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Morphological defects in a Novel *Rdh10* Mutant that has Reduced Retinoic Acid Biosynthesis and Signaling

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Abstract

Retinoic acid (RA) signaling is necessary for proper patterning and morphogenesis during embryonic development. Tissue specific RA signaling requires precise spatial and temporal synthesis of RA from retinal by retinaldehyde dehydrogenases (Raldh) and the conversion of retinol to retinal by retinol dehydrogenases (Rdh) of the short-chain dehydrogenase/reductase gene family (SDR). The SDR, retinol dehydrogenase 10 (RDH10), is a major contributor to retinal biosynthesis during mid-gestation. We have identified a missense mutation in the *Rdh10* gene (*Rdh10*^{m366Asp}) using an N-ethyl-N-nitrosourea (ENU)-induced forward genetic screen that result in reduced RA levels and signaling during embryonic development. *Rdh10*^{m366Asp} mutant embryos have unique phenotypes, such as edema, a massive midline facial cleft, and neurogenesis defects in the forebrain, that will allow the identification of novel RA functions.

Keywords

Retinol dehydrogenase; ENU-induced mutagenesis; embryo development; organogenesis

Introduction

All-*trans*-retinoic acid (atRA) is a biologically active form of vitamin A, which acts through nuclear RA receptors (RAR) to support diverse aspects of vertebrate life, including embryonic development (Maden, 2001; Mark *et al.*, 2006). Retinol is converted to atRA in two distinct steps. The second step involves the oxidation of retinal into RA and is catalyzed by retinaldehyde dehydrogenases (Raldh), of which there are three family members

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expressed during embryogenesis (Raldh1-3; Napoli, 2011). *Raldh2* KO mice have been invaluable for uncovering the biological functions of RA during mouse embryo development, such as anterior-posterior axis formation, limb bud initiation, heart formation, craniofacial development, hindbrain patterning, and bilateral symmetry of somite formation. However, *Raldh2* KO embryos die around E10, precluding analysis and identification of the physiological significances of RA at later stages unless RA is provided exogenously (Niederreither *et al.*, 2002).

Retinol dehydrogenases (Rdh) catalyze the first and rate-limiting reaction of atRA biosynthesis, *i.e.* the oxidation of retinol to retinal, and are essential for controlling retinol homeostasis, atRA biogenesis, and vitamin A function (Napoli, 1999). Since there are over 20 RDH family members (Lidén and Eriksson, 2006), it was unclear whether any one family member would be identified as being essential for embryonic development. Recently, Sandell *et al.*, (2007) identified *Rdh10*, a member of the short-chain dehydrogenase family (Napoli, 2011), as having such an attribute. RDH10 protein primarily oxidizes all-*trans* retinol to all-*trans* retinal (Wu *et al.*, 2002; Belyaeva *et al.*, 2008; Farjo *et al.*, 2009; Takahashi *et al.*, 2009; Farjo *et al.*, 2011). Embryos lacking functional RDH10 protein die around E13 and display limb, craniofacial, and internal organ abnormalities, thereby identifying *Rdh10* as a critical enzyme for the synthesis of retinaldehyde during mid-gestation (*Rdh10^{trax/trax}*: Sandell *et al.*, 2007; *Rdh10^{-/-}*: Rhinn *et al.*, 2011). The *Rdh10^{trax/trax}* mutant was generated in an ENU-induced forward genetic screen and carries a cysteine-to-arginine amino acid substitution in the first β -strand that renders the mutant protein unstable (Sandell *et al.*, 2007; Farjo *et al.*, 2011).

