LncRNA Pathway Involved in Premature Preterm Rupture of Membrane (PPROM): An Epigenomic Approach to Study the Pathogenesis of Reproductive Disorders

Xiu cui Luo1*, Qingxi Shi1*, Yang Gu1, Jing Pan1, Maofang Hua1, Meilin Liu1, Ziqing Dong1, Meijiao Zhang1, Leilei Wang1, Ying Gu1, Julia Zhong2, Xinliang Zhao4, Edmund C. Jenkins3, W. Ted Brown3, Nanbert Zhong1,3,4,5,6.*

1 Center of Translational Medicine for Maternal and Children’s Health, Lianyungang Maternal and Children’s Hospital, Lianyungang, Jiangsu, China, 2 Hunter College High School, New York, New York, United States of America, 3 New York State Institute for Basic Research in Developmental Disabilities, Staten Island, New York, United States of America, 4 Peking University Center of Medical Genetics, Beijing, China, 5 Shanghai Children’s Hospital Affiliated to Shanghai Jiaotong University, Shanghai, China, 6 March of Dimes Global Network of Maternal and Infant Health, White Plains, New York, United States of America

Abstract

Preterm birth (PTB) is a live birth delivered before 37 weeks of gestation (GW). About one-third of PTBs result from the preterm premature rupture of membranes (PPROM). Up to the present, the pathogenic mechanisms underlying PPROM are not clearly understood. Here, we investigated the differential expression of long chain non-coding RNAs (lncRNAs) in placentas of PTBs with PPROM, and their possible involvement in the pathogenic pathways leading to PPROM. A total number of 1954, 776, and 1050 lncRNAs were identified with a microarray from placentas of PPROM (group A), which were compared to full-term birth (FTB) (group B), PTB (group C), and premature rupture of membrane (PROM) (group D) at full-term, respectively. Instead of investigating the individual pathogenic role of each lncRNA involved in the molecular mechanism underlying PPROM, we have focused on investigating the metabolic pathways and their functions to explore what is the likely association and how they are possibly involved in the development of PPROM. Six groups, including up-regulation and down-regulation in the comparisons of A vs. B, A vs. C, and A vs. D, of pathways were analyzed. Our results showed that 22 pathways were characterized as up-regulated 7 down-regulated in A vs. C, 18 up-regulated and 15 down-regulated in A vs. D, and 33 up-regulated and 7 down-regulated in A vs. B. Functional analysis showed pathways of infection and inflammatory response, ECM-receptor interactions, apoptosis, actin cytoskeleton, and smooth muscle contraction are the major pathogenic mechanisms involved in the development of PPROM. Characterization of these pathways through identification of lncRNAs opened new avenues for further investigating the epigenomic mechanisms of lncRNAs in PPROM as well as PTB.

Citation: Luo X, Shi Q, Gu Y, Pan J, Hua M, et al. (2013) LncRNA Pathway Involved in Premature Preterm Rupture of Membrane (PPROM): An Epigenomic Approach to Study the Pathogenesis of Reproductive Disorders. PLoS ONE 8(11): e79897. doi:10.1371/journal.pone.0079897

Editor: Cees Oudejans, VU University Medical Center, The Netherlands

Received June 13, 2013; Accepted September 26, 2013; Published November 27, 2013

Copyright: © 2013 Luo et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This project is granted by a special fund from Lianyungang Maternal and Children’s Hospital, along with support from the March of Dimes Global Network for Maternal and Infant Health (MOD-GNMIH), Peking University Center of Medical Genetics (PUCMG), and New York State Office for People with Developmental Disabilities (NYS OPWDD). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: nanbert.zhong@opwdd.ny.gov

† These authors contributed equally to this work.

