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## A conserved role for retinoid signaling in vertebrate pancreas development

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**Abstract** Retinoic acid (RA) signaling plays critical roles in the regionalization of the central nervous system and mesoderm of all vertebrates that have been examined. However, to date, a role for RA in pancreas and liver development has only been demonstrated for the teleost zebrafish. Here, we demonstrate that RA signaling is required for development of the pancreas but not the liver in the amphibian *Xenopus laevis* and the avian quail. We disrupted RA signaling in *Xenopus* tadpoles, using both a pharmacological and a dominant-negative strategy. RA-deficient quail embryos were obtained from hens with a dietary deficiency in vitamin A. In both species we found that pancreas development was dependent on RA signaling. Furthermore, treatment of *Xenopus* tadpoles with exogenous RA led to an expansion of the pancreatic field.

By contrast, liver development was not perturbed by manipulation of RA signaling. Taken together with our previous finding that RA signaling is necessary and sufficient for zebrafish pancreas development, these data support the hypothesis that a critical role for RA signaling in pancreas development is a conserved feature of the vertebrates.

**Keywords** Pancreas · Liver · *Xenopus* · Quail · Retinoic acid

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### Introduction

Retinoic acid (RA) signaling is essential for normal development of vertebrate embryos. RA is a small, diffusible signaling molecule derived from vitamin A, which is oxidized to retinal, and then to RA through the action of RALDH enzymes. RA signals through retinoic acid receptors (RARs), which interact directly with RA response elements (RAREs) in the regulatory elements of target genes. In the absence of RA ligands, RARs interact with co-repressors; these are replaced by co-activators upon RA binding, leading to transcriptional activation (Germain et al. 2002). Experiments in a variety of vertebrate models demonstrate that endogenous RA plays a vital role during anteroposterior (AP) patterning of the developing embryo. For example, mice and zebrafish mutant for the *RALDH2* gene have disrupted posterior hindbrains and do not form anterior appendages (Begemann et al. 2001; Grandel et al. 2002; Niederreither et al. 2002; Niederreither et al. 2000). Similarly, vitamin A-deficient (VAD) quail embryos, which are unable to synthesize normal concentrations of RA, have multiple defects including loss of the posterior hindbrain and alterations to pharyngeal endoderm (Gale et al. 1999; Maden et al. 1996; Quinlan et al. 2002).

We have recently demonstrated a requirement for RA signaling in specifying pancreas and liver in the zebrafish, *Danio rerio* (Stafford and Prince 2002). Using a zebrafish mutant that prevents RA synthesis, the *neckless* mutant,

and an antagonist of the RA receptors, we established that there is a critical RA-dependent step in pancreas and liver development. By contrast, RA signaling is not required for formation of the endoderm germ layer or for differentiation of other endodermal organs. We also found that exogenous RA has the capacity to transfect anterior endoderm to a pancreatic fate. Furthermore, timed antagonist treatments showed that the RA-dependent step in pancreas specification occurs at the end of zebrafish gastrulation, well before the onset of expression of any known pancreatic development gene.

The zebrafish pancreas fulfills similar exocrine and endocrine functions to the pancreas of tetrapod vertebrates. Many aspects of pancreatic development have been conserved during vertebrate evolution. For example, the developing zebrafish pancreatic primordium expresses many of the same developmental control genes as the chick or mouse pancreas. These include the earliest molecular marker for the pancreas, *pdx1* (Argenton et al. 1999; Milewski et al. 1998), and the endocrine cell markers *islet1* (Ericson et al. 1992) and *pax6* (Biemar et al. 2001). However, one apparent difference between amniote and zebrafish pancreas development has been reported. While Sonic hedgehog (Shh) signaling can activate insulin expression in rat pancreatic cell lines (Thomas et al. 2000), embryological experiments in chick and mouse indicate that absence of Shh expression from the pancreatic primordium is necessary for pancreas development; endodermal Shh signaling negatively regulates pancreas specification and induces intestinal smooth muscle in adjacent mesoderm (Hebrok et al. 1998; Apelqvist et al. 1997). By contrast, experiments in the zebrafish, using both genetic and pharmacological approaches, indicate that Shh signaling is required at embryonic stages for normal zebrafish pancreas development (Roy et al. 2001). This difference suggests that some aspects of pancreas development have diverged during the 400 million years that separate tetrapods (such as chick and mouse) and teleosts (zebrafish).

Here, we have tested whether tetrapod vertebrates share with the teleost zebrafish a requirement for RA signaling in pancreas development. We have investigated the role of RA in pancreas specification of two tetrapod models: the amphibian *Xenopus laevis* and the amniote quail, *Coturnix coturnix japonica*. Our data show that in all three vertebrates examined, RA signaling is required for pancreas development. Furthermore, we find that treatment of *Xenopus* embryos with exogenous RA expands the pancreatic field. In contrast to our previous findings in zebrafish, we find that in RA-signaling-deficient *Xenopus* the ventral pancreatic buds are present, although differentiated pancreas cells do not develop. Moreover, while RA signaling is required for zebrafish liver development, we show that the liver does develop in RA-deficient tetrapods. We conclude that a role for RA in pancreas development is conserved among vertebrates, while mechanisms of liver development have diverged.

## Materials and methods

### Pharmacological treatments of *Xenopus* embryos

Embryos were treated with RA, BMS493, or respective carrier solutions as described (Stafford and Prince 2002), except that working dilutions were made in  $0.1\times$  MBS (Sive et al. 2000). The working concentration of BMS493 was  $5\times 10^{-6}$  M, and embryos were treated from stage 10 onwards. Embryos were pulse-treated with  $10^{-5}$  or  $10^{-6}$  M RA for 1 h, starting at stage 12.

