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There was an error published in *Development* **133**, 949-956.

There was a mistake in the sequence of the raldh2 morpholino, which was missing three nucleotides. The correct sequence is as follows:

5′ GCAGTTCAACTTCACTGGAGGTCAT

The authors apologise to readers for this mistake.

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Retinoids signal directly to zebrafish endoderm to specify *insulin*-expressing β-cells

David Stafford[1], Richard J. White[2,*], Mary D. Kinkel[3,*], Angela Linville[2], Thomas F. Schilling[2] and Victoria E. Prince[1,3,†]

During vertebrate development, the endodermal germ layer becomes regionalized along its anteroposterior axis to give rise to a variety of organs, including the pancreas. Genetic studies in zebrafish and mice have established that the signaling molecule retinoic acid (RA) plays a crucial role in endoderm patterning and promotes pancreas development. To identify how RA signals to pancreatic progenitors in the endoderm, we have developed a novel cell transplantation technique, using the ability of the SOX32 transcription factor to confer endodermal identity, to selectively target reagents to (or exclude them from) the endodermal germ layer of the zebrafish. We show that RA synthesized in the anterior paraxial mesoderm adjacent to the foregut is necessary for the development of *insulin*-expressing β-cells. Conversely, RA receptor function is required in the foregut endoderm for *insulin* expression, but not in mesoderm or ectoderm. We further show that activation of RA signal transduction in endoderm alone is sufficient to induce *insulin* expression. Our results reveal that RA is an instructive signal from the mesoderm that directly induces precursors of the endocrine pancreas. These findings suggest that RA will have important applications in the quest to induce islets from stem cells for therapeutic uses.

KEY WORDS: Pancreas, Zebrafish, Insulin, β-Cell, Endoderm

INTRODUCTION

The vertebrate endoderm gives rise to the epithelial lining of the gut and to organs such as the thymus, liver and pancreas. This remarkable diversity of adult endodermal tissues derives from a homogeneous and multipotent precursor cell population. The later morphology of the gut and associated organs is produced by the precisely orchestrated divergence of subsets of endodermal progenitors towards different developmental fates in response to local inductive signals from adjacent mesoderm (Horb and Slack, 2001; Kim et al., 1997; Kumar et al., 2003; Rossi et al., 2001; Wells and Melton, 2000).

Several signaling molecules have been implicated in induction of specific endodermal cell types. Application of fibroblast growth factor 4 (FGF4) protein in the chick induces posterior endodermal markers in anterior (foregut) endoderm (Wells and Melton, 2000). Activin-βB and FGF2, which function to suppress endodermal Sonic hedgehog (Shh) signaling, are permissive for pancreas formation, similar to the role of the embryonic notochord (Hebrok et al., 1998). Kumar and colleagues (Kumar et al., 2003) used chick explant cultures to show that pancreatic markers are induced in anterior endoderm by lateral plate mesoderm from the pre-pancreatic region. They also showed that activin, bone morphogenetic proteins (BMPs) or retinoic acid (RA) can all induce pancreatic markers in anterior endoderm co-cultured with anterior mesoderm (Kumar et al., 2003). Of these molecules, only RA has also been found necessary for pancreas development. Zebrafish or mice defective in RA synthesis or RA receptor (RAR) function lack pancreatic cell types (Martin et al., 2005; Molotkov et al., 2005; Stafford and Prince, 2002). In addition, exogenous application of RA to zebrafish embryos induces ectopic pancreatic cells throughout the anterior endoderm. Work in amphibian and avian systems has yielded similar results, indicating that the requirement for RA in endocrine pancreas specification is probably a general feature of vertebrate development (Chen et al., 2004; Stafford et al., 2004). Together the gain- and loss-of-function studies suggest that RA plays an instructive role in specifying the position of the pancreas along the anteroposterior (AP) axis of the gut.

The mechanism of RA signal transduction is well known (Bastien and Rochette-Egly, 2004). The rate-limiting step in RA synthesis is conversion of retinaldehyde to RA, catalyzed by retinaldehyde dehydrogenase (RALDH). RA signals are transduced by two types of nuclear hormone receptors: retinoic acid receptors (RARs) and retinoid receptors (RXRs). RARs and RXRs bind as heterodimers to retinoic acid response elements (RAREs) and either activate or repress transcription based on the presence or absence of ligand, respectively. The expression of RALDH enzymes (RALDH1-3) defines the tissues that produce RA; the RA essential for early vertebrate embryogenesis depends on RALDH2 function (Begemann et al., 2001; Grandel et al., 2002; Niederreither et al., 1999). In all vertebrates that have been examined, *Raldh2* is expressed during gastrulation in the mesendoderm, and becomes localized to lateral plate and paraxial mesoderm during segmentation (Begemann et al., 2001; Berggren et al., 1999; Niederreither et al., 1997; Swindell and Eichele, 1999; Wang et al., 1996; Zhao et al., 1996). Similarly, the expression of RARs and RXRs defines the tissues capable of responding to RA, and these exhibit broad, dynamic expression patterns during development (Begemann et al., 2001; Joore et al., 1994; Mollard et al., 2000; Sharpe, 1992). Consistent with these broad expression domains, loss-of-function studies have revealed that RA signaling is important for the development of derivatives of all germ layers (reviewed by Ross et al., 2000). The expression of RARs in a given tissue does not

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necessarily imply active RA signaling, as RARs can act as transcriptional repressors when not bound by RA (Koide et al., 2001).

Although RA clearly plays a crucial role in pancreas development, the precise nature of the inductive interaction is unknown. RA synthesized in the mesoderm could act directly on pancreas precursor cells in the endoderm. However, as RA also patterns other germ layers, its influence on the endoderm may be indirect. Distinguishing between these direct and indirect signaling models is crucial to understanding the molecular mechanism of pancreas specification.