Introduction

Preterm birth (PTB) is a live birth delivered before 37 weeks of gestation (GW), 80% of which are spontaneous (sPTB) [1]. About one-third of PTBs result from the preterm premature rupture of membranes (PPROM) [2]. Although PPROM is usually caused by reproductive genital infections and inflammatory reactions of cytokine and chemokine pathways, and involves the extracellular matrix (ECM) [3–5], the pathogenic mechanisms underlying the remaining PTB are not yet well understood. Infections have been associated with and well characterized in PPROM [6,7]. Approximately 32% of patients with PPROM have amniotic fluid cultures that test positive for microbes at the time of presentation to clinics and 75% of patients have positive cultures at the onset of labor [7]. Proteinases directly secreted by bacteria degrade the collagen in fetal amniotic and chorionic membranes, and in the maternal decidua. Bacteria also produce phospholipase A2 that increases matrix metalloproteinase 1 and 3 (MMP1 and MMP3), leading to ECM degradation [8,9]. Molecular studies on the development of PPROM have been undertaken [10], with a focus on genes that are involved with inflammation [11–14], including TNFα [15–17], FAS, which is member 6 gene of the tumor necrosis factor receptor superfamily [9,18], and Toll-like receptor (TLR) [19,20]. TNF-α induces apoptosis in PPROM, through binding to the TNF receptor [13–15] and activating a proteolytic cascade via the FAS-caspase pathway [9]. An apoptotic pathway associated with PPROM involves the binding of p53 to the MMP2 gene promoter [8]. In PPROM, a strong association has been
observed between haplotypes of TIMP2 (tissue inhibitors of MMP 2) in maternal DNA and COL4A3 in fetal DNA. A “three-locus” model has been identified in a subset of patients of Hispanic origin in Chile [2]. A similar result was obtained from studying a Norwegian cohort, where haplotypes of COL5A2 in fetal DNA and COL5A1 in maternal DNA were found to associate with spontaneous preterm delivery (PTD) [21]. This suggested there are differing genetic predispositions among different ethnic populations, in agreement with previously reported racial-ethnic disparities [22]. Finding a strong association of single nucleotide polymorphisms with PPROM in a particular ethnic population but not in another one strongly suggests that the heterogeneity and complexity of PPROM is greater than what has yet been perceived. Thus, the molecular mechanisms underlying the pathogenic pathways in PPROM appear to be more complicated. These findings lead us to investigate the complement of pathogenic pathways involved in regulating PPROM.

In recent years there has been an increased focus on noncoding RNAs (ncRNAs). Approximately 98% of total human genomic DNA has been found to be transcribed into ncRNA [23,24]. Although the roles of many small ncRNAs (such as siRNA and microRNA) are well defined, long chain ncRNA (lncRNA) is much less well characterized. LncRNAs are transcribed RNA molecules greater than 200 nucleotides in length, which are involved in diverse cellular processes such as cell differentiation, imprinting control, immune responses, human diseases and tumorigenesis [25–29]. These lncRNAs include not only antisense, intronic transcript and large intergenic ncRNA but also promoter-associated lncRNA and UTR (untranslated region)-associated lncRNA. Knockdown and overexpression studies have shown that an increasing number of lncRNAs play important roles in regulating a diverse spectrum of processes, including splicing [30], transcription [31], localization [32] and organization of subcellular compartments [33]. Underscoring the importance of lncRNAs' regulatory roles is their emergence as key players in the etiology of several disease states [34,35]. A number of lncRNAs have been demonstrated to alter expression in human cancers and are regulated by specific oncogenic and tumor-suppressor pathways, such as p53, MYC, and NF-kB [36–38]. ANRIL is a lncRNA that regulates three separate tumor suppressor genes: p16INK4a, p14ARF and p15INK4b, and is an important negative regulator of the cell cycle [39]. Disruptions to the expression of ANRIL have accordingly been associated with the development of several cancer types, including neuroblastoma [40], acute lymphocytic leukemia [41], melanoma [39] and prostate cancer [42]. Overexpression of the lncRNA transcript encoded by the gene HOTAIR has been associated with hepatocellular carcinoma [43], colorectal cancer [44] and breast cancer [45] by deregulation of HOXD cluster genes. Several lncRNAs have been reported to be important moderators of metabolism and endocrine function, such as PINK1 [PTEN (phosphatase and tensin homologue deleted on chromosome 10)-induced putative kinase 1] [46], H19/IGF2 [47] and thyroid growth receptor α2 (ERBα2) [48]. The regulation of the PINK1 locus is altered in obesity, type II diabetes and inactivity. The Delta 5-desaturase (FADS1) and steroidogenic acute regulatory protein (STAR) genes have also reported lncRNAs [49,50]. Dreesen et al. demonstrated that the expression of FADS, and its lncRNA, reverses Delta 5-desaturase was found to be reciprocally regulated by dietary fat content in animal models. Moreover, lncRNAs have been reported to involve in other human diseases, such as neurodegenerative and psychiatric diseases [51], cardiovascular disease [52], immune dysfunction and auto-immunity [53]. PPROM is a result of a pathogenic pregnancy. However, there has yet been no study on the possible involvement of lncRNA in PPROM. We hypothesize that lncRNA may play epigenetic/epigenomic function associated with the pathogenic development of PTB including PPROM. In this study, we have initiated an investigation of the differential expression of lncRNAs in placentas of PPROM, compared to controls, and their possible involvement in the pathogenic pathway of PPROM.