### Microinjection of mRNA into *Xenopus* embryos

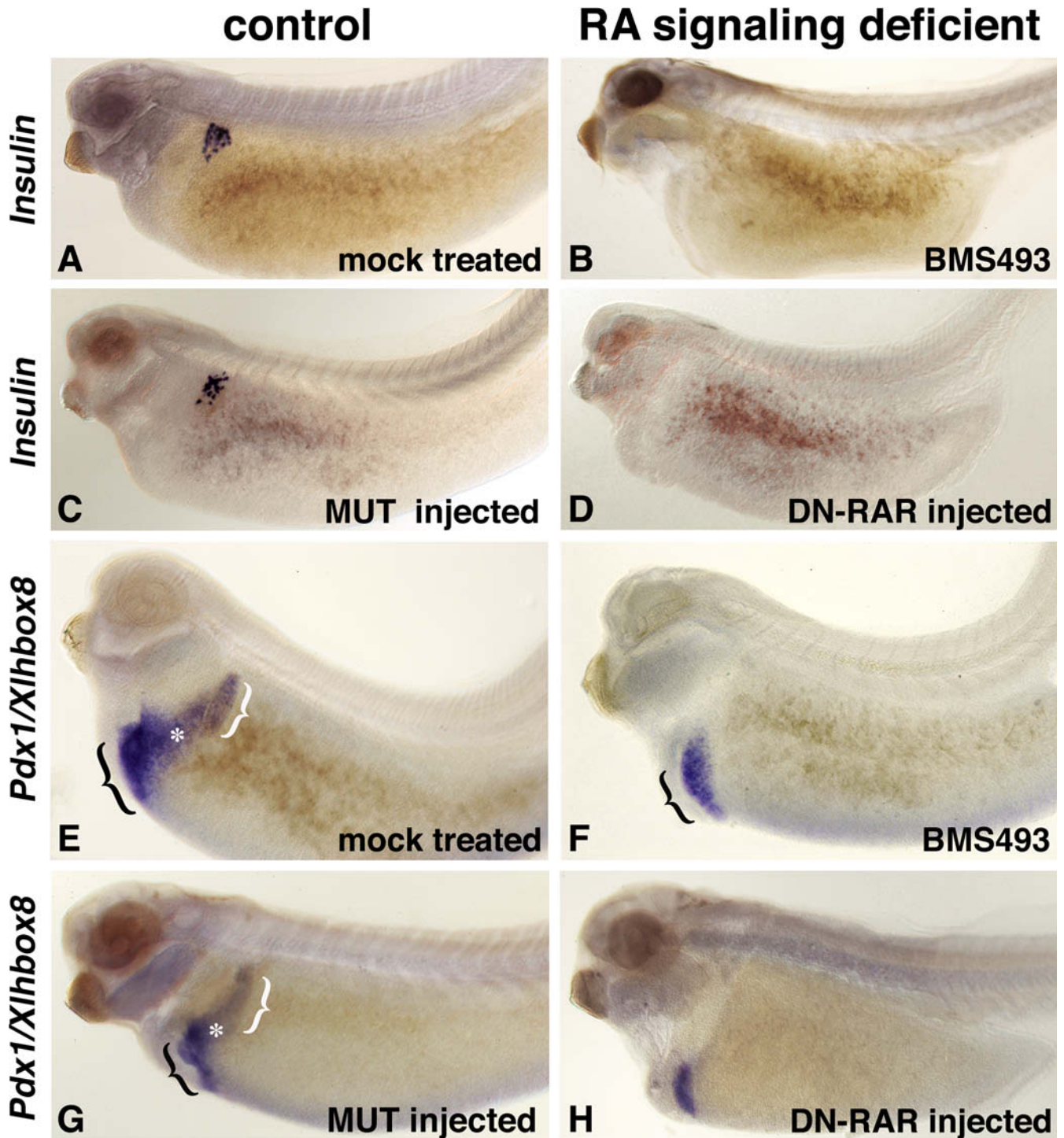
Microinjection of two-cell *Xenopus* blastomeres was performed as described (Leise and Mueller 2002). The 5' methyl-capped mRNA was prepared by in vitro transcription of linearized DN-RAR $\alpha$ 2 (RAR $\alpha$  $\Delta$  393), or DN-RAR $\alpha$ 2-MUT (RAR $\alpha$  $\Delta$  h\*d), as described (Sharpe and Goldstone, 1997). DN-RAR $\alpha$ 2 is a dominant-negative RAR with a C-terminal truncation that allows it to bind to RA response elements but not to transduce the RA signal. DN-RAR $\alpha$ 2-MUT bears a mutation that prevents binding to RA response elements and is thus non-functional.

### In situ hybridization of *Xenopus* embryos

Embryos were processed for whole-mount in situ hybridization as described (Leise and Mueller 2002) with the following modifications. Following rehydration, embryos were bleached in 1% H<sub>2</sub>O<sub>2</sub>, 0.5% formamide, 0.5% SSC, under white light until pigmentation faded (approximately 60 min), washed  $3\times 5$  min in TBS, 0.1% Tween 20 (TTw), treated with proteinase-K (1%) in TTW for 5 min, then immediately post-fixed in MEMPFA (Sive et al. 2000), 0.1% TTW, 0.2% glutaraldehyde for 60 min. Following the color reaction, embryos were dehydrated in a methanol series and then transferred to 2:1 benzyl benzoate:benzyl alcohol. Antisense DIG-labeled riboprobes were generated using the appropriate RNA polymerase from linearized plasmids. The *Insulin* construct was kindly provided by Maya Kumar, all other *Xenopus* probes are referenced in "Results."

