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Differential expression of microRNAs in mouse embryonic bladder

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ABSTRACT

MicroRNAs (miRNAs) are involved in several biological processes including development, differentiation and proliferation. Analysis of miRNA expression patterns in the process of embryogenesis may have substantial value in determining the mechanism of embryonic bladder development as well as for eventual therapeutic intervention. The miRNA expression profiles are distinct among the cellular types and embryonic stages as demonstrated by microarray technology and validated by quantitative real-time RT-PCR approach. Remarkably, the miRNA expression patterns suggested that unique miRNAs from epithelial and submucosal areas are responsible for mesenchymal cellular differentiation, especially regarding bladder smooth muscle cells. Our data show that miRNA expression patterns are unique in particular cell types of mouse bladder at specific developmental stages, reflecting the apparent lineage and differentiation status within the embryonic bladder. The identification of unique miRNAs expression before and after smooth muscle differentiation in site-specific area of the bladder indicates their roles in embryogenesis and may aid in future clinical intervention.

Introduction

Bladder development during embryogenesis is a well-defined process that requires coordination between cell types and the surrounding environment [1]. The molecular mechanisms responsible for bladder development and cellular differentiation are not well understood, although reciprocal interactions between epithelium and mesenchyme play a critical role. Genes such as the sonic hedgehog signaling pathway have recently been discovered to be involved in this process [2–4].

MicroRNAs (miRNAs) were discovered in *Caenorhabditis elegans* in 1993 but have since been identified in many different organisms, including plants, flies, fish, mice and humans [5–14]. MiRNAs are small non-coding RNA molecules that regulate gene expression post-transcriptionally [9]. They are transcribed as long primary transcripts that undergo a series of cleavage events to become functional 21–23 nucleotide (nt) RNAs that incorporate into the RNA-induced silencing protein complex (RISC) [9,13,15]. The miRNA/RISC complex binds to complementary sequences in the 3′-untranslated regions (UTRs) of target mRNAs to inhibit protein synthesis by preventing translation or promoting mRNA degradation. Studies have shown miRNAs are a newly defined class of transcription factors and play diverse roles ranging from developmental patterning and cell differentiation to genome rearrangement and DNA excision by fine-tuning or functioning as master switches that turn genes on and off during development. [9,10,12,13,16].

MiRNA is now recognized as one of the major regulatory gene families in eukaryotic cells. Recently, bioinformatics analysis has predicted that miRNAs comprise ~5% of the transcriptome [17], and may regulate the translation of more than one-third of human mRNA species [18]. An additional independent analysis predicted that 20% of human genes can potentially be regulated by miRNAs. A number of studies suggest one miRNA can target numerous mRNAs, often in combination with other miRNAs, indicating that miRNAs operate highly complex regulatory networks [6,9,10,12,13,16]. The elucidation of the spatial and temporal patterns of their expression is primary for understanding the precise role of the miRNAs in developmental processes. The miRNA expression levels presumably play an important role in keeping tissue/organ identity or functions, and misregulation of them may lead to various kinds of diseases [10,13,19].

Identification and quantification of cellular miRNAs at critical stages of bladder development would provide valuable insight in the role of miRNA's during organogenesis. In the present study, we demonstrate that subpopulations of cell types in the mouse bladder at defined embryonic stages (before, during and after bladder smooth muscle formation) have distinct miRNA expression patterns using printed miRNA microarray and subsequent confirmation by real-time RT-PCR analyses.

Abbreviations: E, embryo day; HE, hematoxylin–eosin; LCM, laser capture microdissection; miRNA, micro-ribonucleic acid; RT-PCR, reverse transcriptase polymerase chain reaction; snRNA, small nuclear ribonucleic acid.

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Material and methods

Animal. FVB mice were purchased from Charles River Laboratories (Wilmington, MA) and housed in separate animal cages. Timed mating was set up according to the experiment schedule. Noon on the day of the vaginal plug was designated as embryonic day 0.5. All animal-related procedures described here were approved by the laboratory animal resource center at University of California, San Francisco.

Laser capture microdissection (LCM). Embryonic mouse bladders were collected at embryo day (E) 12.5 (before smooth muscle), 13.5 (during smooth muscle), and 15 (after smooth muscle), respectively. Frozen embryonic bladders were cryosectioned consecutively into 7 micron sections and stained using HistoGene® solution (HS) (MDS Analytical Technologies, Sunnyvale, CA). The PixCell® IIe laser capture microdissection instrument (MDS Analytical Technologies) was used to localize particular compartments of the bladder mesenchyme in relationship to the urothelium, as previously described [2,3]. Besides epithelium, mesenchymal locations were chosen either next to the serosal layer or next to the urothelium in the E12.5 and E13.5 bladders (Fig. 1A). In the E15 bladders, in which smooth muscle differentiation has already occurred, three locations were captured: the smooth muscle layer, the submucosa layer, and the loosely organized mesenchymal cell layer between (Fig. 1B).

Small RNA extraction, purification and amplification. MiRNA extraction was performed with the PicoPure® microRNA Isolation Kit developed by MDS Analytical Technologies. Each miRNA sample from similar locations at the same gestational stage was pooled from three fetuses from different dams. Two-rounds of linear miRNA amplifications were performed for both test and control miRNAs with Global MicroRNA Amplification Kit from System Biosciences (SBI, Mountain View, CA) according to the manufacturer's protocol.