Here, we take advantage of the ability to transplant cells into specific germ layers in zebrafish to pinpoint the source of RA and to demonstrate that it acts directly on endodermal cells to induce pancreatic fates. To establish the source, we generated chimeric embryos with a mixture of normal and RALDH2-deficient cells, an approach similar to previous studies of neural patterning (Begemann et al., 2001). The presence of wild-type donor cells in anterior mesoderm (somites 1-7) of a host embryo otherwise unable to synthesize RA rescues the formation of *insulin*-expressing cells, demonstrating that the crucial source of RA is the paraxial mesoderm. Conversely, disruption of RAR function in the endoderm with antisense morpholinos (MOs) and dominant-negative receptors blocks β-cell development, whereas loss of RAR function in other germ layers has no effect on embryonic *insulin* expression. In addition, a constitutively active RAR can drive ectopic expression of *insulin* in anterior endoderm. Our studies are the first to demonstrate that β-cell development requires RA produced in the paraxial mesoderm and that pancreatic precursors receive the RA signal directly.

MATERIALS AND METHODS

Cloning of expression constructs

A full-length *rarα2a* cDNA was isolated using PCR primers upstream of the START codon and overlapping the STOP codon (Accession Number NM_131406): RARα2a-START, TTGAATTCGCCAGCCGTTGTCTTG-AGGAAT; RARα2a-STOP, CATCCTCGAGTGATGGTTGTCACGGTG-ACTG. A DN-RARα2a containing a premature stop codon to remove the C-terminal activation domain was modeled on *Xenopus* DN-RARs (Blumberg et al., 1997; Sharpe and Goldstone, 1997). To generate dominant-negative (DN) RARα2a, the above START primer was used with a primer that introduced a STOP codon at amino acid residue 403: DN-RARα2a-403-STOP, CATCCTCGAG CTATGAGCCTGGGATCTCATTT. PCR products were digested with *Eco*RI and *Xho*I and subcloned into pCS2+ (Turner and Weintraub, 1994).

Microinjections

Synthetic capped mRNAs encoding zebrafish SOX32, GFP, RARα2a, DN-RARα2a and *Xenopus* VP16-RARα1 were synthesized using the Ambion Megascript kit according to manufacturer's instructions. mRNA was suspended in 0.2 M KCl and ~100 pl pressure-injected into one-cell stage embryos. Antisense morpholinos were designed by Gene Tools to target the *raldh2* and *rar* genes: raldh2-MO, 5′ GCAGTTCTTCACTGGAGGTCAT; RARα2b-MO, 5′ CCACAACGTCCACGCTCTCGTACAT; RARα2a-MO, 5′ GGTTCACATCCACACTCTCATACAT; RARγ-MO, 5′ CCAGAGC-CTCCATACAGTCGAACAT.

Approximately 100 pl of morpholino was injected at the yolk/blastoderm interface at the one- to two-cell stage, at concentrations ranging from 2 mg/ml to 4 mg/ml in injection buffer (0.25% Phenol Red, 120 mM KCl, 20 mM HEPES-NaOH pH7.5). Reagents were injected at the following final concentrations unless otherwise stated: SOX32 mRNA, 20 ng/μl; GFP mRNA, 15 ng/μl; RARα2a mRNA, 100 ng/μl; DN-RARα2a mRNA, 125 ng/μl; VP16-RARα1, 1 μg/μl; raldh2-MO, 4 mg/ml; RARα2b-MO, 2 mg/ml; RARα2a-MO, 2 mg/ml; RARγ-MO, 2 mg/ml; sox32-MO (Sakaguchi et al., 2001), 5 mg/ml; 40 kDa lysinated fluorescein dextran (Molecular Probes), 1 mg/ml.

Luciferase assay

Zebrafish embryos were injected at the one-cell stage with 25 pg of a RA-responsive reporter (tk-[βRARE]$_2$-luc, a gift from B. Blumberg) either with or without 500 pg DN-RARα2a RNA. Embryos were then treated with various concentrations of RA diluted in embryo medium and harvested at 24 hpf. Six embryos were used for each experimental condition, lysed in 10 μl/embryo lysis buffer (50 mM Tris pH 7.5, 1 mM DTT, 2 mM EDTA) and centrifuged at 16,000 ***g*** to remove cell debris. Lysate (10 μl) was assayed using the Enhanced Luciferase Assay kit (Pharmingen); Monolight 2010 luminometer measurements were performed in duplicate.

In situ hybridization, immunohistochemistry and microscopy

In situ probes were used as previously described (Prince et al., 1998; Stafford and Prince, 2002). GFP protein was detected by immunohistochemistry using rabbit anti-GFP polyclonal antibody (Molecular Probes) at a dilution of 1:2000, processed with the Vectastain Universal ABC kit and visualized with TSA substrate (NEN) according to manufacturer's instructions. Confocal microscopy was performed on a Zeiss LSM510.

Cell-transplantation

Wild-type embryos used in transplants were from the *AB strain or a locally obtained pet store variety. Transplantation was performed as previously described (Ho and Kane, 1990). For transplants to test where RA signals are produced, the host embryos were injected with ~100 pl of 4 mg/ml raldh2-MO, and donor embryos were labeled with 40 kDa fixable fluorescein dextran at the one-cell stage. Groups of 10-15 uncommitted cells from 4 hpf donors were transplanted along the blastoderm margin of equivalent stage host embryos. The embryos were then raised to 24 hpf and *insulin* expression detected to assay for the presence of endocrine β-cells. For transplants to test whether RARs function in mesoderm or ectoderm to specify pancreas, the hosts were injected with RKD reagents (DN-RARα2a at 125 ng/μl plus RARα2b-MO at 2 mg/ml) at the one-cell stage.