### Results

#### Identification of lncRNA differentially expressed in PPROM

A total number of 1968, 1954, and 1050 lncRNAs were identified differentially expressed from placentas of PPROM (group A) compared to FTB (group B), PTB (group C), and PROM (group D), respectively; 449 and 3024 from PTB compared to FTB and PROM, respectively; and 3627 from PROM compared to FTB (Table 1). Differential expression, which was determined by 2-fold change at \( p<0.05 \), was both up- and down-regulated. Data of differentially expressed lncRNAs, generated by the microarray, has been deposited in Gene Expression Omnibus with an accession number GSE 50879 (http://www.ncbi.nlm.nih.gov/geo/info/linking.html). To visualize differential expression between two different conditions, Volcano Plots (Figure 1) were constructed using fold-change (magnitude of change) values and \( p \)-values, thus allowing visualization of the relationship between fold-change and statistical significance, which takes both magnitude of change and variability into consideration. The vertical lines correspond to 2.0-fold up and down, respectively, and the horizontal line represents a \( p \)-value of \( <0.05 \). So the red point in the plot represents the differentially expressed lncRNAs with statistical significance. LncRNAs whose \( p \)-value\(<0.0001 \) were assigned to 0.0001, therefore, those points were located in the Y = 4 axis.

#### Quantitative real-time PCR validation of lncRNAs differentially expressed between PPROM and PTB

Seven up-regulated and nine down-regulated lncRNAs in PPROM (group A) compared with PTB (group C) in microarray assay (Table 2) were randomly chosen for validation, using a technology of real-time quantitative PCR (RT-qPCR). The results revealed that all the up-regulated lncRNAs were increased in PPROM compared to PTB, and six of them rose significantly (Figure 2B). Therefore, the results of the qPCR analysis were consistent with those of the microarray.

#### Metabolic pathways involved in PPROM

A functional analysis of mapping genes to KEGG Pathways was performed with a \( p \)-value \( =0.05 \) as cut-off. The \( p \)-value (EASE-score, Fisher-Plauel or Hypergeometric-Plauel) denotes the significance of the Pathway correlated to the conditions. The lower the \( p \)-value, the more significant is the Pathway. Six groups of pathways were analyzed (Table 3), including up-regulation and down-regulation in the comparison of PPROM vs. FTB (A vs. B); PPROM vs. PTB (A vs. C); PPROM vs. PROM (A vs. D); PTB vs. FTB (C vs. B); PTB vs. PROM (C vs. D), and PROM vs. FTB (D vs. B). Our results showed that 34 pathways were characterized as up-regulated 6 down-regulated in A vs. B, 7 up-regulated and 22 down-regulated in A vs. C, 18 up-regulated and 15 down-regulated in A vs. D; 16 up-regulated and 9 down-regulated in C vs. B, 12 up-regulated and 26 down-regulated in C vs. D; and 28 up-regulated and 15 down-regulated in D vs. B. Pathways with the Top 10 enrichment scores (\( \text{set} \ p<0.05 \) as cutoff) were shown in
Figure 3. When PPROM was compared to PTB (A vs. C), rupture of membrane was the focus; while if compared to PROM (A vs. D), the preterm was the focus. If PPROM compared to FTB, both rupture of membrane and preterm labor would be analyzed.