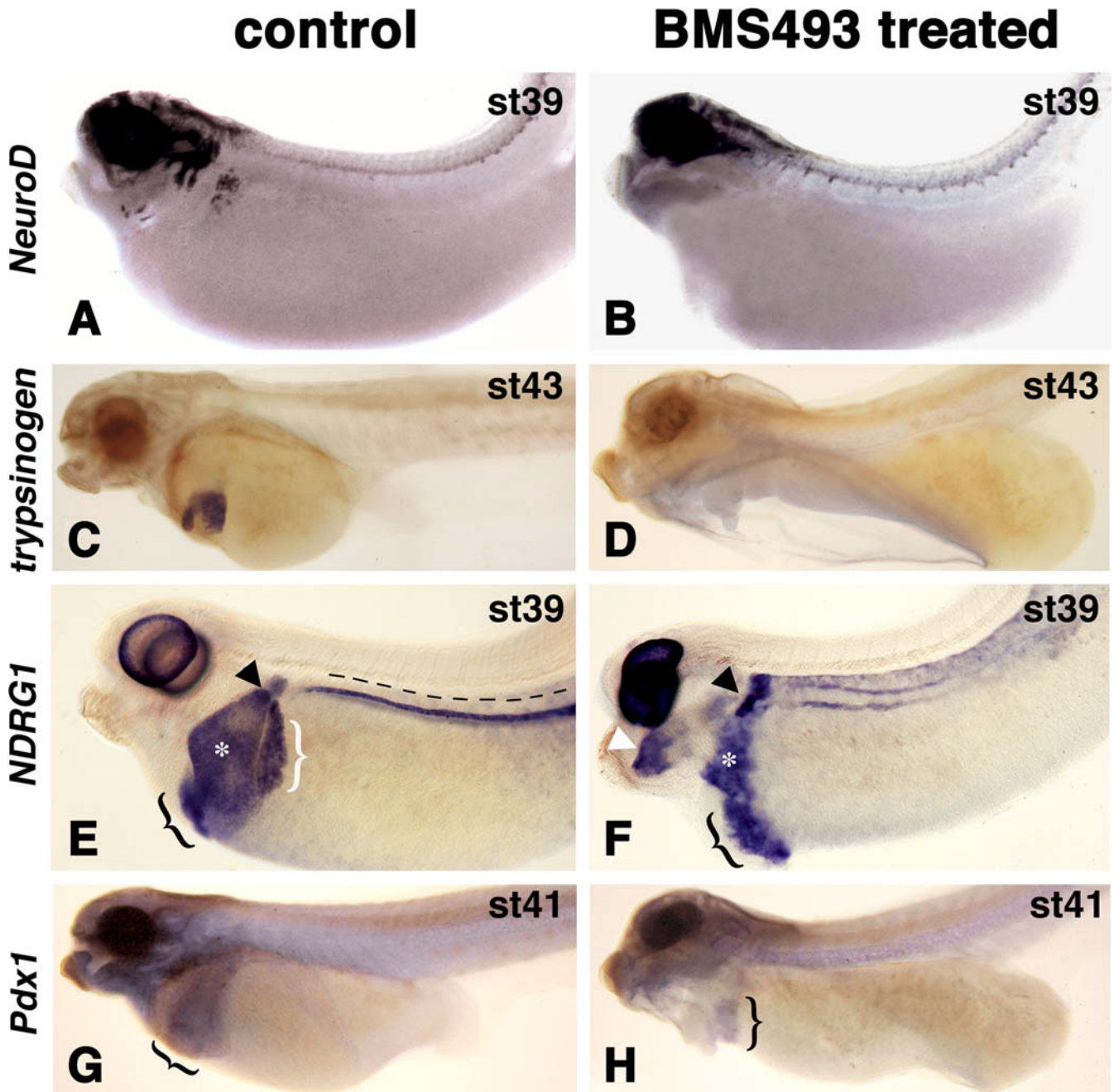
### In situ hybridization of quail embryos

VAD (vitamin A-deficient) quail eggs were obtained from birds fed on a retinoid-deficient defined diet (Gale et al. 1999). Whole-mount in situ hybridization was performed as described (Sanders et al. 2002). Chick *Pdx1* and *Islet1* probes were as described (Kim et al. 1997a, b).



**Fig. 1** Retinoic acid (RA) is required for specification of the dorsal but not ventral *Xenopus* pancreatic buds. **a, c, e, g** Mock-treated or DN-RAR $\alpha$ 2-MUT control RNA-injected embryos (indistinguishable from non-manipulated siblings). **b, d, f, h** RA-signaling-deficient embryos. These were treated with  $5 \times 10^{-6}$  M BMS493 from stage 10 until fixation or injected with 1 ng mRNA-encoding DN-RAR into both blastomeres of two-cell embryos. **a** *Insulin* expression in differentiated  $\beta$  cells. **b** No *Insulin* expression is evident in embryos treated with BMS493. **c** There is no change in *Insulin* expression in embryos injected with DN-RAR $\alpha$ 2-MUT (MUT). **d** *Insulin* expression is not detectable in DN-RAR-injected

embryos. **e** *Pdx1* marks dorsal pancreatic bud (white bracket), ventral pancreatic buds (black bracket), and stomach (asterisk). **f** *Pdx1* expression is absent from the dorsal pancreatic bud and adjacent stomach tissue, but is retained in the ventral domain (black bracket), following BMS493 treatment. **g** Normal *Pdx1* expression appears in the dorsal pancreatic bud (white bracket), ventral pancreatic buds (black bracket), and stomach (asterisk) in embryos injected with DN-RAR $\alpha$ 2-MUT. **h** *Pdx1* expression is specifically lost in the stomach and dorsal pancreatic bud following injection of DN-RAR. Embryos at stage 39 in lateral view, anterior to the left



**Fig. 2** RA is required for dorsal pancreas specification and ventral pancreas differentiation. **a, c, e, g** Mock-treated embryos. **b, d, f, h** Embryos grown in BMS493-containing media. **a** *NeuroD* is expressed in the nervous system and endocrine pancreas. **b** Pancreatic *NeuroD* expression is lost following BMS493 treatment, but is retained in the nervous system. **c** *Trypsinogen* expression in the exocrine pancreas. **d** *Trypsinogen* is not detectable in BMS493-treated embryos (note anterior/ventral edema and aberrant posterior localization of gut). **e** *NDRG1* expression in eyes, pronephros (black arrowhead), pronephric ducts (dashed line), dorsal pancreas (white

bracket), stomach (asterisk), and ventral pancreas (black bracket). **f** No dorsal pancreas expression of *NDRG1* is detectable in BMS493-treated embryos. Expression remains in eyes, pronephros (black arrowhead), pronephric ducts (dashed line), stomach (asterisk), and ventral pancreas (black bracket). Expression also appears in pharyngeal endoderm (white arrowhead). **g** *Pdx1* is expressed in the combined pancreatic buds (bracket) and adjacent gut tissue. **h** A small domain of *Pdx1* expression remains in BMS493-treated tadpoles (bracket). Stages are as indicated. All specimens are shown in lateral view, anterior to the left

## Results and discussion

RA is required for specification of the dorsal but not ventral *Xenopus* pancreatic buds

We have previously established that RA is required for specification of the zebrafish pancreas and liver (Stafford and Prince 2002). To test the hypothesis that RA is similarly required for development of these organs in tetrapod vertebrates, we have assayed expression of endodermal markers in RA-signaling-deficient embryos of the frog *X. laevis*. We blocked RA signaling using the pan-RAR antagonist BMS493, which prevents transduction of the RA signal by stabilizing interactions between RARs and co-repressor molecules (Germain et al. 2002). We also used a previously described dominant-negative *Xenopus* RAR $\alpha$  that cannot bind RA ligand and thus maintains the repressive state of the receptor complex (Sharpe and Goldstone 1997). As both approaches maintain the repressive state, we predicted they would yield similar results.

