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Resource

# Identification of novel retinoic acid target genes

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ABSTRACT

Retinoic acid is required for diverse ontogenic processes and as such identification of the genes and pathways affected by retinoic acid is critical to understanding these pleiotropic effects. The presomitic mesoderm of the E8.5 mouse embryo is composed of undifferentiated cells that are depleted of retinoic acid, yet are competent to respond to the retinoid signal. We have exploited these properties to use this tissue to identify novel retinoic acid-responsive genes, including candidate target genes, by treating E8.5 embryos with retinoic acid and assessing changes in gene expression in the presomitic mesoderm by microarray analysis. This exercise yielded a cohort of genes that were differentially expressed in response to exogenous retinoic acid exposure. Among these were a number of previously characterized retinoic acid targets, validating this approach. In addition, we recovered a number of novel candidate target genes which were confirmed as retinoic acid-responsive by independent analysis. Chromatin immunoprecipitation assays revealed retinoic acid receptor occupancy of the promoters of certain of these genes. We further confirmed direct retinoic acid regulation of the *F11r* gene, a new RA target, using tissue culture models. Our results reveal a significant number of potential RA targets implicated in embryonic development and offer a novel *in vivo* system for better understanding of retinoid-dependent transcription.



## Introduction

Retinoic acid (RA), the principal active metabolite of vitamin A, plays fundamental roles in a number of developmental processes ranging from axial patterning and cranio-facial development to patterning of the central nervous system and organogenesis of multiple systems (Ross et al., 2000; Blomhoff and Blomhoff., 2006; Mark et al., 2006; Niederreither and Dolle, 2008). RA is also required in the adult, where it plays roles in processes such as learning, memory, immune function and reproduction (Blomhoff and Blomhoff, 2006; Altucci et al., 2007).

The pleiotropic effects of RA are mediated by RARα, RARβ and RARγ, members of the nuclear receptor superfamily which function as ligand-dependent transcription factors. RARs form heterodimers with the retinoid X receptors (RXRs), and mediate transcription by binding as RXR–RAR heterodimers to *cis* acting RA response elements (RAREs) found in the promoter or enhancer regions of target genes (Chambon, 1996; Balmer and Blomhoff, 2002, 2005; Blomhoff and Blomhoff, 2006). Canonical RAREs consist of a direct repeat of the sequence 5′-PuG(G/T)(T/A)CA-3′, typically separated by five spacer nucleotides and referred to as a DR5 element (Perlmann et al., 1993; Mangelsdorf and Evans, 1995; Bastien and Rochette-Egly, 2004). Notably, although such DR5 motifs have been described for a number of RA targets, many retinoid-responsive genes harbor variant RAREs (Balmer and Blomhoff, 2005), complicating assignment of direct retinoid regulation by algorithm-based methods.

RA deficiency or excess or genetic disruption of RARs all result in diverse developmental defects (Conlon, 1995; Marshall et al., 1996; Mark et al., 2006, 2009). Together with reporter-based analysis of biologically active retinoid signaling (Rossant et al., 1991), these studies underscore a critical need for tight regulation of RA levels and distribution in the developing embryo. This bio-availability is controlled by the opposing actions of RA biosynthesis, including generation of RA by retinaldehyde dehydrogenase (RALDH) family members, and the degradation of RA by the cytochrome P450 family members CYP26A1, CYP26B1 and CYP26C1 (Maden et al., 1998; Niederreither et al., 1999; Abu-Abed et al., 2003; Duester, 2008; Ross and Zolfaghari, 2011). Notably, the expression patterns of CYP26A1, B1 and C1 are typically complementary to that of the RALDHs (White et al., 1996; Fujii et al., 1997; Ray et al., 1997; Hollemann et al., 1998; Duester, 2008; Uehara et al., 2009), and disruption of retinoid metabolism through targeted deletion of CYP26 or RALDH family members can evoke developmental defects reflective of RA excess or deficiency, respectively.

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Central to understanding the biological roles of RA is the identification of retinoid target genes. Microarray and RNA-seq technologies as well as Chromatin Immunoprecipitation (ChIP)-seq methods, have revealed changes in gene expression evoked by exogenous RA and genomic loci occupied by RARs, although such work has largely been restricted to tissue culture models (Kim et al., 2009; Delacroix et al., 2010; Mahony et al., 2011; Akanuma et al., 2012; Moutier et al., 2012). While such models are useful systems, they do not necessarily reflect the *in vivo* regulatory hierarchies governed by RA, and as such our understanding of the nature of RA target genes involved in development is incomplete.