Infection-inflammatory pathways were the most common ones identified in the up-regulated condition, although they were in the down-regulated condition in A vs. D. The ECM (extracellular matrix) interacted with the receptor present in all the down-regulated groups. This suggested that infection-inflammatory and degradation of ECM were the two major pathogenic mechanisms associated with PPROM, in agreement with previous studies [1,3,5,7–9]. Up-regulated actin cytoskeleton pathway suggested that lncRNA interferes with the normal structure and function of the cytoskeleton. The smooth muscle contraction pathway in the down-regulation of A vs. D strongly indicated that reduced expression of lncRNAs in the smooth muscle contraction pathway is the major driving factor underlying the rupture of membrane.

This could increase the placental smooth muscle contraction inducing the premature labor, in addition to damage of intake membrane by ECM-receptor interaction.

### GO molecular function annotations

The Gene Ontology (GO) website (http://www.geneontology.org) provides a controlled vocabulary to describe gene and gene product attributes in any organism. The ontology covers three domains: Biological Process, Cellular Component and Molecular Function. Fisher’s exact test was used to determine if there is more overlap between the list of PPROM vs. controls and that of GO annotation than expected by chance. The \( p \)-value denotes the significance of GO terms enrichment in the A vs. C, A vs. D, and A vs. B genes. The lower the \( p \)-value (\( p \)-value<0.05 was applied), the more significant is the GO Term. Top 10 gene ontology (GO) molecular function annotations for lncRNAs (Table 4), based on their \( p \) values, showed their relative relationship with each other (Figure 4). Each color labeled box or circle represents a GO functional pathway. Red color labels indicate that these GO pathways are associated with PPROM and yellow labels were not detected from PPROM in this study. The order of the number represents the \( p \) value for how significant it is associated with PPROM.

### LncRNAs shared by two or more conditions

To analyze all lncRNA molecules among the six groups of comparisons, the following lncRNAs involved in PPROM were identified.

1) Cytokine-cytokine receptor interaction pathway and rheumatoid arthritis pathway were shown in both up- and down-

---

**Table 1. Differential expression of lncRNAs in PPROM.**

|            | A vs. B | A vs. C | A vs. D | C vs. B | C vs. D | D vs. B |
|------------|---------|---------|---------|---------|---------|---------|
| Up-regulated | 1233    | 974     | 904     | 136     | 1698    | 1245    |
| Down-regulated | 735     | 980     | 146     | 313     | 1326    | 2382    |
| Total       | 1968    | 1954    | 1050    | 449     | 3024    | 3627    |

A = PPROM, B = FTB, C = PTB, D = PROM.

doi:10.1371/journal.pone.0079897.t001

---

**Figure 1. Volcano Plots of six comparisons.** X-axis is fold change (log 2) and Y-axis is \( p \) value (-log 10). Up-regulated (X axis >0) or down-regulated (X axis <0) lncRNAs (red squares) were identified in an about the same number when fold change was set >2 folds [Log 2 (Fold change)] in PTB vs. PPROM. However, there were more down-regulated lncRNAs in PPROM vs. FTB and more up-regulated lncRNAs in PPROM vs. PROM.

doi:10.1371/journal.pone.0079897.g001
regulated when A vs. C. However, the lncRNA molecules identified from PPROM in these two pathways were not the same at up- or down-regulation.

2) Rad51 and Shc were shared in down-regulation of A vs. C and Av sB.

3) BMP, ECM, TCF/LEF were shared by A vs. C (down-regulation) and A vs. D (down-regulation).

4) IFNγ, MHCI, and/or MHCII were shared by the groups of all up-regulation of A vs. B, vs. C, and vs. D.

5) CLDN, EGFR, IFNγ, MHCI, MHCII, MHCII/II were shared by up-regulation of A vs. C and A vs. D.

6) CD5, CXCL9, CXCL10, FLT1, GZM, GZMB, HLA-DR, IFNG, IFNγ, IL1RAP, IL2RB, MHCI, MHCII, MHCII/II were shared by up-regulation of A vs. B and A vs. D.