The frog pancreas, similar to that of birds or mammals, develops from both dorsal and ventral buds (Horb and Slack 2002; Kelly and Melton 2000). Before these primordia fuse (stage 41), the dorsal bud expresses the endocrine marker *Insulin*. By contrast, the ventral region does not express any endocrine markers at early stages, and is thought to give rise primarily to exocrine tissue (Kelly and Melton 2000). Both *Xenopus* pancreatic primordia express *XLHbox8/Pdx1* (Offield et al. 1996), a transcription factor that is similarly expressed in the pancreatic primordia of all vertebrates examined (Kim et al. 1997a, b; Milewski et al. 1998; Ohlsson et al. 1993; Stoffers et al. 1997), and which has been shown to be required for pancreas development in mouse and zebrafish (Ahlgren et al. 1998; Yee et al. 2001). We investigated expression of both *Insulin* and *Pdx1* in stage 39 (Nieuwkoop and Faber 1994) tadpoles, where RA signaling had been blocked either by incubation with BMS493, or by microinjection of dominant-negative RAR $\alpha$ 2 (DN-RAR). We found that *Insulin*, which marks differentiated  $\beta$  cells in the dorsal pancreas (Fig. 1a), was not expressed in BMS493-treated tadpoles (Fig. 1b, 100%,  $n=64$ ). *Insulin* expression was also sharply reduced or absent in DN-RAR-injected embryos (100% reduced, 63% absent,  $n=38$ , Fig. 1d) compared to control embryos injected with a mutated, non-functional version of the dominant-negative receptor (MUT,  $n=43$ , Fig. 1c). We next compared *Pdx1* expression in RA-signaling-deficient and control embryos. As with *Insulin* expression, both pharmacological and dominant-negative approaches produced similar results. We found that tadpoles deficient for RA signaling retained ventral expression of *Pdx1* (Fig. 1e–h, black bracket), but did not express *Pdx1* in the developing dorsal pancreas (Fig. 1e, white bracket) or stomach (Fig. 1e, g, asterisk) (Fig. 1e–h, 100%,  $n=66$ , BMS493; 70%,  $n=36$ , DN-RAR). This finding suggested to us that RA might only be required for dorsal or endocrine pancreas development in *Xenopus*.

To further test this hypothesis, we compared expression of additional pancreatic markers in normal and RA-signaling-deficient stage 39 embryos. We first examined markers of the dorsal pancreas. *NeuroD* (Fig. 2a) and *Islet1* (Kelly and Melton 2000), which encode transcription factors with roles in endocrine pancreas development, were absent from the dorsal pancreas of BMS493-treated embryos (*NeuroD*, 100%,  $n=40$ , Fig. 2b; *Islet1*, 100%,  $n=22$ , data not shown). However, nervous system expression of *NeuroD* and *Islet1* was retained (Fig. 2a, b; data not shown). Additionally, we tested whether *Insulin* expression might commence at later stages in RA-signaling-deficient embryos; we found no expression in tadpoles raised in BMS493 until stages 40 ( $n=20$ ) or 43 ( $n=20$ ) (data not shown). We conclude that RA is required for development of the frog endocrine pancreas.

