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Wt1 and retinoic acid signaling are essential for stellate cell development and liver morphogenesis

A. Ijpenberg [a,1,2], J.M. Pérez-Pomares [b,2], J.A. Guadix [b], R. Carmona [b], V. Portillo-Sánchez, D. Macías [b], P. Hohenstein [a], C.M. Miles [a,3], N.D. Hastie [a], R. Muñoz-Chápuli [b,*]

[a] MRC Human Genetics Unit, Western General Hospital, Edinburgh, UK
[b] Department of Animal Biology, Faculty of Science, University of Málaga, E-29071 Málaga, Spain

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Abstract

Previous studies of knock-out mouse embryos have shown that the Wilms' tumor suppressor gene (*Wt1*) is indispensable for the development of kidneys, gonads, heart, adrenals and spleen. Using OPT (Optical Projection Tomography) we have found a new role for Wt1 in mouse liver development. In the absence of Wt1, the liver is reduced in size, and shows lobing abnormalities. In normal embryos, coelomic cells expressing Wt1, GATA-4, RALDH2 and RXRα delaminate from the surface of the liver, intermingle with the hepatoblasts and incorporate to the sinusoidal walls. Some of these cells express desmin, suggesting a contribution to the stellate cell population. Other cells, keeping high levels of RXRα immunoreactivity, are negative for stellate or smooth muscle cell markers. However, coelomic cells lining the liver of Wt1-null embryos show decreased or absent RALDH2 expression, the population of cells expressing high levels of RXRα is much reduced and the proliferation of hepatoblasts and RXRα-positive cells is significantly decreased. On the other hand, the expression of smooth muscle cell specific α-actin increases throughout the liver, suggesting an accelerated and probably anomalous differentiation of stellate cell progenitors. We describe a similar retardation of liver growth in RXRα-null mice as well as in chick embryos after inhibition of retinoic acid synthesis. We propose that *Wt1* expression in cells delaminating from the coelomic epithelium is essential for the expansion of the progenitor population of liver stellate cells and for liver morphogenesis. Mechanistically, at least part of this effect is mediated via the retinoic acid signaling pathway.

Keywords: Wt1; Stellate cells; Liver development; RALDH2; Retinoic acid; RXRα

Introduction

The gene *Wt1* encodes a zinc-finger transcription factor involved in the development of several organs and in tumorigenesis. It has been experimentally demonstrated that Wt1 is able to activate and repress the transcription of many genes, but its physiological targets are still uncertain (Davies et al., 1999; Little et al., 1999). In normal development, Wt1 is transiently expressed in several mesodermal tissues, namely in mesothelial and submesothelial cells, derivatives of the intermediate mesoderm such as meso and metanephros, gonads and adrenals (Pritchard-Jones et al., 1990; Pelletier et al., 1991; Armstrong et al., 1993; Rackley et al., 1993), as well as the septum transversum and the limbs (Moore et al., 1998). *Wt1* knock-out embryos have no kidneys, gonads, spleen nor adrenal glands and die at mid-gestation due to heart abnormalities (Kreidberg et al., 1993; Moore et al., 1999; Herzer et al., 1999).

We have studied in detail the visceral phenotype of *Wt1*-null mice, and found a previously undescribed liver hypoplasia with defects in lobe formation, as well as a mal-positioning of the stomach. Interestingly, *Wt1* is expressed in the coelomic epithelium covering these viscerae, as well as in the mesenchymal

* Corresponding author. Fax: +34 952131668.
E-mail address: chapuli@uma.es (R. Muñoz-Chápuli).
[1] Present address: Institute of Stem Cell Research, GSF-National Research Center for Environment and Health, Neuherberg, Germany.
[2] Contributed equally to this work.
[3] Present address: Institute of Human Genetics, International Centre for Life, Newcastle upon Tyne, UK.

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cells that delaminate from this epithelium, invade the septum transversum and the liver primordium and contribute to the diaphragm. Using chick embryos, we have previously shown that these delaminating coelomic cells contribute to the stellate (Ito) cell population, as well as to the sinusoidal endothelium (Perez-Pomares et al., 2004). However, the origin of stellate cells in mammals remains obscure (Cassiman et al., 2006). On the other hand, the expression pattern of *Wt1* in the liver coincides with that of the main retinoic acid (RA) synthesizing enzyme RALDH2. Moreover, since RXRα-null mice also display a hitherto unexplained liver hypoplasia (Sucov et al., 1994) and equally die of cardiac failure, we suspected a functional connection between Wt1 and RA-dependent signaling.

In this paper, we present our detailed analysis of the role of Wt1 and retinoic acid signaling during early hepatic development. Our results provide new molecular evidence for parallels in visceral tissue development, whereby Wt1 expression in mesenchymal cells derived from the coelomic epithelium is essential for the development of the underlying tissue.

Material and methods

All the procedures involving use of animals were compliant with the legal regulations from the countries where these procedures were performed.

Generation of transgenic mice

Wt1 knock-out mice were generated by gene targeting, as previously described (Kreidberg et al., 1993). Embryos were genotyped by PCR on their yolk sacs. E11.5–E13.5 homozygous embryos were fully studied via serial sectioning or 3D virtual reconstruction using OPT (see below). Heterozygous and wild-type littermates were used as controls. All embryos were alive at the moment of their collection. The yWT470LacZ mouse strain (A9.3, line H) expressing LacZ under control of 470 kb of the human Wt1 locus has been previously described (Moore et al., 1999).

The RXRα knock-out mouse line was previously described (Kastner et al., 1994). We used E12.5 and E13.5 embryos for morphological comparison with Wt1-null embryos.