For transplants using SOX32 to target cells to the endoderm, donor embryos were injected at the one-cell stage with SOX32 mRNA. The embryos were co-injected with fixable fluorescein dextran, or with GFP mRNA, to allow visualization of donor cells, and with RKD reagents where appropriate. Approximately 25-30 cells from 4 hpf donors were distributed along the blastoderm margin of stage-matched hosts. Donor embryos die by 9 hpf as a consequence of being composed exclusively of endoderm.

RESULTS

RA produced in anterior somites is essential for pancreas development

Zebrafish embryos mutant in the *raldh2/neckless* gene (Begemann et al., 2001), or treated with a pan-RAR inhibitor (BMS493), lack all pancreatic cell types (Stafford and Prince, 2002). Timed treatments with BMS493 indicate that RA signals required to specify the pancreas occur at the end of gastrulation (9-12 hours post fertilization, hpf) (Stafford and Prince, 2002). As RA production during these stages depends on RALDH2 activity, we examined the distribution of *raldh2* mRNA in endoderm and mesoderm of the region that forms the pancreas in greater detail. *raldh2* is expressed in mesendodermal progenitor cells at 6 hpf (Begemann et al., 2001; Grandel et al., 2002). By 10 hpf, expression appears more restricted to anterior trunk mesoderm but may still include endodermal cells (Fig. 1A). To investigate this, we made use of SOX32/Casanova, a transcription factor expressed specifically in endoderm that has the capacity to confer endodermal fates on transplanted cells (Kikuchi et al., 2001; Sakaguchi et al., 2001). This allowed us to label the endoderm by transplanting fluorescein dextran-labeled cells from donor embryos ubiquitously expressing SOX32 into uninjected hosts, and to test if endodermal cells express *raldh2*. Using confocal analysis, we found co-localization of fluorescein and *raldh2* mRNA in grafted endodermal cells in addition to raldh2 expression in adjacent host endoderm and mesoderm (Fig. 1B-D).

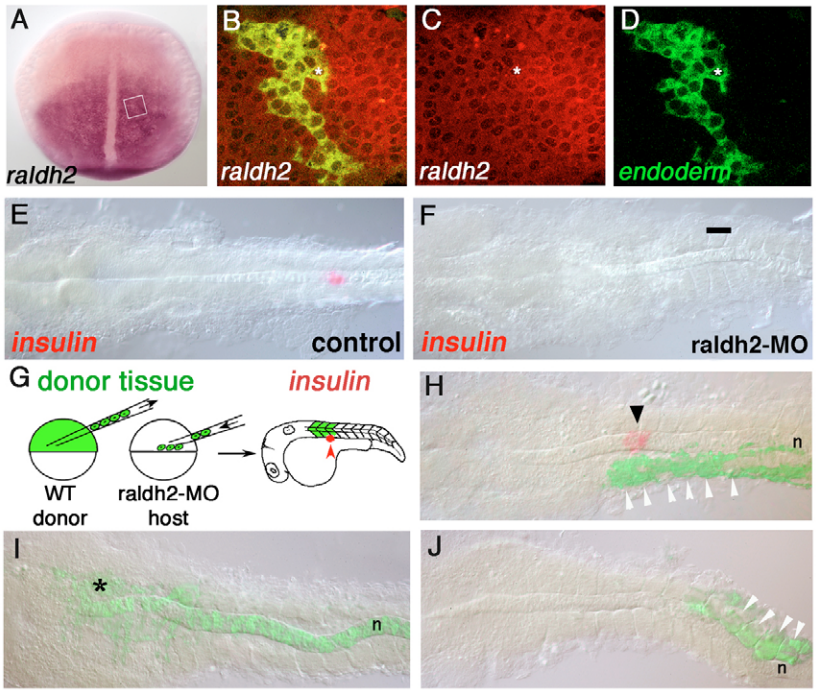

Fig. 1. The RA required for pancreas development is produced in anterior paraxial mesoderm. (**A**) *raldh2* expression in dorsal view (anterior to top) at 10 hpf. (**B-D**) *raldh2* expression in endoderm cells; an example is indicated with an asterisk. Confocal images of *raldh2* expression (red) at 10 hpf, SOX32-positive donor-derived endoderm is labeled with fluorescein dextran (green). Approximate area of image is boxed in A. (E,F) *insulin* expression (red) at 24 hpf. (**E**) Normal *insulin* expression. (**F**) No *insulin* expression appears in *raldh2* morpholino injected embryos (bar indicates normal position of pancreas; 100%; *n*=38). (**G**) Schematic of cell-transplantation approach. Fluorescein dextran-labeled donor cells from sphere stage (4 hpf) embryos are distributed along the blastoderm margin of *raldh2*-MO injected hosts. Donor cells contribute to mesoderm (indicated here in anterior somites). (H-J) Transplanted, *raldh2*-MO injected embryos probed for *insulin* expression at 24 hpf (red). Photographs are composites of bright field and fluorescent images to detect donor tissue (green). (**H**) An embryo in which donor tissue contributed to anterior somites (white arrowheads), rescuing *insulin* expression (arrowhead). (**I,J**) Embryos in which donor tissue contributed to notochord (n) plus overlying neural cells (*) (I) and posterior somites (white arrowheads) (J); no *insulin* expression is detectable.

Embryos injected with an antisense morpholino oligonucleotide (MO) directed against the *raldh2* transcript phenocopy *neckless* (*nls*) mutants (Begemann et al., 2001) and fail to form a pancreas (Fig. 1E,F). We used these embryos as hosts in cell-transplantation experiments to establish which tissue(s) require RALDH2 function in pancreas development. Based on the zebrafish embryonic fate map (Kimmel et al., 1990), we transplanted wild-type cells into the mesoderm or ectoderm of RALDH2-deficient host embryos (schematized in Fig. 1G), and assayed for *insulin*-expressing endocrine β-cells. In the 36 cases in which donor tissue was located in anterior paraxial mesoderm (defined as somites 1-7), 70% developed *insulin*-expressing cells (Fig. 1H). Transplants that contributed to more posterior mesoderm (*n*=11), or to the notochord, floorplate or neural tube, did not induce *insulin* expression (Fig. 1I-J). Similarly, transplants from SOX32-expressing donor embryos that contributed to endoderm only did not induce *insulin* expression (*n*=12; data not shown). We conclude that RA derived from anterior, pre-somitic mesoderm, the site of high levels of *raldh2* expression, is sufficient for pancreas induction.