7) API, CASP1, CCL2, CHMP3, CHMP4, cofilin, CTSB/L/S, Cyclin, Cyclin D, DUB, EEA1, eIF1, eIF3, eIF4G, eIF4y, EphA, E3ligase, FIP, FNR, GPCR, HLA-C, HLA-G, hnRNK, Hsc70, HSP70, HSP72, HSP90, IA-2, IFNAR, IFNAR2, IFNγ, IFNs, IFNsr, IL-2r, IP10, IRF9, ITGB1, JAK1/Tyk2, JAK1/2, JAK1/3, LARG, LTBP1, MCP1, MHC, MHCII, MHCII/II, NFAT, Npf4, OSTs, PERK, PKR, p107, p120, Rab5, Rab22, Ras, GAP, ROCK, ROCK1, RTK, Sec62/63, Sema6, Smurf1/2, Smurf2, SRPK1, STAM, SUMO, TAP1/2, THOC2, TNFα, TRAIL, TRAM, Upf2, Upf3, Wnt have been determined as the up-regulation of A vs. B and A vs. C.

### Differential expression of lncRNAs embedded in coding sequence

Thirteen differentially expressed lncRNAs have been identified from PPROM in these two pathways were not the same at up- or down-regulation.

2) Rad51 and Shc were shared in down-regulation of A vs. C and Av sB.

3) BMP, ECM, TCF/LEF were shared by A vs. C (down-regulation) and A vs. D (down-regulation).

4) IFNγ, MHCI, and/or MHCII were shared by the groups of all up-regulation of A vs. B, vs. C, and vs. D.

5) CLDN, EGFR, IFNγ, MHCI, MHCII, MHCII/II were shared by up-regulation of A vs. C and A vs. D.

6) CD5, CXCL9, CXCL10, FLT1, GZM, GZMB, HLA-DR, IFNG, IFNγ, IL1RAP, IL2RB, MHCI, MHCII, MHCII/II were shared by up-regulation of A vs. B and A vs. D.

7) API, CASP1, CCL2, CHMP3, CHMP4, cofilin, CTSB/L/S, Cyclin, Cyclin D, DUB, EEA1, eIF1, eIF3, eIF4G, eIF4y, EphA, E3ligase, FIP, FNR, GPCR, HLA-C, HLA-G, hnRNK, Hsc70, HSP70, HSP72, HSP90, IA-2, IFNAR, IFNAR2, IFNγ, IFNs, IFNsr, IL-2r, IP10, IRF9, ITGB1, JAK1/Tyk2, JAK1/2, JAK1/3, LARG, LTBP1, MCP1, MHC, MHCII, MHCII/II, NFAT, Npf4, OSTs, PERK, PKR, p107, p120, Rab5, Rab22, Ras, GAP, ROCK, ROCK1, RTK, Sec62/63, Sema6, Smurf1/2, Smurf2, SRPK1, STAM, SUMO, TAP1/2, THOC2, TNFα, TRAIL, TRAM, Upf2, Upf3, Wnt have been determined as the up-regulation of A vs. B and A vs. C.
which was an unique one present only in the group of A vs. C; similarly for the up-regulated sense lncRNA of QSOX1 and antisense lncRNA of SYNPO2L, ADAM12, and ADH6 in the group of A vs. D. Two down-regulated lncRNAs, TP73 and HOMER3, were identified in the group of A vs. B only. The remaining lncRNAs were presented in two or three groups (Table 5).

**Discussion**

Previous studies of PPROM have suggested it is associated with genital infections and inflammatory reactions of cytokine and chemokine pathways, and involves the extracellular matrix (ECM). However, the molecular pathogenic mechanisms underlying the pathogenic pathways in PPROM appear to be more complicated,
and the involvement of the complement of pathogenic pathways in regulating PPROM has been unclear. Recently, increasing evidence has confirmed lncRNAs are one of the most important factors controlling gene expression [54], and they have important roles in imprinting control, cell differentiation, immune responses, human diseases, tumorogenesis and other biological processes [25,26,55–57]. In this study, we identified 1,968 lncRNAs that were differentially expressed in PPROM when compared to FTB, 1954 in PPROM when compared to PTB, and 1,050 in PPROM when compared to PROM. To our knowledge, there is no report up to now describing lncRNAs expression in either normal or pathological placentas, and there has been no study on the association of differentially expressed lncRNA with the molecular mechanisms underlying the pathogenic pathways in preterm birth including PPROM.