The pre-somitic mesoderm (PSM) of the developing mouse embryo represents a progenitor zone that is composed largely of poorly differentiated tissue destined to contribute to the elongating axis. As development progresses, the anterior PSM condenses at regular intervals to form somites, with tissue being replenished by the addition of new cells provided by a proliferative precursor population in the tail bud (Aulehla and Pourquie, 2010; Benazeraf and Pourquie, 2013). The proliferation and subsequent differentiation of the PSM is controlled by a network of factors including RA which is generated in the trunk region just anterior to the undifferentiated PSM (Ribes et al., 2009). The cells in the caudal progenitor cell region, in contrast, express *Cyp26A1* which is believed to protect this population against precocious exposure to RA (Fujii et al., 1997; Iulianella et al., 1999; Swindell et al., 1999). This is underscored by the finding that loss of *Cyp26A1* phenocopies many of the developmental defects that are elicited by exogenous RA administered at E8.5 (Abu-Abed et al., 2001; Sakai et al., 2001). Moreover, while *Cyp26A1* does not require RA for basal expression, as evidenced by its expression in RALDH2 mutants (Molotkova et al., 2005), it is directly regulated by exogenous RA in an RARγ-dependent manner, suggesting a feedback mechanism to strictly regulate posterior RA levels (Lohnes et al., 1994; Abu-Abed et al., 2003). In addition to *Cyp26A1*, RA also impacts expression of a number of additional retinoid target genes in the caudal embryo, such as *Cdx1* (Houle et al., 2000, 2003). The finding that *Cdx1* expression, but not *Cyp26A1,* decreases significantly in the caudal region of *Raldh2*−/− mutant embryos (Zhao and Duester, 2009) further suggests at least two classes of RA targets in the PSM; those that are expressed at a basal level independent of retinoid signaling, but induced by RA, such as *Cyp26A1*, and those that absolutely require RA for expression, such as *Cdx1.*

The above observations indicate that the E8.5 caudal precursor population is poised to respond to RA and therefore appears to be a viable population to exploit for identification of target genes relevant to retinoid-dependent developmental programs. To this end, we used microarray analysis to identify genes impacted by RA in the PSM 3 h post-exposure. This analysis revealed a number of genes previously shown to be RA-responsive, including known direct target genes. In addition, we recovered a number of genes that have not been previously identified as RA-responsive. We confirmed RA-regulation of several such genes, and used a newly generated anti-RARγ antibody to demonstrate RARγ occupancy of certain of these candidate targets. Our findings reveal a number of potential new direct retinoid targets, and support the use of this model system to identify RA targets relevant to development.

## Material and methods

### *Mice*

CD1 mice were mated overnight and noon of the day of the vaginal plug was taken as embryonic day (E) 0.5. Pregnant females were treated by oral gavage with either RA (100 mg/kg body weight) or DMSO in corn oil at E8.5 and embryos harvested 3 h post-treatment.

### *Microarray analysis*

Total RNA was prepared from tissue caudal to the most recently formed somite of stage-matched embryos (determined by somite number) by Trizol extraction and RNA amplification performed as previously described (Hoffmann et al., 2002). Three independent amplified RNA samples for each condition were hybridized to Affymetrix MO430_2.0 gene chips. Signal quantification and normalization was performed using MAS5, RMA and GC-RMA algorithms, and a *t*-test performed to compare data sets and to provide a measure of variance. False Discovery Rate Confidence Interval (FDRCI) analysis was used to establish significance testing based on fold-change and variance.

### *Whole mount in situ hybridization*

Whole mount in situ hybridization was performed as previously described (Savory et al., 2009a). Probes for in situ hybridization were generated from the following ESTs: *Abtb2* (ATCC EST, IMAGE 6493840); *Dgkz* (ATCC EST, IMAGE 3668704); *F11r* (ATCC EST, IMAGE 3985305); *Grsf1* (ATCC EST, IMAGE9834831); *Irx3* (ATCC EST, IMAGE 30539191); *Lhx1* (ATCC EST, IMAGE 30363447); *Msi2h* (ATCC EST, IMAGE 40045350); *Pim1* (ATCC EST, IMAGE 5694941); *Ptprz* (ATCC EST, IMAGE 6403687); *Ret* (ATCC EST, IMAGE 6849696); *Ror2* (ATCC EST, IMAGE 4159363); *Spsb4* (ATCC EST, IMAGE 6412566); *Tshz1* (ATCC EST, IMAGE 5363241).

A

| | FDRCI Analysis (# passed) | | |
|---|---|---|---|
| | Fold Change | | |
| Algorithms | 1.5 | 1.8 - 2 | >2 |
| RMA | 154 | 54 | 30 |
| GC-RMA | 787 | 267 | 164 |
| MAS5 | 5,698 | 3,930 | 3,210 |

B

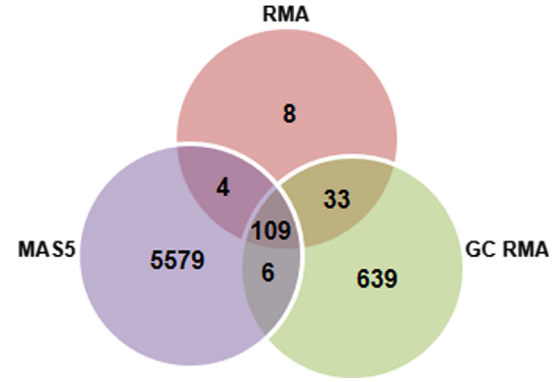


**Fig. 1.** False Discovery Rate Confidence Interval (FDRCI) analysis. (A) For all data sets a *t*-test was performed using three different algorithms to compare data sets and to provide a measure of variance. FDRCI was done to obtain significance testing based on fold change and variance. A *p*-value of $< 1$ was considered significant for the FDRCI analysis. (B) VENN diagram summarizing the results of the FDRCI analysis with the three algorithms. Note the 109 common genes were determined to be differentially regulated by RA with all three algorithms.