Pancreas development depends on RAR function

RA signals are transduced by RARs; three zebrafish genes encoding RARs have previously been described (*rara2a*, *rara2b* and *rarg*) (Begemann et al., 2001; Joore et al., 1994; Stafford and Prince, 2002). At 10 hpf, *rara2b* is expressed at higher levels than *rara2a* in the presumptive pancreatic region (Fig. 2A,B). Using the endoderm-labeling method described above and confocal analysis, we found that *rara2b* is expressed in both endoderm and mesoderm (Fig. 2D-F), while *rara2a* expression is excluded from endoderm (data not shown). These findings are consistent with our previous analysis of sections, which also showed that *rarg* is localized to endoderm (Stafford and Prince, 2002).

To attempt to address the specific roles of individual RARs in pancreatic development, we designed morpholinos targeted against the 5′ coding regions. Morpholinos targeted against *rara2a* and *rara2b* did not cause a significant reduction in the number of *insulin*-expressing cells when injected alone or together (Fig. 2C,H; data not shown). Injection of morpholino targeted against *rarg* caused a minor reduction in number of *insulin*-expressing cells (Fig. 2H), and this effect was exacerbated by co-injection of *rarg* with *rara2b* morpholino (Fig. 2H), although even in co-injected specimens the pancreas retained more than 50% of its normal size. By contrast, injection of a dominant-negative (DN) RARα2a lacking the C-terminal activation domain (Blumberg et al., 1997; Sharpe and Goldstone, 1997) caused severe defects. Zebrafish embryos injected with synthetic mRNA encoding DN-RARα2a exhibited hindbrain and ear defects similar to those typically caused by reductions in RA signaling, as well as a dose-dependent reduction in size of the endocrine pancreas (as assayed by *insulin* expression; Fig. 2G). Furthermore, we found that co-injection of *rara2b*-morpholino with DN-RARα2a mRNA produced a significantly more severe pancreas phenotype than injection of the DN-RAR alone (Fig. 2H,I).

To confirm the specificity of the DN-RAR, we made use of several assays. We microinjected zebrafish embryos with a reporter construct in which luciferase expression is under RARE control and, as expected, found that this reporter was unable to respond to RA treatments in the presence of DN-RAR (Fig. 3A). To test further the specificity of the DN-RAR in vivo, we attempted to rescue pancreas development by co-injection of full-length RARα2a mRNA. However, this mRNA had no effect in the presence of the DN-RAR, and when injected alone led to a reduction in pancreas size (data not shown). Excess RAR protein has been shown to bind RA independently of RXR dimerization and DNA binding, which may reduce the amount of RA available to interact with RARs bound to RAREs (Blumberg et al., 1997). As an alternative test of specificity, we therefore examined the ability of our DN-RAR to block activation of Hox gene transcription by exogenous RA (Bel-Vialar et al., 2002; Conlon and Rossant, 1992). Treatment of 9 hpf embryos with 10^{-6}M RA for 1 hour induced ectopic *hoxa4a* expression in the anterior of the embryo (Fig. 3B,C), suggesting that *hoxa4a* is likely to be a direct RA target. Our DN-RAR blocked early expression of *hoxa4a* (Fig. 3D), and in addition we found that the ability of RA to induce ectopic *hoxa4a* expression in DN-RAR-injected embryos was severely abrogated (Fig. 3E), indicating that this reagent acts in