ENU is an efficient chemical mutagen that induces single nucleotide substitutions, thereby making it an ideal reagent for performing unbiased forward genetic screens in mice to study specific phenotypes with a corresponding genetic basis for morphological defects. We have reported implementation of a focused ENU-screen that generated multiple mouse mutants with defects in cortical development (Zarbalis *et al.*, 2004). Here we describe the genetic basis for one of these mutants (mouse mutant line 366) as a missense mutation in the *Rdh10* gene (*Rdh10^{m366Asp}*). The *Rdh10^{m366Asp}* allele is a strong hypomorph that allows embryos to survive until embryonic day 17.5 (E17.5), providing a model to analyze RA functions throughout mid to late gestation.

Results

Morphological defects in line 366 mutants

To identify novel molecular regulators of cortical development, we carried out a focused ENU-induced forward genetic screen for recessive mutations that disrupt corticogenesis (Zarbalis *et al.*, 2004). One of the mutant lines generated in this screen, line 366, dies around E17.5, although a few embryos were collected near the end of gestation (E18.5) and one live birth (P0) was observed. Numerous morphological defects are easily identifiable. The entire eye is smaller, ventral retina tissue is absent, and eyes are displaced medially (Fig. 1a,b,d,e). There is also a very prominent and consistent midline facial cleft (Fig. 1a-c), signs of which are first evident at E10.5 when the medial nasal prominences (MNP) fail to enlarge. Line 366 mutant embryos display signs of edema as early as E12.5, a condition that continues to

worsen over time (Fig. 1f,g). Limb defects are largely restricted to the forelimbs, which are shorter and have missing digits. Remaining digits are frequently fused together (Fig. 1h). Mild interdigital webbing is evident in both fore- and hindlimbs (Fig. 1i). The forebrain has a dome-shaped appearance with a significantly shorter anterior-posterior axis and enlarged ventricles (Fig. 1a,b,j,k). The cerebral cortex is thin and elongated, the ganglionic eminences have a flattened appearance and the olfactory bulbs are missing, all of which are consistent with defects in neurogenesis (Fig. 1j,k).

Cloning of the line 366 mutation

We mapped the line 366 mutation to a 100 MB interval on chromosome 1 (D1Mit296-D1Mit169; Zarbalis *et al.*, 2004). Fine mapping identified a missense mutation (C to T) in the *Rdh10* gene (*Rdh10*^{m366Asp}) that causes an alanine-to-valine substitution at amino acid residue 196 in the substrate-binding pocket (A196V; Fig. 2a). Alanine¹⁹⁶ is highly conserved, as are the flanking 14-15 amino acids on either side. Deviations in flanking amino acids only occur in zebrafish and *Xenopus* *Rdh10* homologs (Fig. 2b).

Characterization of the *RDH10*^{m366Asp} mutation on RA biosynthesis *in vitro* and *in vivo*

Genetic alterations that result in either RA depletion or RA excess have very similar phenotypes (McCaffery *et al.*, 2003), thus we sought to determine how the *RDH10*^{m366Asp} mutation affects RA biosynthesis *in vitro* and *in vivo*. To do this, wild-type *RDH10* or *RDH10*^{m366Asp} cDNA constructs were co-transfected with retinaldehyde dehydrogenase 2 (RALDH2) into CHO cells. Cell extracts were assayed for atRA production one hour after the addition of the substrate all-*trans*-retinol. *RDH10*^{m366Asp} protein produced 5-fold less atRA compared to wild-type *RDH10* (Fig. 3a).

Our *in vitro* studies suggest that *Rdh10*^{m366Asp} mutant embryos have reduced RA levels during development. To test this notion, two independent methods were employed to assay for RA levels *in vivo*. First, RA levels in whole embryos were assayed using a sensitive LC (liquid chromatography)/MS (mass spectrometry)/MS assay (Kane *et al.*, 2005). We found that atRA levels are approximately 23%, 66% and 50% reduced in mutant embryos compared to wild-type littermates at E10.5, E12.5, and E14.5, respectively (Fig. 3b). Our second approach was to cross *Rdh10*^{m366Asp} mice to a mouse strain in which *lacZ* reporter gene expression is controlled by a RA response element (RARE-LacZ mice) to identify changes in bioactive RA in a tissue-specific manner (Rossant *et al.*, 1991). At E10.5, E12.5 and E14.5, there were significant reductions in LacZ staining in *Rdh10*^{m366Asp} embryos (Fig. 3d; also see Fig 4a,b), and at E10.5 changes in LacZ staining closely overlap with regions of *Rdh10* expression (compare Fig. 3c with Fig. 3d-E10.5). Collectively, these data indicate that the *RDH10*^{m366Asp} protein has a reduced ability to synthesize all-*trans*-retinal from all-*trans*-retinol, and that *RDH10* has a crucial role in RA biosynthesis during mouse embryo development.