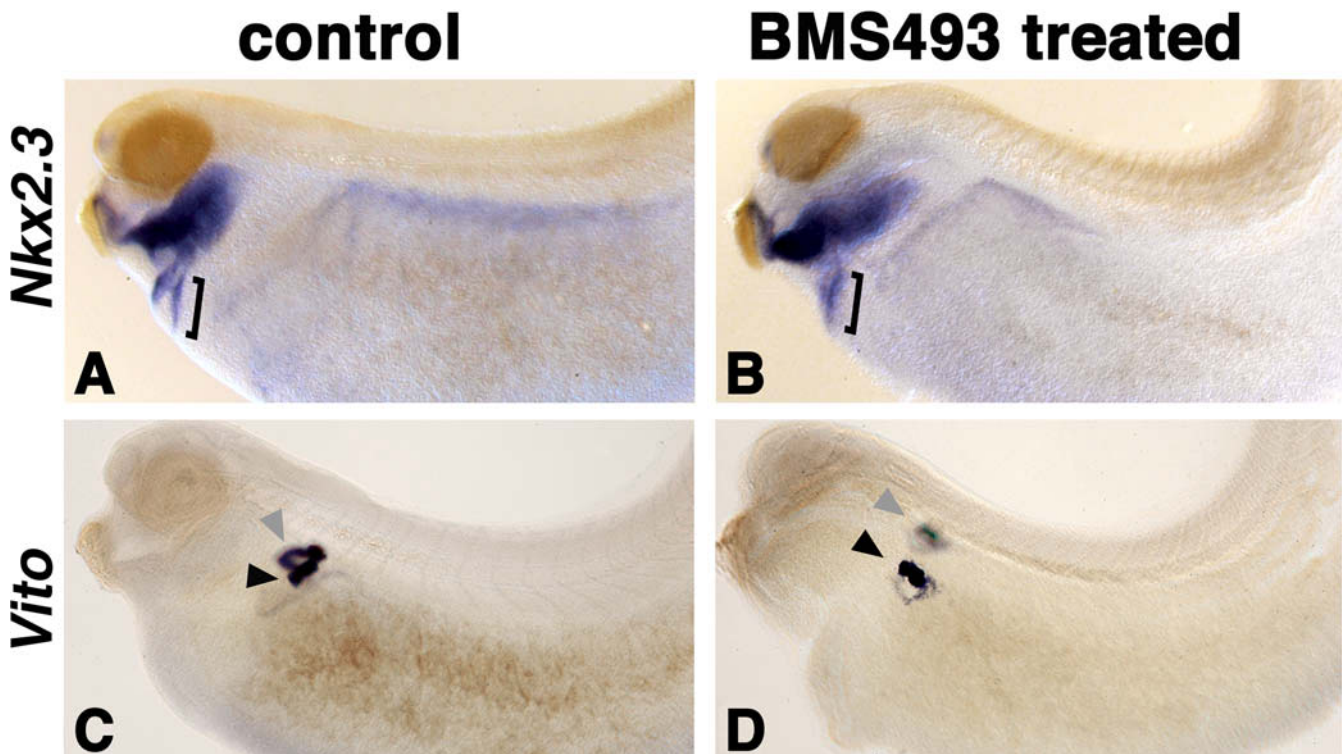
In normal *Xenopus* embryos, markers of differentiated exocrine tissue, such as *Trypsinogen* (Horb and Slack 2002), initiate expression at stage 41 in the ventral pancreatic bud. We were unable to detect expression of *Trypsinogen* at stage 41 ( $n=16$ , data not shown) or stage 43 ( $n=28$ , compare Fig. 2c, d) in BMS493-treated tadpoles. We found that the digestive tracts of RA-deficient tadpoles became severely dysmorphic from stage 40 onwards, with normal elongation and intestinal looping not occurring (Fig. 2d). Consistent with this observation, RA has previously been implicated in gut looping (Zeynali and Dixon 1998). The absence of differentiated exocrine cells could potentially reflect a lack of an inductive signal to the ventral pancreatic bud; for example, normal gut morphogenesis may be necessary to allow appropriate mesoderm to interact with exocrine progenitor cells. Although differentiated exocrine pancreatic cells were not observed, we confirmed that initial specification of the ventral pancreatic buds occurs in the absence of RA signaling. *Xenopus NDRG1* (Costa et al. 2003) is expressed in multiple tissues derived from all three germ layers; at stage 39 endodermal *NDGR1* expression can be detected in both dorsal (Fig. 2e, white bracket) and ventral (Fig. 2e, black bracket) pancreatic primordia, as well as in adjacent stomach tissue (Fig. 2e, white asterisk) and dorsal posterior gut. In RA-signaling-deficient embryos, we found that no dorsal pancreatic *NDGR1* expression was evident, but ventral pancreas and stomach expression was retained ( $n=30$ , Fig. 2f). Although we were unable to detect markers of differentiated exocrine cells in BMS493-treated tadpoles, *Pdx1* continued to be expressed at stage 41 (67%,  $n=15$ , Fig. 2g, h). Taken together, our results suggest that RA signaling is not required for initial specification of the *Xenopus* ventral pancreas, but is required later for exocrine cell differentiation. This differs from our findings in zebrafish, where we have not observed any expression of *pdx1* in RA-signaling-deficient embryos (Stafford and Prince 2002). By contrast, our results indicate that the requirement for RA signaling in endocrine pancreas development is a conserved feature of frogs and zebrafish (Stafford and Prince 2002).

Other *Xenopus* organs are specified in the absence of RA signaling

We wished to determine whether RA signaling is required specifically for *Xenopus* dorsal pancreas development, or whether the specification of organs in the dorsal trunk is more generally blocked. While endocrine pancreas never formed in RA-signaling-deficient tadpoles, the pharyngeal endoderm, heart, and pronephros were all specified. We observed *Nkx2.3* expression in the pharyngeal endoderm and heart (Sparrow et al. 2000) of BMS493-treated embryos, but noted minor changes in patterning (compare Fig. 3a, b, brackets). We also investigated the pronephros (embryonic kidney), a mesoderm-derived bilateral organ that initially develops close to the dorsal pancreas. We used the *NDRG1* and *Vito* genes as molecular markers of pronephros and pronephric ducts at stage 39 (Figs. 2e, f, 3c, d; Costa et al. 2003) and found that these structures do develop in the absence of RA signaling, although again with altered morphology (*NDRG1*, 100%,  $n=30$ ; *Vito*, 100%,  $n=17$ ). We conclude that while disruption of RA signaling may compromise patterning of a variety of organs, it does not merely block initial specification of all organs in the dorsal trunk, but rather is specifically required for endocrine pancreas.

It should be noted that the presence of pronephros in RA-deficient tadpoles does not preclude other alterations

to regionalization of the mesoderm, which may in turn influence patterning of adjacent endoderm. Indeed, an instructive role for lateral plate mesoderm in pancreas specification has previously been demonstrated based on explant experiments in the chick (Kumar et al. 2003). Furthermore, genetic and embryological experiments in the zebrafish have suggested that mesoderm is the primary source of RA during early embryonic patterning: the *RALDH2* gene that encodes the enzyme critical for RA synthesis is expressed in anterior paraxial mesoderm, and wild-type paraxial mesoderm cells can rescue RA deficiency defects in the adjacent neural tissue (Begemann et al. 2001). Neither our data from zebrafish (Stafford and Prince 2002) nor *Xenopus* (this study) addresses whether absence of pancreatic cells is a direct consequence of lack of RA signaling within pancreatic precursor cells versus an indirect consequence of lack of signaling in an adjacent tissue. RA may act within the mesoderm to modify mesodermal identity adjacent to the future pancreas, with a secondary signal then being relayed to adjacent endoderm. We are currently using a transplantation approach in the zebrafish to determine where RA signals are transduced to allow pancreas development.



**Fig. 3** *Xenopus* pharyngeal endoderm, heart, and pronephros can develop in the absence of RA signaling. **a, c** Mock-treated embryos. **b, d** Embryos grown in BMS493-containing media, as above. **a** *Nkx2.3* expression marks pharyngeal endoderm and developing heart (bracket). **b** *Nkx2.3* expression in pharyngeal endoderm and developing heart (bracket) is retained in BMS493-treated embryos.