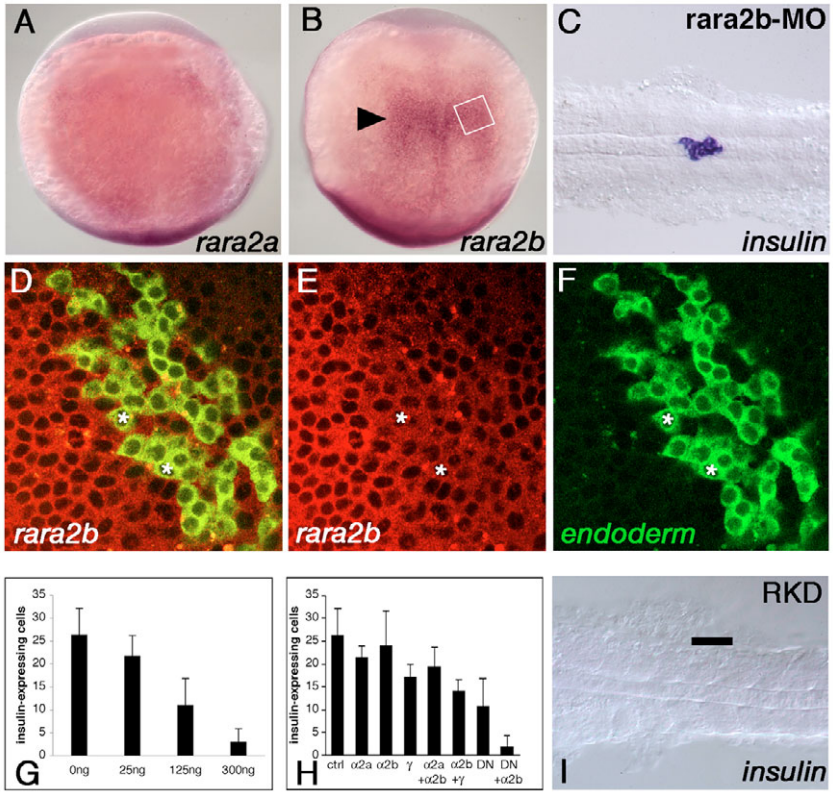

Fig. 2. Pancreas development is dependent on RAR function. (**A**) *rara2a* expression in dorsal view at 10 hpf. (**B**) *rara2b* expression in dorsal view at 10 hpf; arrowhead indicates the approximate AP position where the pancreas will develop. (**C**) *insulin* is expressed at 24 hpf following morpholino knock-down of RARα2b (indistinguishable from controls; data not shown). (**D-F**) *rara2b* expression in endoderm cells; examples are indicated by asterisks. Confocal images of *rara2b* expression (red) at 10 hpf, SOX32-positive donor-derived endoderm is labeled with fluorescein (green). Approximate area of image is boxed in B. (**G**) DN-RARα2a mRNA blocks *insulin* expression in a dose-dependent fashion (concentrations injected in ng/μl). (**H**) Histogram showing number of *insulin*-expressing cells in uninjected control embryos (*n*=44) compared with embryos injected with RARα2a-MO (*n*=11), RARα2b-MO (*n*=12), RARγ-MO (*n*=12), RARα2a-MO plus RARα2b-MO (*n*=10), RARα2b-MO plus RARγ-MO (*n*=7), DN-RARα2a mRNA (125 ng/ml; *n*=11) and DN-RARα2a mRNA (125 ng/ml) plus RARα2b-MO (RAR knock-down, RKD; *n*=85). Error bars indicate standard deviation. (**I**) Co-injection of DN-RARα2a mRNA and RARα2b-MO blocks *insulin* expression.

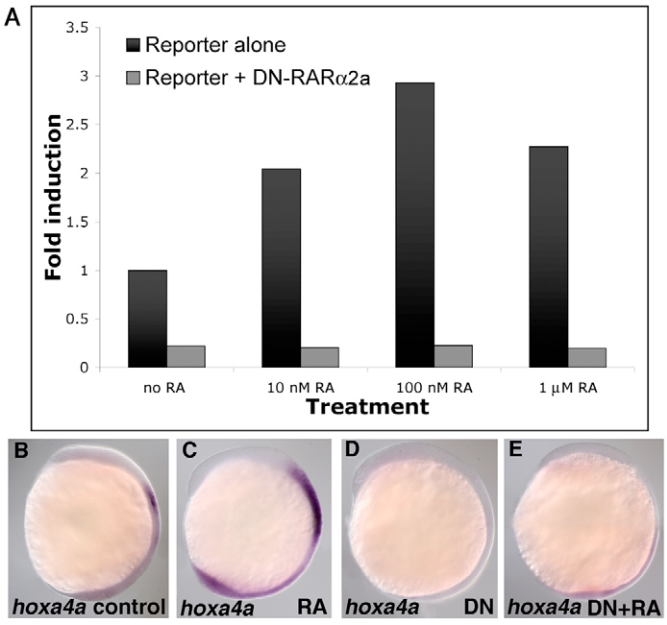

Fig. 3. The DN-RARα2a acts in a dominant negative fashion. (**A**) DN-RARα2a is able to block induction of an RA-responsive luciferase reporter gene (tk-[βRARE]$_2$-luc). Co-injection of DN-RARα2a RNA completely abolishes the response of the reporter gene to RA. Graph shows fold-induction relative to reporter alone; six embryos were used for each experimental condition and assays were carried out in duplicate and averaged. (**B-E**) *hoxa4a* expression, 10 hpf, lateral view. (B) Normal embryo. (C) *hoxa4a* expression is upregulated and extended towards the anterior after 1 hour treatment with RA. (D) DN-RAR mRNA injection blocks *hoxa4a* expression. (E) DN-RAR blocks the ability of RA to activate *hoxa4a* expression.

a dominant-negative capacity. Similarly, we found that the capacity of RA to induce ectopic *insulin*-expressing cells was lost in DN-RAR-injected embryos (data not shown).

Embryos injected with both DN-RARα2a and *rara2b*-MO, hereafter referred to as RAR knock-down (RKD), were used in subsequent experiments as a source of RAR-deficient cells. The inability of *rara2b*-MO to block pancreas development in the absence of DN-RAR may indicate that absence of receptor leads to derepression of downstream targets, as has been observed in *Xenopus* (Koide et al., 2001). In addition, our DN-RAR reagent may block function of RARγ or of other RARs that have not yet been isolated from the zebrafish. In summary, although our results do not directly address which RAR subtype(s) are required for pancreatic development, the RKD reagents allow us to generate embryos that are unable to transduce RA signals while nevertheless expressing the RA synthetic enzyme *raldh2*.

Pancreas development requires RAR function in endoderm and not in surrounding tissues

Although RA is required for pancreas development between 9 and 12 hpf (Stafford and Prince, 2002), the onset of expression of the earliest known pancreatic marker, *pdx1*, is not until ~14 hpf. Thus, there is sufficient time for RA to induce an intermediate signal rather than acting directly on the endoderm itself. Potential sources of this intermediate factor(s) are axial mesoderm or neuroectoderm, or paraxial mesoderm itself. To test the hypothesis that RA acts indirectly on the endoderm, we transplanted wild-type cells into RKD-injected hosts. Labeled donor cells were placed into presumptive paraxial mesoderm in somites 1-7, but in contrast to RALDH2-deficient embryos, these never rescued *insulin* expression (Table 1). Likewise, transplants of axial mesoderm or overlying neural ectoderm never caused a significant increase in the number of *insulin*-expressing cells. Thus, it is unlikely that a primary

Table 1. RAR function is required in endoderm but not in ectoderm or mesoderm for pancreas rescue