MiRNA array printing. For analyzing miRNA expression, miRNA microarrays used in the study were printed at the Gladstone Genomics Core facility at University of California, San Francisco (http://www.gladstone.ucsf.edu/gladstone/site/genomicscore).

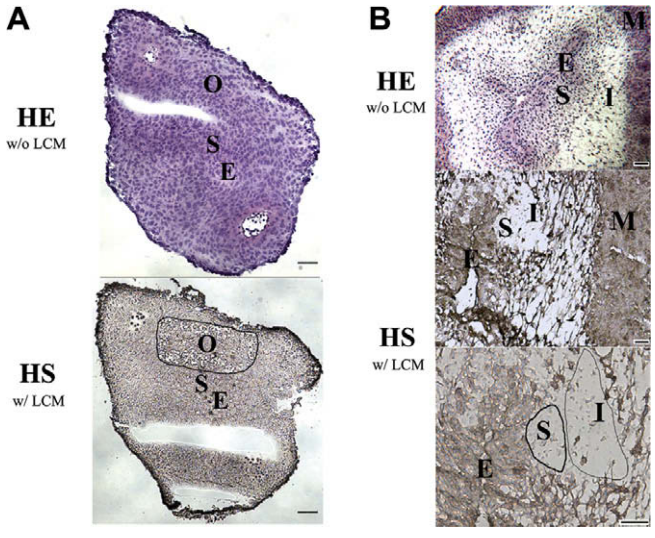

Fig. 1. Laser-assisted microdissection. (A) Embryo day (E) 12.5 mouse bladder. HE w/o LCM: Morphology with hematoxylin–eosin (HE) staining on serial section before laser capture microdissection (LCM). HS w/LCM: Morphology with HistoGene® solution (HS) staining after laser capture microdissection. E, epithelium; S, submucosa; O, outer layer mesenchyme. Scale bar: 50 μm. (B) E15 mouse bladder. I, intermediate loosely organized connective tissue; M, bladder smooth muscle cell.

The arrays contained probes spotted in duplicate. The probes represent 255 known human-specific miRNAs, 183 mouse-specific miRNAs, 240 both human and mouse homologus miRNAs, and 40 controls, as well as 1034 new miRNAs which were unknown in mouse (Sanger miRBase http://microrna.sanger.ac.uk, release 10.0, 2007). The complete list of probes is given in Supplementary Table 1.

Microarray data analysis. Fluorescence ratios (sample/reference) were calculated using GenePix® software. MiRNA array data were analyzed by using Acuity 4.0 (http://www.moleculardevices.com/pages/software/gn_acuity.html, MDS Analytical Technologies). To limit the measurement error, Lowess normalization program was used for raw data process. Further, miRNA spots were filtered based on those where expression levels differed by at least two-fold in at least three arrays. Finally miRNAs passed the filtering criteria were used for unsupervised hierarchical clustering analysis. The clustering algorithm grouped both miRNAs and samples into clusters based on overall similarity in miRNA expression pattern without prior knowledge of sample identity. Significance analysis of microarrays (SAM) was then performed as described previously [15].

Quantitative real-time RT-PCR. The microarray findings were validated using real-time RT-PCR through the QuantiMir RT Kit Small RNA Quantitation System (SBI). After in vitro transcription, amplified miRNA samples were generated to faciliate cDNAs to use for real-time RT-PCR analysis. The forward primers for target miRNAs were designed according to mature miRNAs sequences and synthesized by Integrated Device Technology Corporation (IDT, San Jose, CA) (Table 1). Assays were carried out in triplicate for each sample with FastStart Universal SYBR Green PCR Master Mix (Roche Diagnostics Corp., Indianapolis, IN). A three-step quantitative PCR was performed in an ABI 7600 Thermocycler (Applied Biosystems, Foster City, CA). Values for each gene were normalized to the expression levels of mouse U6 small nuclear RNA (snRNA). PCR data were analyzed as previously described [3].

Results

MiRNA expression profiles in mouse embryonic bladder

Global miRNA expression profiles were performed on 10 cell-specific samples from mice bladders from different embryonic stages. Using significance analysis of microarrays (SAM), we identified 187 miRNAs that correlated with each cell type and embryonic stage, including 16 known miRNAs. The 187 miRNAs that met the filtering criteria were subjected to hierarchical clustering in an unsupervised manner. Clustering based on the 187 miRNAs revealed substantive distinctions in overrepresented and underrepresented miRNAs among samples (Fig. 2A). Sixteen known miRNAs were clustered as Fig. 2B.

Generally the miRNAs expression patterns in bladder epithelial cells, submucosal cells and outer layer mesenchymal cells were more similar with each other at E12.5 prior to smooth muscle differentiation compared to the two older time points. As

Table 1
Forward primers for real-time RT-PCR.

MiRNAs	Forward primers (5′–3′)
hsa/mmu-miR-137	TTA TTG CTT AAG AAT ACG CGT AG
hsa-miR-202	AGA GGT ATA GGG CAT GGG AA
mmu-miR-503	TAG CAG CGG GAA CAG TTC TGC AG
mmu-miR-669a	AGT TGT GTG TGC ATG TTC ATG T
mmu-miR-674*	GCA CTG AGA TGG GAG TGG TGT A
mmu-miR-709	GGA GGC AGA GGC AGG AGG A
mouse U6 snRNA	TGG CCC CTG CGC AAG GAT G