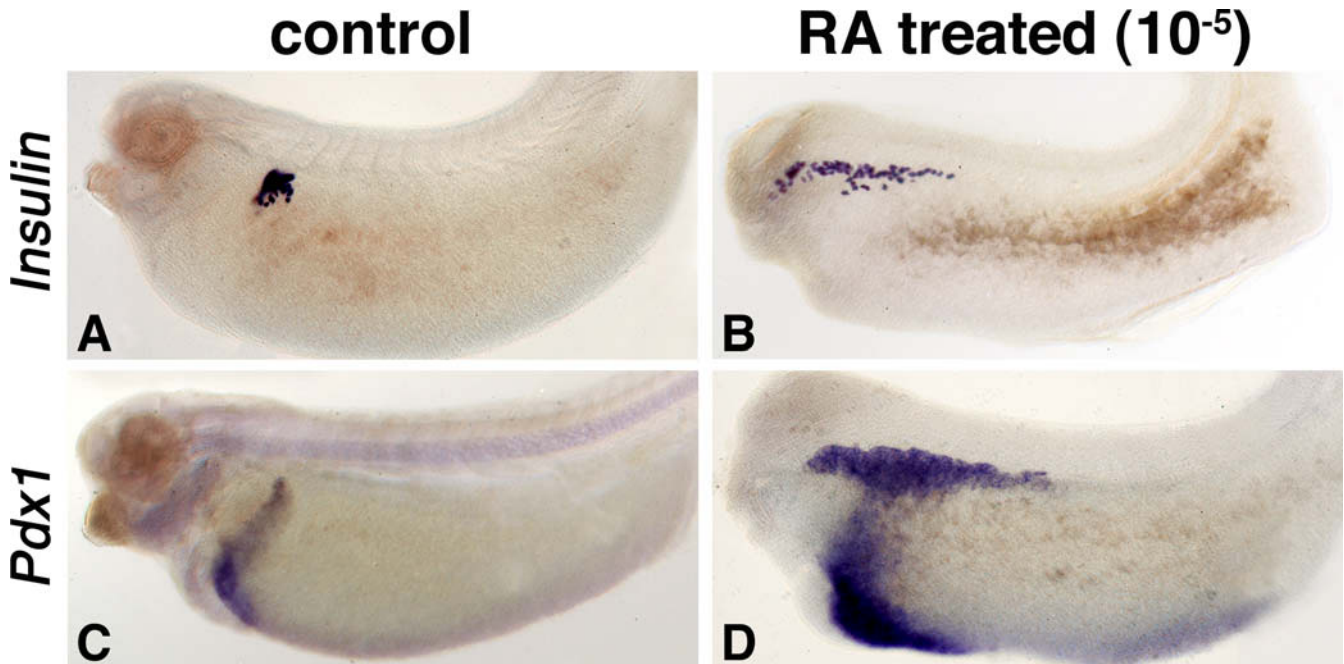
**c, d** *Vito* expression in the bilateral pronephros (left side, black arrowheads; right side, grey arrowheads). **c** Mock-treated control. **d** *Vito* expression is present in the pronephros of BMS493-treated tadpoles. All specimens are stage 39 and shown in lateral view, anterior to the left

## Exogenous RA expands the expression domains of *Xenopus* pancreatic markers

Having established that RA signaling is necessary for *Xenopus* pancreas development, we tested whether exogenous RA is sufficient to induce ectopic pancreas tissue. We pulse-treated *Xenopus* embryos with  $10^{-5}$  M RA for 1 h, starting at stage 12, and found that the *Insulin* expression domain expanded significantly along the AP axis (100%,  $n=14$ , Fig. 4a, b). Treatment with  $10^{-6}$  M RA led to a less dramatic expansion (100%,  $n=25$ , data not shown). Expression of the pancreas primordium marker *Pdx1* was similarly expanded in both its dorsal and ventral domains (100%,  $n=15$ , Fig. 4c, d), as was pancreatic *NDRG1* expression (100%,  $n=15$ , data not shown). Although our data demonstrate that the ventral bud does not require RA signaling for specification (Figs. 1, 2), this domain can nevertheless expand in response to exogenous RA. Our findings are consistent with a report that *Xenopus* dorsal lips treated with RA express pancreatic markers (Moriya et al. 2000). However, our results differ from a previous report that *Pdx1* expression is unaltered by RA treatment (Zeynali and Dixon 1998). An explanation for this discrepancy may be that the embryos used in our studies were treated with RA at a significantly earlier stage of development—stage 12 versus stage 25. As we have previously shown that exogenous RA has the capacity to induce ectopic zebrafish pancreatic tissue (Stafford and Prince 2002), we conclude that the ability of RA to expand

the pancreatic field is conserved between the amphibian *Xenopus* and the teleost zebrafish.

RA signaling plays a critical role in specifying identity along the AP axis of developing embryos (Begemann et al. 2001; Grandel et al. 2002; Gale et al. 1999; Niederreither et al. 2000, 2002; Quinlan et al. 2002). Accordingly, we have found that application of exogenous RA to both zebrafish (Stafford and Prince 2002) and *Xenopus* embryos transforms anterior endoderm into pancreatic tissue. However, we have also found that loss of RA signaling does not lead to the formation of ectopic pancreas from posterior endoderm, instead pancreatic development is severely compromised. Thus, the absence of RA signaling does not lead to a simple shifting of identity along the AP axis. While we have been unable to determine what tissue type, if any, forms in place of the pancreas in RA-signaling-deficient embryos, our results suggest that RA plays an active role in specifying pancreatic fate and not just AP identity. Alternatively, additional factor(s) present in the posterior region of the embryo may limit the possible structures that can be formed. While the resolution of this question awaits further study, our published (Stafford and Prince 2002) and current results suggest that only anterior embryonic domains are competent to form pancreas in frogs and zebrafish.



