-144.

FROESCHLÉ, A., ALRIC, S., KITZMANN, M., CARNAC, G., AURADÉ, F., ROCHETTE-EGLY, C. and BONNIEU, A. (1998). Retinoic acid receptors and muscle b-HLH proteins: partners in retinoid-induced myogenesis. *Oncogene* 16: 3369-3378.

FUJII, H., SATO, T., KANEKO, S., GOTOH, O., FUJII-KURIYAMA, Y., OSAWA, K., KATO, S. and HAMADA, H. (1997). Metabolic inactivation of retinoic acid by a novel P450 differentially expressed in developing mouse embryos. *EMBO J* 16: 4163-4173.

GALE, E., ZILE, M. and MADEN, M. (1999). Hindbrain respecification in the retinoid-deficient quail. *Mech Dev* 89: 43-54.

GOULDING, M., LUMSDEN, A. and PAQUETTE, A.J. (1994). Regulation of Pax-3 in the dermomyotome and its role in muscle development. 1994. *Development* 120: 957-971.

GROSS, M.K., MORAN-RIVARD, L., VELASQUEZ, T., NAKATSU, M.N., JAGLA, K. and GOULDING, M. (2000). Lbx1 is required for muscle precursor migration along a lateral pathway into the limb. *Development* 127: 413-424.

HALEVY, O. and LERMAN O. (1993). Retinoic acid induces adult muscle cell differentiation mediated by the retinoic acid receptor-alpha. *J Cell Physiol* 154: 566-572.

HAMADE, A., DERIES, M., BEGEMANN, G., BALLY-CUIF, L., GENÊT, C., SABATIER, F., BONNIEU, A., and COUSIN, X. (2006). Retinoic acid activates myogenesis *in vivo* through Ffg8 signalling. *Dev Biol* 289: 127-140.

HAMBURGER, V. and HAMILTON, H.L. (1951). A series of normal stage sin the development of the chick embryo. *J Morphol* 88: 49-92.

HAYASHI, K. and OZAWA, E. (1995). Myogenic cell migration from somites is induced by tissue contact with medial region of the presumptive limb mesoderm in chick embryos. *Development* 121: 661-669.

HILL, J., CLARKE, J.D., VARGESSON, N., JOWETT, T. and HOLDER, N. (1995). Exogenous retinoic acid causes specific alterations in the development of the midbrain and hindbrain of the zebrafish embryo including positional respecification of the Mauthner neuron. *Mech Dev* 50: 3-16.

JACOB, M., CHRIST, B. and JACOB, H.J. (1978). On the migration of the myogenic stem cells into the prospective wing region of chick embryos. A scanning and transmission electron microscope study. *Anat. Embryol. (Berl.)* 153: 179-193.

KARDON, G. (1998). Muscle and tendon morphogenesis in the avian hind limb. *Development* 125: 4019-4032.

KOTCH, L.E., DEHART, D.B. and ALLES, A.J., (1992). Pathogenesis of ethanol-induced limb reduction defects in mice. *Teratology* 46: 323-332.

LANGILLE, R.M., PAULSEN, D.F. and SOLURSH, M. (1989). Differential effects of physiological concentrations of retinoic acid *in vitro* on chondrogenesis and myogenesis in chick craniofacial mesenchyme. *Differentiation* 40: 84-92.

MADEN, M., GRAHAM, A., ZILE, M. and GALE, E. (2000). Abnormalities of somite development in the absence of retinoic acid. *Int J Dev Biol* 44: 151-159.

MADEN, M. (2001). Retinoid signalling in the development of the central nervous system. *Nature Rev Neurosci* 3: 843- 853.

MANKOO, B.S., COLLINS, N.S., ASHBY, P., GRIGORIEVA, E., PEVNY, L.H., CANDIA, A., WRIGHT, C.V., RIGBY, P.W. and PACHNIS, V. (1999). Mox2 is a component of the genetic hierarchy controlling limb muscle development. *Nature* 400: 49-73.