Exogenous RA rescues defects in *Rdh10*^{m366Asp} mutants

To verify that the morphological defects in developing *Rdh10*^{m366Asp} mutant embryos result from depleted endogenous RA levels, we attempted to rescue the mutant phenotypes. Pregnant females were switched from a normal chow diet to one supplemented with atRA

starting at E7.5 and maintained until E14.5. With RA supplementation, the limb, face, eye, limb and forebrain defects were all rescued (Fig. 4). The only morphological feature distinguishing *Rdh10*^{m366Asp} mutants from wild-type embryos was the presence of edema, which was not rescued by the method we employed.

Discussion

RDH10 is a primary regulator of the first enzymatic step in RA biosynthesis during embryogenesis despite earlier predictions that this initial rate-limiting step was shared by multiple RDH enzymes, some of which are ubiquitously expressed (Zhang *et al.*, 2001). This discovery, and the generation of two independent *Rdh10* mutants, highlights the power of the ENU-induced mutagenesis procedure for identifying critical regulators of mammalian development. The *Rdh10*^{m366Asp} mutant has a very conservative alanine-to-valine substitution, as both amino acids are hydrophobic, non-polar, uncharged and are relatively similar structurally. The detriment of this mutation on RDH10 function appears to be due to its localization in the ligand binding pocket, which is highly susceptible to even minor perturbations. *In vitro* studies found that mutating Ser¹⁹⁷ in human RDH10 (Takahashi *et al.*, 2009) or other SDR family members (Cols *et al.*, 1997; Filling *et al.*, 2002), a key member of a highly conserved Asn-Ser-Tyr-Lys non-linear tetrad of residues within the pocket, abolishes enzymatic activity. Specifically, the Ser¹⁹⁷ residue is responsible for stabilizing substrate in the catalytic center. Our mutant disrupts an adjacent amino acid suggesting that the Alanine¹⁹⁶ to Valine¹⁹⁶ missense mutation also disrupts substrate stability. Mutations at Ser¹⁹⁷ however, do not affect protein stability (Takahashi *et al.*, 2009), consistent with our findings for the RDH10^{m366Asp} protein *in vivo*, indicating that the instability we observed in our *in vitro* studies is probably related to the N-terminal FLAG tag.

Our RA measurements have provided us with a global view of how RA levels are altered over developmental stages due to significantly depleted RDH10 function. At (early) E10.5, there is a 23% reduction in RA levels in whole embryos. Shortly after, there is a noticeable reduction in RA reporter signal and initial morphological defects are apparent (truncated MNPs). This data suggests that RA levels are being maintained near threshold levels and modest reductions lead to morphological changes, and presumably, corresponding and underlying molecular changes. At E12.5, there is a 66% reduction in RA levels, indicating that RDH10 provides a substantial portion of total RA at E12.5 (*i.e.* greater than 66%, since the *Rdh10*^{m366Asp} mutant is a hypomorphic allele). Others have shown that impaired *Rdh10* function leads to embryonic lethality prior to E13 (Sandell *et al.*, 2007; Rhinn *et al.*, 2011). Here we show quantitatively that this lethality correlates with a steep, approximately 3-fold, reduction in RA levels from E10.5 to E12.5 due to RDH10 “absence”. At 14.5, RA levels slightly recover to 50%, indicating that RDH10 continues to generate most of the *all-trans*-retinal that is used to generate RA at this stage. *Rdh10*^{m366Asp} mutants maintain survival under such RA deprivation. The reduced RA levels in the *Rdh10*^{m366Asp} mutant correlate with previous studies where a 50% reduction in RA levels in the developing cortex, and 20% reduction in the meninges, of a *Foxc1* mutant lead to significant defects in corticogenesis, and a 42-53% decrease in fetal retinoids at E12.5 resulted in aberrant enteric nervous system precursor cell migration by reducing Pten accumulation (Fu *et al.*, 2010).