Histology and immunohistochemistry

For histology and immunohistochemistry, embryos were fixed in 4% buffered paraformaldehyde in PBS, in methanol-acetone-water (2:2:1) or in Dent's fixative (methanol-DMSO 4:1), dehydrated and paraffin-embedded. Some embryos were fixed through cryosubstitution, snap frozen in liquid nitrogen and kept in methanol at −80 °C for 5 days. Subsequently, the embryos were kept at −20 °C for 6 h, washed twice with methanol at 4 °C (45 min) and twice with methanol at room temperature. Dehydration was completed in methanol/butanol 1:1 (20 min) and pure butanol (20 min). yWT470lacZ Transgenic embryos were fixed o/n in 2% PFA at 4 °C. After washing in PBS, the embryos were stained with Xgal, washed and stored in 1% PFA until further use. Immunoperoxidase and double immunofluorescence staining were performed as previously described (Guadix et al., 2006).

The monoclonal mouse anti-smooth muscle cell (SMC) α-actin antibody (clone 1A4, Sigma) was used at a 1:2000 dilution for immunoperoxidase staining and at 1:100 for immunofluorescence. Polyclonal rabbit anti-RALDH2 (a gift of Dr. Peter McCaffery, Eunice Shriver Center, University of Massachusetts) was used at a 1:5000 dilution. Polyclonal rabbit anti-RXRα (Sc-553, Santa Cruz) and anti-HNF1 (Sc-8986, Santa Cruz) were used at a 1:300 and 1:400 dilutions for immunoperoxidase, respectively, or at a 1:100 for immunofluorescence. For RXRα immunofluorescence, tyramide signal amplification (TSA Kit, Perkin-Elmer) was necessary. Monoclonal mouse anti-desmin (clone DE-U-10, Sigma) was used at 1:200 for immunoperoxidase and 1:100 for immunofluorescence. Polyclonal goat anti-GATA-4 (Sc-1237, Santa Cruz) was used at 1:50 dilution for immunoperoxidase and, with tyramide amplification, for immunofluorescence. Polyclonal rabbit anti-phosphohistone H3 (#06-570, Upstate) was used at a 1:200 dilution. Monoclonal rat anti-Ter119 (Sc-19952, Santa Cruz), monoclonal mouse anti-PCNA (Sigma) and polyclonal rabbit anti-bovine epidermal cytokeratin (Z622, Dakopatts, Denmark) were used at 1:100 dilution.

OPT analysis

Wt1 wild-type and null embryos for morphological analysis were fixed for 2 h in 4% PFA at 4 °C. The embryos were dehydrated using sequential MeOH/PBS wash steps (25%, 50%, 75%), and stored in 100% MeOH at −20 °C until further use. Before OPT embedding (see below), the embryos were slowly rehydrated using several wash steps.

All embryos were embedded in 1% low-melting point agarose, immersed in Murray's Clear, and scanned through 360° as previously described (Sharpe et al., 2002). Morphological differences between Wt1 wild-type and mutant embryos were detected by scanning for the auto-fluorescence emitted by all tissues. Using a back-projection algorithm, the 400 captured digital images were independently reconstructed, and high-resolution representations of sections through the specimen were produced. Alternatively, 3D iso-surfaces (contours that connect all regions above a certain threshold intensity) were generated to illustrate the shapes of the tissues in which they were expressed.

Image analysis

Co-localization analysis using confocal images was performed through image processing with ImageJ software. Briefly, we obtained images composed of the non-black pixels present at the same time in the red and green channels as previously described (Carmona et al., 2007). Quantification of the signal in these and other immunoperoxidase images was also performed with ImageJ software. Comparisons were always made on equivalent images, obtained during the same experiment and captured under the same conditions. A gray level threshold was manually established for the selection of the positive cells, and the same threshold was applied to all the images. Then, the surface of the selected cells was measured and expressed as percentage of the total surface. Comparison of mean values for each group of measurements was made using the Student's *t*-test.

RALDH2 inhibition and in vitro culture of quail liver explants

RA-synthesis was inhibited by two competitive inhibitors of aldehyde dehydrogenases, DEAB (4-diethylaminobenzaldehyde, Sigma) and citral (Fluka). Stock solutions were made in DMSO (DEAB, 150 mM; citral, 100 mM). For injection, the competitive inhibitor stocks were further diluted with Tyrode solution to obtain a final concentration of 0.4 mM.

Chick eggs were incubated for 48–72 h. After windowing, 2 ml of albumin was aspirated, after which 2 ml of either DEAB or citral solution was injected per egg. The eggs were re-incubated for 24 h. Then, a further 1 ml of the inhibitor solution was injected into the egg, after which it was incubated again for 24 h. Control eggs were injected with the same volume of DMSO diluted 1:250 with Tyrode.

In order to check the effects of RA and RA inhibitors on *in vitro* growth of liver explants, we dissected the hepatic primordia of HH16-HH17 quail embryos, and we cultured them in four-well plates (Nunc) with DMEM (Gibco) supplemented with 10% fetal bovine serum (Paa), 2% chick serum (Sigma), 100 IU penicillin/streptomycin (Gibco) and Plasmocin (Invivogen) at 37 °C, 5% CO_2. Two explants were placed in each well and treated with 10 μM citral, 1 μM RA, both substances or vehicle (DMSO) only. After 24 h, the explants from each well were pooled and trypsinized, and the cells were pelleted, washed twice in cold PBS+0.1% fetal bovine serum, fixed in ice-cold 70° ethanol for 1 h, washed again in PBS, treated with 100 μg/ml Rnase-A and stained with propidium iodide (40 μg/ml) for 1 h at 37 °C. Cell fluorescence was analyzed using a MoFlo flow cytometer and the results were quantified with Summit 4.0 software. The experiment was performed by duplicate.