MANKOO, B.S., SKUNTZ, S., HARRIGAN, I., GRIGORIEVA, E., CANDIA, A., WRIGHT, C.V., ARNHEITER, H., P.W. and PACHNIS, V. (2003). The concerted action of Meox homeobox genes is required upstream of genetic pathways essential for the formation, patterning and differentiation of somites. *Development* 130: 4655-4664.

MARSHALL, H., NONCHEV, S., SHAM, M.H., MUCHAMORE, I., LUMSDEN, A. and KRUMLAUF, R. (1992). Retinoic acid alters hindbrain Hox code and

induces transformation of rhombomeres 2/3 into a 4/5 identity. *Nature* 360: 737-741.

MIC, F.A. and DUESTER, G. (2003). Patterning of forelimb bud myogenic precursor cells requires retinoic acid signaling initiated by Raldh2. *Dev Biol* 264: 191-201.

MOMOI, T., MIYAGAWA-TOMITA, S., NAKAMURA, S., KIMURA, I. and MOMOI, M. (1992). Retinoic acid ambivalently regulates the expression of MYOD1 in the myogenic cells in the limb buds of the early developmental stages. *Biochem and Biophy Res Comm* 187: 245-253.

MORRISS-KAY, G.M., MURPHY, P., HILL, R.E. and DAVIDSON, D.R. (1991). Effects of retinoic acid excess on expression of Hox-2.9 and Krox-20 and on morphological segmentation in the hindbrain of mouse embryos. *EMBO J* 10: 2985-2995.

PEASE, C.N. (1962). Focal retardation and arrestment of growth of bones due to vitamin A intoxication. *J Am Med Assoc* 182: 980-985.

PERZ-EDWARDS, A., HARDISON, N.L and LINNEY E. (2001). Retinoic acid-mediated gene expression in transgenic reporter zebrafish. *Dev Biol* 229: 89-101.

RAY, W.J., BAIN, G., YAO, M. and GOTTLIEB, D.I. (1997). CYP26, a novel mammalian cytochrome P450, is induced by retinoic acid and defines a new family. *J Biol Chem* 272:18702-18708.

REIJNTJES, S., GALE, E. and MADEN, M. (2003). Expression of the retinoic acid catabolising enzyme CYP26B1 in the chick embryo and its regulation by retinoic acid. *Gene Expr Patterns* 3: 621-627.

REIJNTJES, S., BLENTIC, A., GALE, E. and MADEN, M. (2005). 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., STRICKER, S. and MANKOO, B.S. (2007a). A comparative analysis of Meox1 and Meox2 in the developing somites and limbs of the chick embryo. *Int J Dev Biol* 51: 753-759.

REIJNTJES, S., RODAWAY, A. and MADEN, M. (2007b). The retinoic acid metabolising gene, CYP26B1, patterns the cartilaginous cranial neural crest in zebrafish. *Int J Dev Biol* 51: 351-360.

ROBSON, L.G., KARA, T., CRAWLEY, A. and TICKLE, C. (1994). Tissue and cellular patterning of the musculature in chick wings. *Development* 120: 1265-1276.

RODRIGUEZ-GUZMAN, M., MONTERO, J.A., SANTESTEBAN, E., GAÒAN, Y. MACIAS, D. and HURLE, J.M. (2006). Tendon-muscle crosstalk controls muscle bellies morphogenesis, which is mediated by cell death and retinoic acid signalling. *Dev Biol* 302: 267-280.

SCHWEITZER, R., CHYUNG, J., MURTAUGH, L.C., BRENT, A.E., ROSEN, V., OLSON, E., LASSAR, A. and TABIN, C.J. (2001). Analysis of the tendon cell fate using Scleraxis, a specific marker for tendons and ligaments. *Development* 128: 3855-3866.