Crossing *Rdh10^{trrx}* mutants or an *Rdh10* null (Sandell *et al.*, 2007; Rhinn *et al.*, 2011) with a RA-reporter has provided insight into where tissue-specific alterations in RA signaling occur at stages E8.5 and E9.5. The reduction in RA signaling from such a cross is far less severe than that observed in *Raldh2^{-/-}* embryos, and is more on par with *Raldh2^{-/-}* mice rescued by RA supplementation (Mic *et al.*, 2002). Here we have performed a similar cross with *Rdh10^{m366Asp}* mutants but analysis was performed at stages E10.5-E14.5. Whole embryo staining clearly identifies a global reduction in RA-reporter signal at all stages. Most dramatic changes include the facial mesenchyme and eye tissue. There is also significant signal reduction in the limb and trunk mesenchyme, but some reporter activity persists in the spinal cord and forebrain. Our quantitative RA measurement studies identified a steep drop in RA levels from E10.5 to E12.5 that is clearly evident in our RA-reporter studies. This data identifies tissues to which RDH10 contributes greatest RA signal and these tissues closely correlate with morphological defects in *Rdh10^{m366Asp}* mutants. Interestingly, and in contrast to earlier stages (E8.5-9.5), at E10.5 and E12.5 RA-reporter signal is far more reduced in *Rdh10^{m366Asp}* mutants than in partially rescued *Raldh2^{-/-}* embryos (Niederreither *et al.*, 2002), indicating that RDH10 has a far more prevalent role in regulating RA synthesis, and thus RA signaling (between E10.5 and E12.5), than does RALDH2.

The *Rdh10^{m366Asp}* mutant has many phenotypes that are morphologically very similar to those in the *Rdh10^{trrx/trrx}* mutant and *Rdh10^{-/-}* embryos, such as eye, internal organ, and limb defects (Sandell *et al.*, 2007; Farjo *et al.*, 2011; Rhinn *et al.*, 2011; Cunningham *et al.*, 2011), however it also has unique defects that will further our understanding of RA biology. For example, the *Rdh10^{m366Asp}* mutant consistently has a very prominent midline nasomaxillofacial cleft, which is also present in retinoic acid receptor (RAR) α and γ double knockout mice (Lohnes, 1994) but is variably seen in *Rdh10^{trrx/trrx}* mutants (Sandell *et al.*, 2007; Farjo *et al.*, 2011). *Rdh10^{-/-}* embryos display severe nasal/facial clefting, however it rarely survives to E12.5 allowing only limited analysis of the facial skeletal (Rhinn *et al.*, 2011). The edema phenotype suggests a role for RDH10 in cardiac or circulatory function. RA very clearly functions in heart development, for example cardiac looping, ventricular maturation, atrial septation, and myocardial trabeculation (Niederreither *et al.*, 2001; Rhinn *et al.*, 2011), however the late stage survival and commonly seen areas of pooled blood suggest that vascular defects may be the underlying problem in *Rdh10^{m366Asp}* mutants. Analysis of *Raldh2^{-/-}* embryos identified a role for RA in vascular morphogenesis (Bohnsack *et al.*, 2004). Malformation of the forebrain is also prevalent, and to the best of our knowledge, the complete absence of olfactory bulbs is novel to the *Rdh10^{m366Asp}* mutant. Another phenotype in the forebrain, specifically the observed defects in corticogenesis, was the catalyst for studies that identified RA as a critical inducer of neurogenesis in the cortex via signaling from the meninges (Siegenthaler *et al.*, 2009). Chatzi *et al.*, (2011) have suggested that neurogenesis defects in *Rdh10^{m366Asp}* mutants are secondary to neural crest-derived craniofacial defects that distort the cranium. The ideal model to clarify this matter is the *Rdh10^{m366Asp}* mutant, where this defect was first identified, by temporarily providing RA supplementation during neural crest cell formation/migration and then analyzing corticogenesis. Finally, the survival of *Rdh10^{m366Asp}* embryos to later stages in development allows analysis of unique developmental processes, and together with the potential analysis of the phenotypes mentioned, the *Rdh10^{m366Asp}* mutant

