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Dynamic expression of Lrp2 pathway members reveals progressive epithelial differentiation of primitive endoderm in mouse blastocyst

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Abstract

Mesenchyme to epithelium transitions are crucial to embryonic development. The early mouse embryo offers an excellent model to study epithelium formation as during the first three days of development two epithelia are formed, the trophectoderm (TE) and the primitive endoderm (PrE). We have previously shown that PrE cells are determined within the blastocyst ICM long before epithelium formation. In this work, we isolated Lrp2 as a novel PrE precursor (pre-PrE) marker by using a microarray strategy that combines a transcriptome analysis of three stem cell lines and early embryos. A detailed expression analysis shows that Lrp2 expression is induced in late E3.5 embryos indicating that pre-PrE cells are progressively maturing prior to polarization into an epithelium. Furthermore, the subcellular location of Lrp2, Disabled-2 (Dab2) and Collagen-IV shows that the epithelial structure is acquired in individual cells through successive steps.

Keywords: Blastocyst; Epithelium; Cell differentiation; Primitive endoderm; Cell polarization; Microarray

Introduction

During embryonic development, cells are subjected to dynamic changes in their morphology, position and interactions with their environment. In particular, the transition between two different cell states, mesenchymal and epithelial, is fundamental to organogenesis. In early mouse development, two distinct epithelia, the trophectoderm (TE) and the primitive endoderm (PrE), have differentiated by the embryonic day 4.5 (E4.5), whereas the epiblast (Epi) remains as a mass of undifferentiated cells (Yamanaka et al., 2006). TE and PrE are extraembryonic tissues that are required for nutrient exchange and have also been shown to play important roles in the induction of the anteroposterior axis (Ang and Constam, 2004; Rossant and Tam, 2004). The mechanism of epithelium formation is different in the formation of the TE and PrE (Johnson and McConnell, 2004; Yamanaka et al., 2006). After fertilization, all blastomeres of the developing embryo are equipotent until the eight-cell stage when compaction occurs. During this process, the cells polarize by maximizing their contacts through E-cadherin binding, producing the first proto-epithelium of development. During the subsequent two rounds of mitosis each cell either divides asymmetrically producing an inner and an outer cell or symmetrically producing two outer cells. Outer cells remain polarized and generate the TE epithelium with adherens and tight junctions. The etiology of PrE differentiation is not known but recent data has shown that PrE cells are already determined by the blastocyst stage (E3.5) (Chazaud et al., 2006). However, at this early stage, PrE precursor (pre-PrE) cells, expressing the PrE marker Gata6, have not formed an epithelium but are scattered throughout the inner cell mass, intermingled with other cells of the future Epi expressing Nanog. Lineage tracing experiments have shown that individual cells of the ICM contribute only to the Epi or PrE, suggesting that between E3.5 and E4.5 pre-PrE

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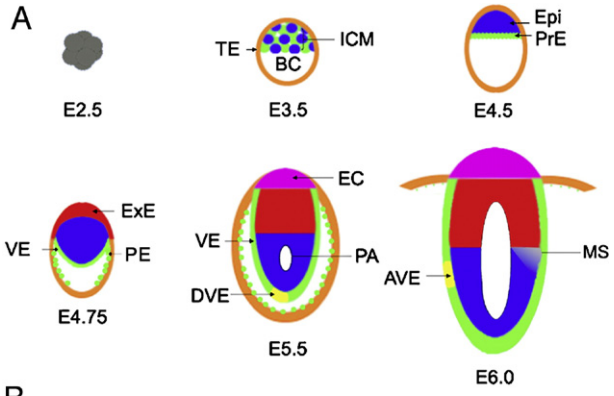
cells move toward the surface of the ICM to form the epithelium (Chazaud et al., 2006). The mechanisms involved in these morphogenetic movements are currently unknown, but cell adhesion mechanisms are certainly implicated (Yamanaka et al., 2006).

Low-density lipoprotein receptor-related proteins (Lrp) are transmembrane receptors that share homology with mammalian LDL-receptor (Fisher and Howie, 2006; Nykjaer and Willnow, 2002). One Lrp family member, Lrp2, also known as gp330 or Megalin (Saito et al., 1994), has been shown to be expressed during fetal development in the neuroepithelium (Assemat et al., 2005a) and the endodermal portion of the yolk sac (Assemat et al., 2005b; Drake et al., 2004; Maurer and Cooper, 2005; Yang et al., 2007). In adults, Lrp2 expression can be detected in various tissues but the protein is mainly found in absorptive epithelia, such as renal proximal tubules, gallbladder or mammary epithelia (Fisher and Howie, 2006). Historically, Lrp family members were generally thought to only be involved in receptor-mediated endocytosis. Newer research has shown Lrp2 to have a wide variety of ligands, such as lipoproteins, proteases and protease/inhibitor complexes, plasminogen and their activators, albumin and some drugs like Gentamycin (Christensen and Birn, 2002; Hussain et al., 1999). Analysis of *Lrp2* mutant mice has highlighted an important role during embryo development (Willnow et al., 1996), implicating this receptor in the binding and regulation of morphogenic signaling pathways such as Sonic Hedgehog (Shh) and BMP4 (McCarthy et al., 2002; Spoelgen et al., 2005).