SOLURSH, M., DRAKE, C. and MEIER, S. (1987). The migration of myogenic cells from the somites at the wing level in avian embryos. *Dev Biol* 121: 389-396.

STANDEVEN, A.M., DAVIES, P.J.A., CHANDRARATNA, R.A.S., MADER, D.R., JOHNSON, A.T. and THOMAZY, V.A. (1996). Retinoid-induced epiphyseal closure in guinea pigs. *Fundam Appl Toxicol* 34: 91-98.

SUNDIN, O. and EICHELE, G. (1992). An early marker of axial pattern in the chick embryo and its respecification by retinoic acid. *Development* 114: 841-852.

SWINDELL, E.C., THALLER, C., SOCKANATHAN, S., PETKOVICH, M., JESSELL, T.M. and EICHELE, G. (1999). Complementary domains of retinoic acid production and degradation in the early chick embryo. *Dev Biol* 216: 282-296.

TANAKA, M., TAMURA, K. and IDE, H. (1996). Citral, an inhibitor of retinoic acid synthesis, modifies chick limb development. *Dev Biol* 175: 239-47.

THALLER, C. and EICHELE, G. (1987). Identification and spatial distribution of retinoids in the developing chick limb bud. *Nature* 327: 625-628.

TICKLE, C., ALBERTS, B., WOLPERT, L. and LEE, J. (1982). Local application of retinoic acid to the limb bud mimics the action of the polarizing region. *Nature* 296: 564-566.

VASYUTINA, E., STEBLER, J., BRAND-SABERI, B., SCHUIZ, S., RAZ, E. and BIRCHMEIER, C. (2005). CXCR4 and Gab1 cooperate to control the development of migrating muscle progenitor cells. *Genes Dev* 19: 2187-98.

WHITE, J.A., GUO, Y.D., BAETZ, K., BECKETT-JONES, B., BONASORO, J., HSU, K.E., DILWORTH, F.J., GONES, G. and PETKOVICH, M. (1996). Identification of the retinoic acid-inducible all-trans-retinoic acid 4-hydroxylase. *J Biol Chem* 271: 29922-29927.

WHITE, J.A., BECKETT-JONES, B., GUO, Y.D., DILWORTH, F.J., BONASORO, J., JONES, G. and PETKOVICH, M. (1997). cDNA cloning of human retinoic acid-metabolizing enzyme (hP450RAI) identifies a novel family cytochromes P450. *J Biol Chem* 272: 18538-18541.

WHITE, J.A., RAMSHAW, H., TAIMI, M., STANGLE, W., ZHANG, A., EVERINGHAM, S., CREIGHTON, S., TAM, S.P., JONES, G. and PETKOVICH, M. (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 USA* 97: 6403-6408.

WHITE, J.C. HIGHLAND, M., KAISER, M. and CLAGETT-DAME, M. (2000). Vitamin A deficiency results in the dose-dependent acquisition of anterior character and shortening of the caudal hindbrain of the rat embryo. *Dev Biol* 220: 263-284.

WILLIAMS, B.A. and ORDAHL, C.P. (1994). Pax3 expression in the segmental mesoderm marks the early stages in myogenic cell specification. *Development* 120: 785-796.

WILLIAMS, S.S., MEAR, J.P., LIANG, H., POTTER, S.S. ARONOW, B.J. and COLBERT, M.C. (2004). Large-scale reprogramming of neural crest gene expression by retinoic acid exposure. *Physiol Genomics* 19: 184-197.

WILSON, J.G., ROTH, C.B. and WARKANY, J. (1953). An analysis of the syndrome of malformations induced by maternal vitamin A deficiency. Effects of restoration of vitamin A at various times during gestation. *Am J Anat* 92: 189-217.

WILSON, L., GALE, E., CHAMBERS, D. and MADEN, M. (2004). Retinoic acid and the control of dorsoventral patterning in the avian spinal cord. *Dev Biol* 269: 433-446.