lncRNAs have been recognized to have comprehensive functions in biological processes through various mechanisms [58], and to play important roles in both normal development and disease [59]. The lncRNAs that have been identified may be involved in the development and progression of PPROM and provide novel approaches to better understand the molecular basis of PPROM. Almost every step in the life cycle of genes—from transcription to mRNA splicing, RNA decay, and translation—can be influenced by lncRNAs [34]. Through distinct mechanisms

### Table 3. PPROM pathways identified from differentially expressed lncRNAs.

| Number of pathway: Up-regulated | Number of pathway: Down-regulated |
|--------------------------------|----------------------------------|
| **A vs. B** 22: Infection-inflammation | 2: Apoptosis |
| 5: Signaling | 2: Signaling |
| 3: Cell metabolism | 1: Misregulation of transcription |
| 1: RNA transport | 1: Glycerolipid metabolism |
| 1: Protein processing | 1: ECM-receptor interaction |
| 1: Regulation of actin cytoskeleton | 1: Protein processing |
| **A vs. C** 2: DNA synthesis | 11: Infection-inflammation |
| 2: Signal transduction | 4: Signaling |
| 1: Glycosphingolipid biosynthesis | 2: Metabolism |
| 1: Focal adhesion | 2: Neurological related |
| 1: ECM-receptor interaction | 1: RNA transport |
| 1: Regulation of actin cytoskeleton | 1: RNA transport |
| **A vs. D** 11: Infection-inflammation | 9: PLC and IL8 involved Immuno-inflammatory |
| 4: Metabolism | 5: Smooth muscle contraction |
| 1: Cell adhesion molecules | 1: ECM-receptor interaction |
| 1: Aldosterone-regulated sodium reabsorption | 1: Hematopoietic cell lineage |
| **C vs. B** 11: Infection-inflammation | 3: Infection-inflammation |
| 3: Metabolism | 3: Metabolism |
| 1: Cell adhesion molecules | 1: Neuroactive ligand-receptor interaction |
| 1: Complement and coagulation cascades | 1: Complement and coagulation cascades |
| 1: Transcriptional misregulation | 1: Transcriptional misregulation |
| **C vs. D** 5: Signaling | 13: Infection-inflammation |
| 4: Infection-inflammation | 6: Signaling |
| 1: Neuroactive ligand-receptor interaction | 4: Metabolism |
| 1: Complement and coagulation cascades | 1: Smooth muscle contraction |
| 1: Hematopoietic cell lineage | 1: Transcriptional misregulation |
| 1: Cell adhesion molecules | 1: Cell adhesion molecules |
| **D vs. B** 10: Metabolism | 5: Signaling |
| 6: Smooth muscle contraction | 4: Metabolism |
| 4: Signaling | 2: Infection-inflammation |
| 4: Infection-inflammation | 2: Cell adhesion molecules |
| 3: Cell adhesion molecules | 1: Smooth muscle contraction |
| 1: ECM-receptor interaction | 1: Neuroactive ligand-receptor interaction |

A = PPROM, B = FTB, C = PTB, D = PROM.
doi:10.1371/journal.pone.0079897.t003

LncRNA Pathways Associate with PPROM

PLOS ONE | www.plosone.org 6 November 2013 | Volume 8 | Issue 11 | e79897
Figure 3. Metabolic pathways characterized from the lncRNAs differentially expressed in PPROM. Six groups of pathways (each group has up- and down-regulated) were characterized with KEGG functional analysis. Three p values, the EASE-score, Fisher-Pvalue and Hypergeometric-Pvalue were integrated for the analysis. The bar plot shows the top Enrichment Score [-log10(Pvalue)] value of the significant enrichment pathway. If there were more than 10 pathways whose Enrichment Score is >0.05, only the top 10 pathways are shown here. The higher Enrichment Score indicates the more lncRNA molecules are involved in this pathway.

doi:10.1371/journal.pone.0079897.g003
by which lncRNAs regulate gene expression, they may regulate different metabolic pathways and participate in a wide repertoire of biological processes [58]. In this study, we identified lncRNAs that are localized in untranslated regions (UTRs) and embedded in protein-coding regions in human placentas from patients with PPROM (group A) and compared them to FTB (group B), PTB without premature rupture of membranes (group C), and PROM (group D). Comprehensive in-depth analysis of the expression profiles of lncRNAs in these group comparisons (A vs. B, A vs. C, A vs. D, C vs. B, C vs. D, and D vs. B) was carried out in order to understand the role of lncRNAs in PPROM, which may help in uncover both preterm delivery and rupture of membrane.