Here we show that Lrp2 is an early marker of PrE differentiation. A microarray-based screen identified this gene and other members of this signaling pathway as potential PrE specific genes. Detailed expression analysis confirms that Lrp2 is expressed in pre-PrE cells as early as E3.5. Interestingly, the timing of expression, as well as the changes in subcellular localization of Lrp2, highlights a progressive maturation of pre-PrE cells to finally form the PrE epithelium.



B

GO-ID	GO Description	Genes in test set	Corrected p-value
42995	cell projection	PSEN1, **LRP2,** TPM3, KIF5B, HIF1A, SMO, ARHGAP1, MAPK8IP3, PHR1, ITGA5, CTTN, ROBO1	0,004
5905	coated pit	**LRP2,** CLTC, **DAB2,** AP1S1, COPG	0,003
5783	endoplasmic reticulum	EDEM1, PSEN1, ZDHHC16, GCS1, TLOC1, **LRPAP1,** CAML, GANAB, MGAT1, CAI, DNAJC10, EXT1, SSR3, SOAT1, MCFD2, SEC61A1, **LRP2,** RCN1, TRAM1, HMOX2, SEC23IP, CALU, TMEM4, PLD1, NUCB2, PDIR, GRP58, TXNDC7, MAPK8IP3, MBTPS1, RCN2, SRPR, TXNDC5, GOSR2, ATP2A2, FKBP9, ITM1, RRBP1, KDELR2, SRPRB, DAD1, CANT1, P4HA1, P4HB, ALG3	0,000
16192	vesicle-mediated transport	LIN7C, VPS4A, VDP, SNAP23, MAPK8IP3, **DAB2,** AP1S1, MCFD2, **LRP2,** GOSR2, SYNJ2BP, KDELR2, ARFGAP3, RABEP1	0,003

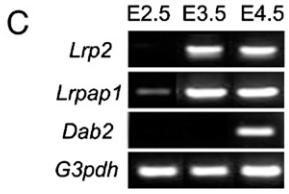


Fig. 1. (A) Representation of the stages studied in the microarray. AVE, anterior visceral endoderm; DVE, distal visceral endoderm (yellow); EC, ectoplacental cone (pink); Epi, epiblast (blue); ExE, extraembryonic ectoderm (red); MS, mesendoderm (light gray); PA, proamniotic cavity; PE, parietal endoderm (green dots); PrE, Primitive endoderm (green); TE, trophectoderm (orange); VE, visceral endoderm (green). (B) Table displaying candidate genes enriched for GO terms. (C) Expression analysis of *Lrp2*, *Lrpap1* and *Dab2* by RT–PCR at indicated stages. *G3pdh* is used as reference for sample normalization.

Materials and methods

Embryo microarray

Embryos from each stage were pooled and mRNA extracted with the Micro-Fast Track isolation kit (Invitrogen, 45-0036). Two independent pools (for two independent experiments) were made for each stage, on average, 106 embryos at E2.5, 61 at E3.5, 47 at E4.5, 28 at E4.8, 17 at E5.5 and 19 at E6.5 (see Fig. 1A for stages description). SMART (Clontech) reverse transcription and PCR was adapted for each developmental stage to amplify cDNAs. To generate probes for array hybridization, 1 μg of cDNA was labeled by incorporation of either Cy5 or Cy3-dCTP during random hexamer-primed primer extension in the presence of Klenow DNA polymerase (Roche) according to Livesey et al. (2000). Poly-L-lysine-coated slides spotted with probe sequences from the NIA mouse 15K cDNA library (Ko et al., 2000) were purchased from the University Health Network Microarray Centre (http://www.microarrays.ca). Labeled probes were hybridized according to Wigle et al. (2002). Slides were scanned with a Genepix Axon 4000 microarray scanner. Spot intensities were quantified and median back ground corrected with the supplied Genepix software and exported as tables. Duplicate samples were analyzed and probe spot intensities were averaged. Expression data was set as a $\log_2$ ratio of expression at E2.5. Data have been deposited at GEO under accession number (GSE8339).

Stem cell microarray

Stem cell microarray data on Affymetrix MGU74x2A chips was obtained from the GEO database (http://www.ncbi.nlm.nih.gov/geo/) for XEN cells (GDS1763), ES and TS cells (GSE3766). Cel files were downloaded and processed in GCOS ver1.4 software (Affymetrix). Expression data of XEN cells were taken as ratios of ES and TS cells using GCOS ver1.4 software (Affymetrix).

Data mining

TS, XEN and ES cell expression ratios were filtered to obtain XEN enriched genes based on the fold change call and expression call. A gene was considered enriched if it was called as present in both XEN cell data sets and had increased expression compared to both TS and ES cells. Data filtering was preformed in Microsoft Excel.