will be an invaluable tool for furthering our understanding of retinoid metabolism and the biological functions of retinoids during fetal development.

Materials and Methods

Histological Sections

Mouse embryos were dissected in cold PBS and fixed overnight in 4% paraformaldehyde (PFA) at 4°C. The following day, PFA was washed away and embryos processed using a graded ethanol series to 100% ethanol. Xylene washes then removed ethanol, and embryos were subsequently embedded in Paraplast wax. Serial sections were collected at 10µm thickness, mounted onto glass slides and stained using cresyl violet (Nissl stain).

Cell Transfection

CHO-K1 cells were co-transfected with 1 µg each of either pBSK-N-terminal FLAG-mRDH10 or pBSK-N-terminal FLAG-mRDH10^{m366Asp} and pBSK-RALDH2. Cells were seeded at ~60% confluency into 6-well plates and transfected the next day with Lipofectamine reagent (Invitrogen). Each transfection was done in triplicate wells. A luciferase control vector (Promega) was transfected into all wells to normalize for transfection efficiency. Luciferase activity was measured using the Luciferase assay system (Promega). Medium was replaced with fresh medium without serum 24 hr after transfection. HPLC-purified retinol was added at 2.0 µM to the medium under yellow lights and incubated at 37 °C for 1 hr. Medium was removed and cells were lysed in 1 ml of reporter lysis buffer (Promega). After a freeze-thaw cycle, cells were combined with their medium and extracted. Protein concentrations were determined by the Bradford Assay (Bio-Rad).

Western Blotting

For *in vitro* studies, 10 µg of cell lysate was electrophoresed on 10% SDS-PAGE gels, transferred to nitrocellulose membranes, and blocked with PBS containing 5% nonfat milk. The membrane was then incubated with anti-flag antibody (Sigma) diluted 1:2000 for 1 hr at room temperature. After washing, the membrane was incubated with an anti-mouse secondary antibody conjugated to horseradish peroxidase (Sigma) for 1 hr. Staining for β-actin (antibody from Sigma) was used to control for loading. Signals were detected using an enhanced chemiluminescent substrate (Pierce) and were quantified by densitometry. A similar approach was used for Western blotting using embryo tissues (Ashique *et al.*, 2009). The anti-RDH10 polyclonal antibody used was raised in a rabbit using the C-terminal peptide (327-341aa) of RDH10.

Tissue Preparation for Retinoid Extraction

Samples were processed under yellow light. Mouse embryos were dissected using a Nikon SMZ-10A dissection microscope equipped with a Volpi (Auburn, NY) NCL 150 light source with a yellow filter. Whole embryo bodies were immediately placed on dry-ice and kept frozen until they were homogenized in 1 mL ice-cold 0.9% NaCl with a ground-glass hand homogenizer. Protein concentrations were determined by the Bradford Assay (Bio-Rad) and retinoid concentrations are expressed per g total protein.