To validate the reliability of microchip technology, 16 lncRNAs, including 7 up-regulated and 9 down-regulated, that represent the differential expression of lncRNAs in PPROM were randomly selected and subjected to RT-qPCR validation (Table 2). Our results demonstrated that the differential expression of 86% (6 out of 7) up-regulated and 89% (8 out of 9) down-regulated lncRNAs were statistically significant (Figure 2). Among the significantly expressed 14 lncRNAs, 10 (71%) were significant at \( p < 0.001 \) and 4 (29%) were at \( p < 0.05 \). 12.5% (2 out of 16) lncRNAs, one from each up-regulated and down-regulated group, were not significant although the RT-qPCR showed their expression was strikingly altered. This provided an overall concordance of 87.5% of the lncRNAs identified from this study with the microchip technology to be reliable. We believe that an increase of the sample size of validation would increase the analysis power and that a high degree of reliability may be achieved.

Instead of investigating the individual pathogenic function of each lncRNA involved in the pathogenic mechanism underlying PPROM, we have focused on investigating the biological pathways and their functions in order to explore what is the likely association and how they are possibly involved in the development of PPROM. Our results showed (at \( p \leq 0.05 \)) that there were 29 biological pathways, including 7 up-regulated and 22 down-regulated, in comparisons of PPROM vs. PTB (A vs. C); 33 pathways, including 18 up-regulated and 15 down-regulated, identified in A vs. D (PROM), and 40 pathways, including 33 up-regulated and 7 down-regulated, in A vs. B (FTB). Because both PPROM and PTB are delivered at \( \leq 35 \) gestational weeks, the only variation between these two groups was the premature rupture of membrane (PROM). From this comparison, our attention was focused on the lncRNAs that are involved in
membrane rupture, which could be a result of premature labor [60], different from the D vs. B where the rupture is the primary factor. However, when PPROM was compared to PROM, the focus was preterm labor since both PPROM and PROM share the common feature of premature membrane rupture. Normal birth at 39–40 gestational weeks without rupture of membrane provided two variation factors for PPROM, which are both preterm and membrane rupture. Therefore, A vs. B may show a combination of pathways presented in both A vs. C and A vs. D. In addition, we have shown 30 pathways in C vs. B, 30 pathways in C vs. D, and 43 pathways in D vs. B, which provided more detailed information to complement the PPROM pathways (Table 3).

Among the PPROM pathways, immunoreactions related to infection and inflammation, including chemokine and cytokine pathways, were the most common pathways within the top-10 list (Figure 3), which were up-regulated in all A vs. C, A vs. B, and A vs. D comparisons and in down-regulated A vs. D. This supported and explained the consensus of most researchers that PPROM is usually caused by genital infections, inflammatory reactions of cytokine and chemokine pathways [61,62]. In the immune processes caused by pathogenic infections, leukocytes secrete different cytokines, among which pro-inflammatory cytokines (including IL-1β, IL-6 and TNF-α) activate host inflammatory response and were closely associated with PPROM [62,63]. TNF-α can bind the receptor TNFR and induce activation of apoptosis, MMPs (matrix metalloproteinases) and caspase, and degradation of extracellular matrix (ECM) in fetal membranes, which play a major role in promoting PPROM [62]. Infection and inflammatory pathway is also seen in the list of top-10 pathways of non-PPROM groups, in both up- and down-regulation, although it may not be the most common pathway in the group of C vs. D and D vs. B.
Pathway of ECM-receptor interaction is presented in all PPROM down-regulated comparisons in this study, although there was only one pathway in each comparison. PPROM was closely associated with the remodeling of ECM, because weakening of the amniochorion ECM is one of the key events predisposing to membrane rupture [62,64]. The down-regulated focal adhesion and ECM-receptor interaction may further provide evidence supporting the previous clue [62,64] that the remodeling of ECM is a key component in the pathogenesis of PPROM. In this study, ECM-receptor interactions were involved in down-regulation of lncRNAs of collagens, laminin, OPN, VLAα10, α/βDG, and α6β1, which suggested that these lncRNAs may be critical for the decreased synthesis of mRNA and resulted in weakness of the extra cellular matrix. Up-regulation of lncRNA involved in ITGA and ITGB suggested a regulatory function that increased lncRNA could reduce the synthesis of ITGA and ITGB. The up-regulated pathway of ECM-receptor interaction in A vs. C and D vs. B but down-regulated in A vs. B and A vs. D may suggest different mechanisms of regulation that lncRNAs involved, which deserved further investigations.