Manipulation	*n*	Mean number of IECs	More than six IECs (mean)
1. Unmanipulated controls	44	26.3±5.8	100% (26.3)
2. RKD everywhere	85	1.8±2.6	6% (8.6)
3. RKD host; donor cells to somites	20	2.25±3.42	15% (9.3)
4. RKD host, donor cells to notochord and neural tube	17	1.41±2.83	12% (8.0)
5. Sox32-MO injected (no endoderm)	29	0	0% (0)
6. Sox32-MO hosts; Sox32-expressing donor endoderm	37	17.5±11.9	81% (21.4)
7. Sox32-MO hosts; RKD + Sox32-expressing donor endoderm	26	2.69±3.06	8% (9.0)
8. RKD host; Sox32-expressing donor endoderm	26	8.9±7.5	54% (14.8)
9. Wild-type host; xVP16-RARα1-expressing endoderm	21	32.2±5.2	100% (32.2)

Pancreas development was scored by counting *insulin*-expressing cells (IECs) at 22 hpf. For each manipulation, mean IECs±s.d. is shown. In the far right column, the percentage of specimens with seven or more IECs is shown; in parentheses the mean number of IECs within that subclass is given. Normal donor tissue in the mesoderm or ectoderm of RKD hosts is not sufficient to restore pancreas: compare row 2 with rows 3 and 4. The number of IECs in either class of transplanted embryos was not significantly different from non-transplanted RKD control embryos, as determined by ANOVA and Bonferroni's multiple comparison post-test. Only in endoderm is RKD as effective at preventing pancreas development as RKD throughout the embryo: compare rows 2 and 7. By contrast, RAR function in endoderm only is sufficient to allow pancreas development (compare rows 2 and 8).

response to RA occurs in the mesoderm or ectoderm. As our transplantation techniques did not target lateral plate mesoderm, we did not test the possibility that this tissue relays RA signals. However, our results with endoderm (below) suggest that if such a relay exists, it acts in parallel with RA that signals to endoderm.

To target reagents exclusively to the zebrafish endoderm, we grafted SOX32-expressing donor cells into wild-type or SOX32-deficient hosts. SOX32 functions downstream of the Nodal receptor Taram-A, and is necessary and sufficient for endoderm formation (Fig. 4A) (reviewed by Ober et al., 2003). *casanova* (*cas/sox32*) mutants or embryos injected with a SOX32-targeted MO completely lack endoderm and display no expression of *insulin*, *nkx2.3* (Lee et al., 1996), or the posterior endodermal marker *foxa3* (Odenthal and Nüsslein-Volhard, 1998) (data not shown and Fig. 4B,C). Cells transplanted from donor embryos injected with *sox32* mRNA into SOX32-MO hosts restored development of large regions of the endoderm (Fig. 4D-H). Similar targeting of cells to the endoderm can be achieved by expressing Taram-A in donors (David and Rosa, 2001); however, in our hands larval-stage endoderm morphology appeared more normal in transplants using SOX32 (data not shown).

To determine how well the transplantation method itself restores normal endodermal patterning, we examined *insulin* expression. Endodermal morphology at 24 hpf appeared identical to untransplanted controls in these mosaics, forming a strip along the ventral midline that widened towards the anterior (Fig. 4E,F). In mosaic embryos, however, there were on average only 17.5 *insulin*-expressing cells (*n*=37; Fig. 4E; Table 1), compared with 26.3 (*n*=44; Table 1) in controls. These fell into two classes: 19% with fewer than seven *insulin*-expressing cells, whereas the other 81% averaged 23 *insulin*-expressing cells per embryo. The failure to form pancreas in some cases may reflect the dorsoventral position along the blastoderm margin where donor cells were grafted and their subsequent migration during gastrulation. Transplanted embryos expressed the posterior endoderm marker *foxa3* (*n*=9; Fig. 4F) and the pharyngeal endoderm marker *nkx2.3* (*n*=5; Fig. 4G,H) in the