Retinoid Extraction

Cell and tissue samples were extracted as previously described using a liquid-liquid extraction (Kane *et al.*, 2005). Only glass containers, pipettes, and calibrated syringes were used to handle samples, because retinoids adhere to plastic, which can cause losses up to 40%. Resuspended samples were analyzed immediately.

LC/MS/MS Analysis of RA

We measured atRA in embryo bodies with a highly sensitive and specific LC/MS/MS assay that distinguishes atRA from its isomers, and provides definitive mass identification (Kane *et al.*, 2005; Kane and Napoli, 2010). An internal standard (4,4-dimethyl-atRA) was used routinely and established recovery of endogenous atRA at 84%. Briefly, resolution of atRA from its isomers was effected with a Supelco ABZ+PLUS 100 × 2.1 mm, 3 μm C-16 alkylamide column.

Data were collected with an Applied Biosystems API-3000 LC/MS/MS system equipped with an atmospheric pressure chemical ionization (APCI) source operated in positive ion mode (Kane *et al.*, 2005). All data were collected in SRM (selected reaction monitoring) mode, in which the following SRM transitions were monitored (precursor ion to product ion): RA, m/z 301.1 to 205.0; 4,4-dimethyl RA, m/z 329.4 to 151.3. RA isomer retentions were verified with authentic standards. Retinoid standards purchased from Sigma-Aldrich (St. Louis, MO) were prepared fresh on the day of use and concentrations were verified spectrophotometrically from ε values (Dawson *et al.*, 1981; Barua and Furr, 1998).

RA rescue

The standard normal chow diet (NCD) of pregnant females was changed to one supplemented with atRA starting at E7.5. Mice were transferred to a fresh cage. The RA supplemented diet was given sparingly, although in excess of daily requirement, which mice could consume ad libidinum. Fresh food was provided daily and cages were protected from excess light since RA is light sensitive. RA dosage was based on previous reports (Niederreither *et al.*, 2002). At E7.5, NCD was supplemented with RA at a concentration of 100 μg/g. From E8.5 to E14.5, RA was supplemented at a dose of 250 μg/g.

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Figure 1. Morphological defects in ENU-induced mutant line 366 embryos

Wild-type (WT) (a) and mutant (Mut) (b) embryos at E14.5 expressing a *Dlx-LacZ* reporter (Zerucha *et al.*, 2000) that was used in the mutagenesis screen (see Zarbalis *et al.*, 2004). (c) Frontal view of a mutant embryo at (E14.5) displaying very prominent midline facial cleft, while the lower jaw forms normally. (d,e) Transverse sections through the developing eyes at E12.5. (f,g) Whole embryos at E16.75. Note the inflated skin in the mutant that is characteristic of edema (g). (h) Left forelimbs from mutant compared to wild-type limb at E14.5 showing example of limb defects, which are variable. (i) Right hindlimb from mutant and wild-type limb showing mild interdigital webbing (asterisk). (j,k) Sagittal section through the brain at E16.5. Asterisks marks the position of the absent olfactory bulbs in mutant (k).

a*Rdh10/Rdh10^{m366Asp} Mutant*

Base pair #588 CATTGTGACGGT**T**CGCGAGCTCCCTGGGACT
 Amino acid #196 --I--V--T--**V**--A--S--S--L--G--L

b

RDH10 ^{m366Asp}	LPTMLEINHG	HIVTV V SSLG	LFSTAGVEDY
Mammalian	LPTMLEINHG	HIVTVASSLG	LFSTAGVEDY
Zebrafish	LPKMLELNHG	HIVTVASSLG	LF T TAGVEDY
Xenopus	LPK M MEMNHG	HIVSVASSLG	LFSTAGVEDY

Figure 2. Mutant line 366 carries a mutation in the *Rdh10* gene

(a) Base-pair sequence and corresponding amino acid sequence of a region of the *Rdh10* gene and RDH10 protein, respectively. Highlighted are the mutated nucleotide and corresponding amino acid. The alanine-to-valine missense mutation is a conservative change, with valine having a pair of methyl groups replacing two hydrogen atoms on the amino acid side chain of the alanine residue. (b) Amino acid #196 of the RDH10 protein is highly conserved amino acid residue. Also highly conserved are the residues surrounding Alanine¹⁹⁶. In the segment of RDH10 protein depicted, there is 100% conservation among humans, bovine, rat and mouse (summarized as mammalian). Some divergence begins to appear in amino acids nearby Alanine¹⁹⁶ in zebrafish and *Xenopus*.