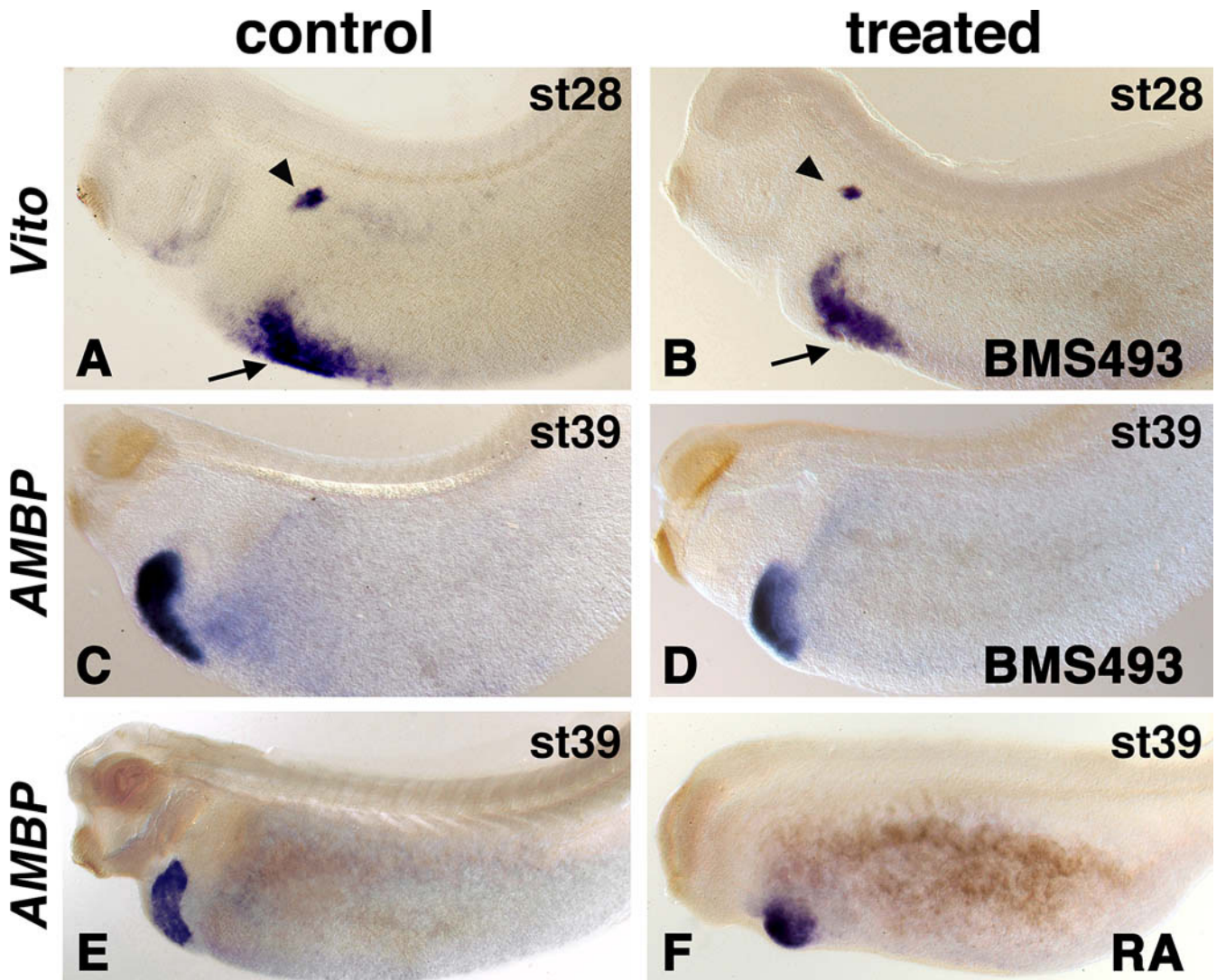
**Fig. 4** Exogenous RA expands the expression domains of *Xenopus* pancreatic markers. **a, c** Mock-treated embryos. **b, d** RA-treated embryos. Embryos were treated with  $10^{-5}$  M RA at stage 12, removed to  $0.1\times$  MBS after 1 h, and fixed at stage 39. Embryos exhibit characteristic loss of anterior structures (e.g. eye, cement gland) associated with RA treatment. **a** *Insulin* expression in  $\beta$  cells

of the dorsal pancreatic bud. **b** *Insulin* expression expands along the anteroposterior axis following RA treatment. **c** *Pdx1* expression in both dorsal and ventral pancreatic buds as well as adjacent gut tissue. **d** *Pdx1* is expressed at high levels in an expanded domain in both dorsal and ventral pancreas. All specimens are shown in lateral view, anterior to the left

*Xenopus* liver specification is independent of RA signaling

In zebrafish, liver development is dependent upon RA signaling: liver does not form in the absence of RA signaling and exposure to exogenous RA leads to the formation of ectopic liver (Stafford and Prince 2002). We therefore investigated whether liver development was responsive to RA signaling in *Xenopus* embryos. In stark contrast to zebrafish, *Xenopus* liver development initiates normally in the absence of RA signaling. The presumptive *Xenopus* liver marker *Vito* (Costa et al. 2003) is expressed in both control and BMS493-treated stage 28 embryos (100%,  $n=15$ , Fig. 5a, b). Furthermore, the liver differentiation markers  $\alpha 1$ -microglobulin/bikunin precursor (AMBP) and *Fibrinogen* (Zorn and Mason 2001) are expressed in both control and BMS493-treated stage 39

tadpoles (Fig. 5c, d; data not shown), suggesting that later-stage liver development is also independent of RA signaling. The retention of both the liver and the ventral pancreatic bud in RA-signaling-deficient tadpoles suggests that these two structures may have a common origin in frogs, as has been demonstrated in mice (Deutsch et al. 2001). We performed the reciprocal experiment to test whether exogenous RA could expand the region expressing the *Xenopus* liver differentiation marker AMBP. Again, we found that perturbation of RA signaling had little effect on liver development. In RA-treated embryos, non-expanded AMBP expression was observed (100%,  $n=19$ , compare Fig. 5e, f). As RA is required for liver development in zebrafish but not in frog, we suggest that the mechanisms that pattern the liver may have diverged during the approximately 400 million years that separate zebrafish and *Xenopus*.