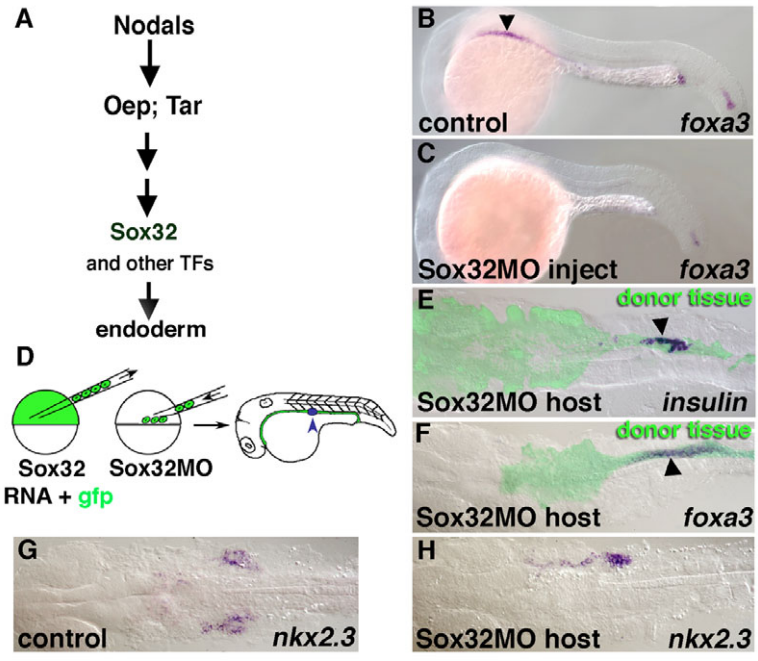

Fig. 4. A transplantation technique using SOX32 to target reagents to specific germ layers. (**A**) Genetic pathway leading to endoderm formation. SOX32 acts downstream of Nodals, their receptors (TAR) and receptor co-factors (OEP) in endodermal specification. (**B**) Lateral view of *foxa3* expression in a 24 hpf control embryo. Endodermal *foxa3* expression extends from slightly anterior of the AP level of somite one (arrowhead) towards the posterior, there is also expression in the posterior tip of the notochord. (**C**) *foxa3* is not expressed in the endoderm of SOX32-MO injected embryos, while notochordal expression in the tail is retained. (**D**) Schematic of the transplantation technique using *sox32* to target donor cells to the endoderm. Donor cells from embryos co-injected with SOX32 and GFP mRNA are placed along the blastoderm margin of a SOX32-MO injected host. Following gastrulation, all endoderm is derived from donor cells, which distribute along the ventral AP axis and exhibit regionalized expression of an array of endoderm-specific markers, including *insulin* (blue arrowhead). (E-H) Transplanted specimens in dorsal view at 24 hpf. (**E**) *insulin* expression (arrowhead) in donor-derived endoderm (green). (**F**) Normal 24 hpf *foxa3* expression (arrowhead) in donor-derived endoderm (green). (**G**) Normal pharyngeal *nkx2.3* expression. (**H**) *nkx2.3* expression in donor-derived endoderm; donor tissue has contributed to only the right side in this example but AP patterning is normal.

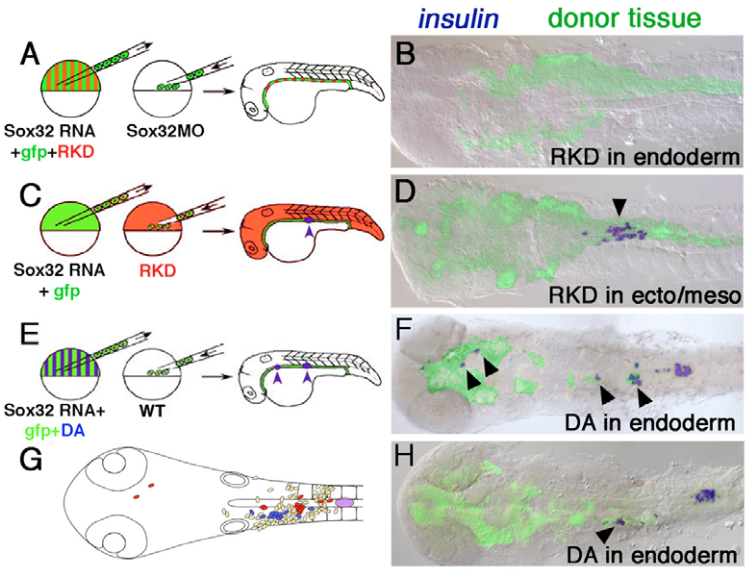

Fig. 5. RAR function is required in endoderm but not mesoderm or ectoderm for pancreas development. (**A**,**B**) RAR function is required in endoderm for pancreas specification. (A) Schematic illustrating transplantation approach, which is essentially the same as that shown in Fig. 3D, except that donor embryos are additionally injected with RKD reagents at the one-cell stage. (B) Dorsal view of 24 hpf embryo generated as in A; no *insulin* expression is present (compare with Fig. 3E). (**C**,**D**) RAR function is not required in mesoderm or ectoderm for pancreas specification. (C) Schematic illustrating transplantation approach. (D) Dorsal view of 24 hpf embryo generated as in C; *insulin* expression is present (blue; arrowhead). (E-H) A constitutively active RAR is able to induce ectopic *insulin*-expressing cells in anterior endoderm. (**E**) Schematic illustrating transplantation approach; DA is dominant active RAR (xVP16-RARα1). (**F**,**H**) Dorsal views of 24 hpf embryos generated as in E. Ectopic donor-derived *insulin*-expressing cells (blue; arrowheads) are found anterior to the first somite. Photographs are bright-field/fluorescent composite images. (**G**) Summary diagram showing locations of all *insulin*-expressing cells in 21 embryos generated as in E; red cells represent embryo in F, blue cells represent embryo in H, yellow cells are from 19 additional specimens.

appropriate AP locations. Thus, targeted transplantation using SOX32 restores pancreas development and does not disrupt regionalization of the endoderm.

To test the hypothesis that RAR function is required in endoderm for pancreas development, we targeted RKD-expressing cells to the endoderm of SOX32-deficient hosts. Donors were co-injected with SOX32 mRNA and RKD (schematized in Fig. 5A). These transplants also formed endoderm with approximately normal morphology that expressed markers such as *nkx2.3* (data not shown). However, *insulin* expression in these mosaic embryos was either absent or severely reduced (Fig. 5B). Of 26 transplanted embryos, 24 had fewer than seven *insulin*-expressing cells (Table 1). This resembled the frequency of β-cell defects observed when RKD reagents were delivered to the entire embryo (94%; Table 1). Thus, disrupting RAR signal transduction in the endoderm alone blocks pancreas as effectively as doing so throughout the entire embryo, suggesting that RAR function in the endoderm is necessary for normal pancreas development.

RAR function is sufficient to bias cells towards an endocrine pancreatic fate

To determine if RAR function in the endoderm is sufficient for pancreas formation, we transplanted endoderm cells into RKD hosts (schematized in Fig. 5C). Fifty-four percent had seven or more *insulin*-expressing cells (n=26; Fig. 5D; Table 1), indicating that the presence of endoderm capable of transducing RA signals is sufficient to allow pancreas specification. We conclude that RAR function need only be present in the endoderm for pancreas specification to occur, strengthening our hypothesis that the signal is direct.