WOOD, H., PALL, G. and MORRISS-KAY, G. (1994). Exposure to retinoic acid before or after the onset of somitogenesis reveals separate effects on rhombomeric segmentation and HoxB gene expression domains. *Development* 120: 2279-2285.

WOLBACH, S.B. (1947). Vitamin-A deficiency and excess in relation to skeletal growth. *J Bone Joint Surg* 29: 171-192.

XIAO, Y., GRIESHAMMER, U. and ROSENTHAL, N. (1995). Regulation of a muscle-specific transgene by retinoic acid. *J Cell Biol* 129: 1345-1354.

YANG, X.M., VOGAN, K., GROS, P. and PARK, M. (1996). Expression of the met receptor tyrosine kinase in muscle progenitor cells in somites and limbs is absent in Splotch mice. *Development* 122: 2163-2171.

ZAKERI, Z. and AHUJA, H.S. (1994). Apoptotic cell death in the limb and its relationship to pattern formation. *Biochem Cell Biol* 72: 603-613.

ZUCKER, R.M., HUNTER, S. and ROGERS, J. (1998). Confocal laser scanning microscopy of apoptosis in organogenesis stage mouse embryos. *Cytometry* 33: 348-354.

ZUCKER, R.M., HUNTER, S. and ROGERS, J. (1999). Practical confocal microscopy and the evaluation of system performance. *Methods* 18: 447-458.

## Further Related Reading, published previously in the *Int. J. Dev. Biol.*

See our recent Special Issue ***Fertilization***, in honor of David L. Garbers and edited by Paul M. Wassarman and Victor D. Vacquier at:
http://www.ijdb.ehu.es/web/contents.php?vol=52&issue=5-6

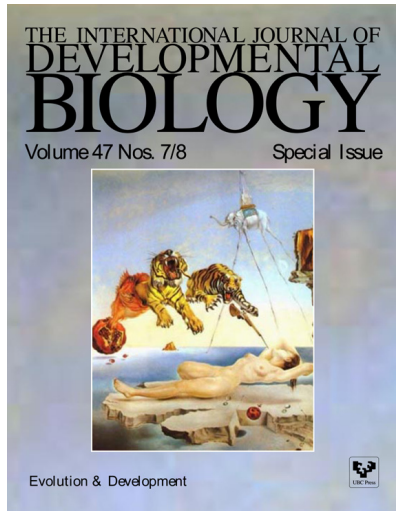
**Comparative expression analysis of Pax3 and Pax7 during mouse myogenesis**
David Horst, Svetlana Ustanina, Consolato Sergi, Gregor Mikuz, Herbert Juergens, Thomas Braun and Eugene Vorobyov
Int. J. Dev. Biol. (2006) 50: 47-54

**Changes in the proportion and number of Pax(7+ve) and MF20(+ve) myoblasts during chick myogenesis in the head and limb**
Antonio S J Lee, Ming Zhang and Darrell J R Evans
Int. J. Dev. Biol. (2004) 48: 31-38

**Myogenic potential of mouse primordial germ cells**
Francesca-Gioia Klinger, Maria-Lucia Scaldaferri, Anna Di Carlo, Marta Baiocchi, Marcello Coletta, Giulio Cossu and Massimo De Felici
Int. J. Dev. Biol. (2003) 47: 303-305

**Limb muscle development**
Bodo Christ and Beate Brand-Saberi
Int. J. Dev. Biol. (2002) 46: 905-914

**Determination, diversification and multipotency of mammalian myogenic cells**
G Cossu, L De Angelis, U Borello, B Berarducci, V Buffa, C Sonnino, M Coletta, E Vivarelli, M Bouche, L Lattanzi, D Tosoni, S Di Donna, L Berghella, G Salvatori, P Murphy,M G Cusella-De Angelis and M Molinaro
Int. J. Dev. Biol. (2000) 44: 699-706




**5 yr ISI Impact Factor (2008) = 3.271**