**Fig. 5** *Xenopus* liver development is not RA signaling dependent. Embryos were treated with BMS493 or RA as previously described. **a** *Vito* expression at stage 28 in liver primordia (arrow), and pronephros (arrowhead). **b** In BMS493-treated embryos, *Vito* expression is retained in the developing liver (arrow) and proneph-

ros (arrowhead). **c**, **e**  $\alpha 1$ -microglobulin/bikunin precursor (AMBP) expression in the liver at stage 39. **d** AMBP is expressed in the liver of BMS493-treated tadpoles. **f** AMBP expression does not expand in response to RA treatment. All specimens are shown in lateral view, anterior to the left



RA is required for dorsal pancreas development in the avian quail

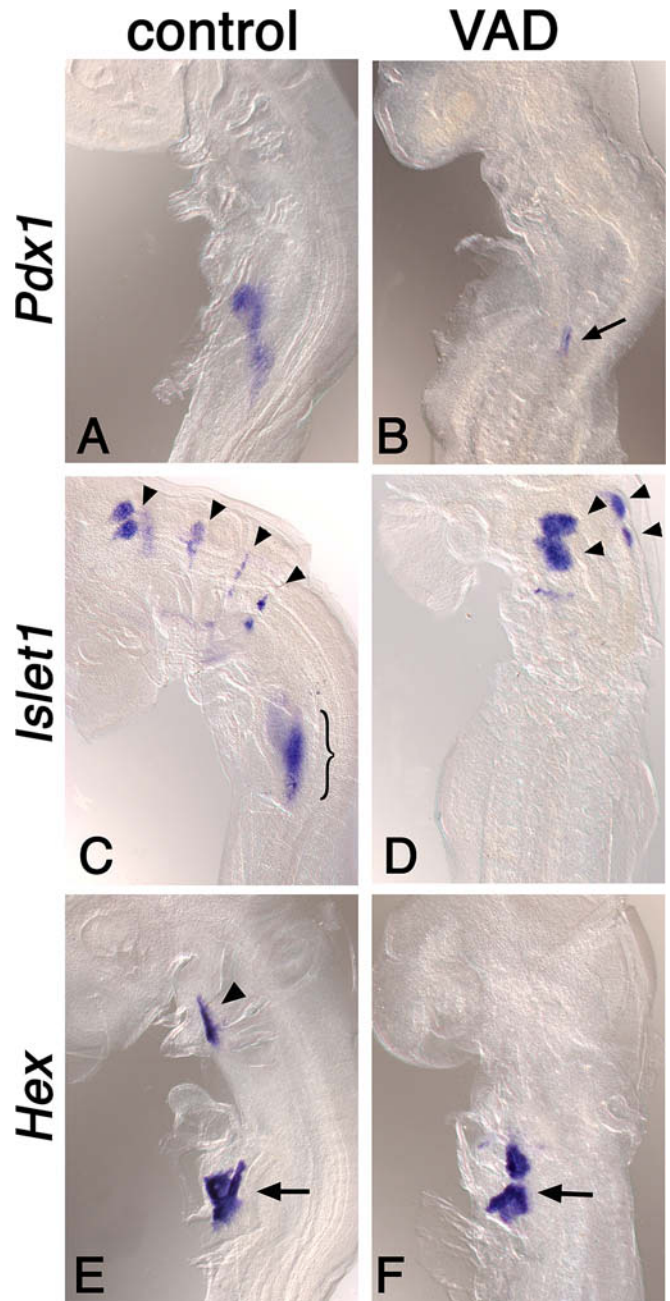
To investigate whether RA is required for pancreas and liver development in amniote vertebrates, we made use of VAD Japanese quail embryos. Quail embryos derived from hens with a dietary deficiency in vitamin A, the precursor of RA, have defects in the hindbrain and pharyngeal development similar to RALDH2 deficient mice and zebrafish (Gale et al. 1999; Quinlan et al. 2002). As the VAD embryos rarely developed beyond HH stage 16 (Hamburger and Hamilton 1951), we were only able to assay early markers of the dorsal pancreatic bud (Kim et al. 1997b). We found that, as for zebrafish and *Xenopus*, *Pdx1*, and *Islet1* pancreatic expression was severely disrupted in quails lacking normal RA-signaling (*Pdx1*, 94%,  $n=16$ ; *Islet1*, 100%,  $n=9$ , Fig. 6a–d). We conclude that in the amniote quail, as in the amphibian *Xenopus* and the teleost zebrafish, RA signaling is critical for normal development of the pancreas.

We also analyzed expression of quail *Hex*, a marker of both liver and thyroid progenitors (Yatskievych et al. 1999). In VAD animals *Hex* expression was maintained (100%,  $n=6$ , Fig. 6e, f) in the normal location of the developing liver, immediately posterior to the developing heart. Our data suggest that the liver can be specified in the absence of RA signaling in both quails and *Xenopus*. These findings may indicate that a requirement for RA signaling in initiation of liver development is a feature specific to teleosts such as zebrafish.

**Conclusion:** RA plays a critical role in pancreas development in vertebrates

Our results reveal a requirement for RA signaling in the development of the pancreas of both amphibian and avian embryos. Taken together with our previous finding that RA is required for pancreas development in the zebrafish, our data support the hypothesis that RA has a critical early role in pancreas development of all vertebrates. By contrast, while RA signaling is also required for zebrafish liver development, this role is not conserved in the tetrapods. The extensive conservation of vertebrate pancreas patterning mechanisms confirms that non-mammalian systems represent valuable models for the study of human pancreas development. Furthermore, our demonstration that the requirement for RA signaling is a general early feature of pancreas specification may be of importance for the development of protocols to achieve in vitro differentiation of human stem cells into mature pancreatic cell types.

**Note added in proof** Similar results were recently reported by Chen et al. Dev Biol 271:144–160



**Fig. 6** RA is required for dorsal pancreas development in the avian quail. Embryos were fixed at stage 16 (based on somite number), anterior to top of page, lateral view. Vitamin-A-deficient (*VAD*) embryos exhibited characteristic morphological defects in the anterior nervous system and pharynx. **a** *Pdx1* expression in developing pancreas. **b** *Pdx1* expression is absent or radically reduced in VAD embryos. The sole example of an embryo exhibiting *Pdx1* expression is shown (arrow). **c** *Islet1* is expressed in pancreatic endoderm and its adjacent mesenchyme (bracket) and the cranial nerves (arrowheads). **d** No pancreatic *Islet1* expression was detected in VAD embryos; cranial nerve expression is retained (arrowheads). **e** *Hex* expression in the thyroid (arrowhead) and liver (arrow). **f** *Hex* is expressed in the liver of VAD embryos

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