Figure 3. The *Rdh10^{m366Asp}* mutation reduces RA biosynthesis

(a) On top is a representative western blot of N-terminal-FLAG-tagged RDH10 and N-terminal-FLAG-tagged mutant RDH10^{m366Asp} after transfection of CHO cells. Mutant protein levels were consistently 40% less than wild-type, presumably because of either reduced translation and/or stability. At bottom, CHO cells co-transfected with FLAG-tag WT-RDH10 or FLAG-tagged Mut-RDH10^{m366Asp} and RALDH2 biosynthesize atRA. Retinol was incubated with transfected cells for 1 hr (n=3 wells assayed independently). Background atRA values produced by cells transfected with empty vectors and RALDH2 were subtracted to provide net pmol RA. Net pmol RA values for RDH10^{m366Asp} mutant and wild-type RDH10 proteins were normalized to differences in protein levels. A significant difference between wild-type RDH10 and mutant RDH10^{m366Asp} was found, **p*

= 0.008. Comparable data were reproduced at least three times under identical conditions. **(b)** On top is a representative western blot of RDH10 protein from whole wild-type or *Rdh10*^{m366Asp} embryos at E10.5 and E13.5. The RDH10 protein migrates at ~39kDa, as expected. Unlike our *in vitro* studies, we did not detect any appreciable difference in RDH10 protein levels from whole embryos as a result of the A196V mutation. At bottom, RA levels in whole wild-type and *Rdh10*^{m366Asp} embryos at three embryonic stages expressed as pmol/g protein $\pm$ SEM. Since morphological defects in *Rdh10*^{m366Asp} mutants are first apparent after E10.5, as indicated by hypoplasia of the MNPs, we wanted to assay for differences in RA levels just prior to seeing these initial defects. We also examined subsequent stages (E12.5 and E14.5) to determine whether changes in RA levels persist or recover. Number of embryos analyzed at each embryonic age (wild-type/mutant): E10.5, 11/13; E12.5, 6/4; E14.5, 5/5; **p* < 0.04. **(c)** *Rdh10* expression at E10.5. Note how closely the expression of *Rdh10* overlaps with endogenous RA signaling **(d)** at E10.5. **(d)** Sagittal view of whole embryos at E10.5 and E12.5, and E14.5 head, stained for β -galactosidase activity after crossing the *Rdh10*^{m366Asp} mutant to a RA-reporter strain (Rossant *et al.*, 1991). Reduced β -galactosidase staining is observed at all stages. See also Figure 4a,b for anterior view of head at E14.5 after β -galactosidase staining.

Figure 4. *Rdh10*^{m366Asp} mutant phenotype is rescued by RA supplementation

(a) Top, anterior view of head shows complete formation of facial midline in wild-type mice at E14.5. Bottom, forelimb and hindlimbs. (b) *Rdh10*^{m366Asp} embryos displaying face, brain and limb phenotypes. (c) *Rdh10*^{m366Asp} embryos fed a RA supplemented diet. The severe limb and craniofacial defects were rescued to such an extent that they were indistinguishable from wild-type embryos at E14.5. Limb outgrowth and digit patterning were morphologically normal. The midline facial cleft was non-evident as the snout formed normally. In addition, the eyes were the same size as wild-type embryos, and *Rdh10*^{m366Asp} mutant embryos no longer had a domed forebrain region.