Although we have previously shown that exogenous RA treatment is sufficient to induce anterior endoderm cells to take on endocrine pancreas fates (Stafford and Prince, 2002), such global RA application probably alters positional information in all three germ layers. Thus, this experiment does not discriminate between RA acting as an instructive signal, which confers positional information directly to endoderm, or as a permissive signal, which allows previously specified endoderm cells to complete their differentiation program. To distinguish between these possibilities, we activated RA signal transduction only in the endoderm germ layer by using a constitutively active RAR (*Xenopus* VP16-RARα1) (Blumberg et al., 1997). As expected, this activated receptor expands expression of a RA reporter transgene (RARE-YFP) when microinjected into zebrafish embryos (data not shown) (Perz-Edwards et al., 2001). When we transplanted endoderm cells expressing the constitutively active receptor into otherwise normal embryos (Fig. 5E), we found *insulin*-expressing cells differentiated within anterior endoderm (Fig. 5F-H). All embryos formed a normal-sized pancreas in the wild-type position (Table 1). However, in 21 out of 43 cases, ectopic donor-derived *insulin*-expressing cells were found anterior to the first somite (cell locations summarized in Fig. 5G). These studies confirm that RA signal transduction in the endoderm is able to promote an endocrine pancreatic fate even when adjacent mesoderm is of anterior character.

DISCUSSION

Our study has shown that RA derived from the anterior paraxial mesoderm signals directly to adjacent endoderm to induce pancreatic progenitors. Importantly, our results suggest that RA can act as an instructive signal; RA may therefore play a role not only in specification of the pancreas, but also in its localization along the AP axis. The finding that RA induces β-cell fates directly may have important implications for islet stem cell biology and diabetes.

We have used cell transplantations to show that the RA signal required for zebrafish β-cell development is produced in the anterior paraxial mesoderm. This finding is consistent with previous zebrafish transplantation experiments, which revealed a similar reliance of neural ectoderm regionalization on paraxial mesoderm-derived RA signals (Begemann et al., 2001). It should be noted that although most of the zebrafish β-cells derive from the dorsal pancreatic bud, a few β-cells differentiate at later stages from the ventral bud (Field et al., 2003); the stages at which our experiments were analyzed do not allow us to make conclusions about the role of RA in development of this later differentiating population of β-cells. The requirement for mesodermal RALDH2 function in pancreas development is conserved between fish and mouse (Martin et al., 2005; Molotkov et al., 2005): mice mutant for *Raldh2* do not form a dorsal pancreatic bud. The expression pattern of mouse *Raldh2*

suggests that the splanchnic component of the lateral plate mesoderm is the crucial source of RA for pancreas specification in this species, as this mesoderm is juxtaposed with the dorsal pre-pancreatic endoderm at the onset of expression of the pancreas progenitor marker *Pdx1* (Martin et al., 2005; Molotkov et al., 2005). Similarly, explant studies in chick have suggested a crucial role for the lateral plate mesoderm in conferring pancreatic identity (Kumar et al., 2003). By contrast, our transplants reveal that RA produced in paraxial mesoderm is sufficient to induce the zebrafish pancreas. As our transplants did not target lateral plate mesoderm, RA from this source might also be capable of inducing pancreas in zebrafish. Alternatively, this apparent discrepancy between species may reflect differences in the relative locations of germ layer derivatives during gastrulation of different vertebrates**.**

During the course of this study, we developed a novel transplantation technique using activity of the SOX32 transcription factor to direct or exclude reagents from the endoderm germ layer. Our transplantations of RAR-deficient cells into the gut show that the RA signal required for zebrafish pancreas specification is received directly by the endodermal cells. These data rule out an indirect model for RA-mediated pancreas induction in which RA first confers positional information to an intermediate tissue, such as the paraxial mesoderm, which subsequently signals to the endoderm. Although RA had previously been shown to play a role in endoderm regionalization (Chen et al., 2004; Kumar et al., 2003; Martin et al., 2005; Molotkov et al., 2005; Stafford et al., 2004; Stafford and Prince, 2002), this is the first direct demonstration that mesoderm-derived RA signals directly to endoderm cells to induce pancreatic fates. Consistent with our finding, reporters driven by RA response elements (RAREs) are expressed in the mouse pancreatic endoderm (Martin et al., 2005; Molotkov et al., 2005). Martin and colleagues (Martin et al., 2005) have also reported low level RARE reporter activity in adjacent pancreatic mesenchyme, suggesting that RA signals are additionally received by the mesoderm. However, our transplantation results show that, at least in zebrafish, RA receptor function in mesoderm is not required for pancreas specification.

Signals required for pancreas development may function in either a permissive or an instructive fashion: for our purposes we define permissive signals as allowing cells to continue along a pre-specified differentiation program, whereas instructive signals confer positional information. We have shown that a dominant active RA receptor is capable of conferring β-cell fate on anterior endoderm, suggesting that RA acts as an instructive signal. However, it should be noted that in these experiments only a small proportion of the anterior endoderm cells expressing active RA receptor go on to express *insulin*, and further that the majority of these *insulin*-positive cells are located posterior to the level of the otic vesicle. These findings may imply that additional signals normally act at foregut level to further bias progenitor cells towards a β-cell fate, and that these signals are lacking from the most anterior part of the embryo. Alternatively, they may merely suggest that the *Xenopus* dominant-active receptor used does not have full function in the zebrafish. Explant studies in chick (Kumar et al., 2003) have shown that RA is not able to induce pancreatic markers in explants of anterior endoderm cultured in the absence of mesoderm, revealing that a second mesoderm-derived signal in addition to RA is necessary for pancreas specification. Our experiments suggest that mesoderm anterior to the first somite can supply this second signal, implying that the signal is RA independent and permissive in nature.

A major determinant of pancreas position may be the localized source of mesoderm-derived RA. This source is dependent on activity of both the synthetic RALDH2 enzyme and the RA degrading Cyp26 enzymes (Kudoh et al., 2002; Sakai et al., 2001). In future studies, it will be important to determine the precise locations of the pancreatic progenitors during gastrulation stages, in order to deduce whether localized activity of RA is sufficient, in turn, to localize the pancreas. Finally, as an instructive signal, RA is likely to prove a crucial component of protocols to induce stem cells to differentiate into pancreatic β-cells. Consistent with this, a novel three-step approach to inducing β-cells from stem cells, using both Activin A and RA, has recently been reported (Shi et al., 2005).

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