Although the second most common pathway in PPROM is the signaling since there were 13 pathways involved in both up- and down-regulation of A vs. C and A vs. B, the pathways of regulation of actin cytoskeleton in A vs. C and that of smooth muscle contraction in A vs. D are more relevant to the premature labor. LncRNAs of ERM, IQGAP, ROCK, RhoGEF, F2RCD14, GF, RTK, Ras, FN1, ITG, MLCK, PAK, CFN, and GÖy identified from our discovery study of microarray were differentially expressed in the pathway regulating actin cytoskeleton, through activation of actin stress fiber, actin polymerization, and actomyosin assembly contraction in addition to adherens junction, focal adhesion, and MARK signaling (Figure 5A). In A vs. D, the regulation of lncRNA on smooth muscle contraction could be through calcium signaling by PLC, s-GC, CRLR, MHC, DHPR, TnI, TPM, ACTA1/CyR2, and TCF/LEF; or by ECM-receptor interactions through SGCD, Desmin, and Lamin A/C, as shown in an example of the pathway in Figure 3B.

Thirteen differentially expressed lncRNAs embedded in coding sequences, among which, six are sense strand and seven are antisense strand, have been identified (Table 5). The lncRNA embedded in the coding locus of AKAP8 was the one identified from A vs. C only; QSOX1, SYNO2L, ADAM12, and ADH6 were unique from A vs. D; and TP73 and HOMER3 were unique from A vs. B [65–68]. These unique differentially expressed lncRNA molecules may lead us to further explore their possible pathogenic function in the development PPROM. Two antisense lncRNAs were particularly interesting in this study, the ADAM12 (ADAM metallopeptidase domain 12) [69,70] and PGF (placental growth factor) [71]. ADAM12 (a disintegrin and metalloprotease) is a member of a family of multidomain proteins with structural homology to snake venom metalloproteases. This family possesses extracellular metalloprotease and cell-binding functions, as well as intracellular signaling capacities. It is an active metalloprotease, and has been implicated in insulin-like growth factor (IGF) receptor signaling, through cleavage of IGF-binding proteins, and in epidermal growth factor receptor (EGFR) pathways, via ectodomain shedding of membrane-tethered EGFR ligands. These proteolytic events may regulate diverse cellular responses, such as altered cell differentiation, proliferation, migration, and invasion. ADAM12 may also regulate cell-cell and cell-extracellular matrix contacts through interactions with cell surface receptors—integrins and syndecans—potentially influencing the actin cytoskeleton. Interacting with several cytoplasmic signaling and adaptor molecules through its intracellular domain, ADAM12 thereby directly transmits signals to or from the cell interior [69,70]. These ADAM12-mediated cellular effects appear to be critical events in both biological and pathological processes in PPROM, as well as in PROM and PTB. The PGF was identified from A vs. B and A vs. C. It is a placental vascular endothelial growth factor-related protein, activating angiogenesis and endothelial cell growth, stimulating their proliferation and migration. It binds to the receptor FLT1/VEGFR-1 [71]. It is likely that reduction of lncRNA could reduce the mRNA of PGF in the placenta and lead to further investigations.

### Table 5. Differentially expressed lncRNA in coding sequences.

| A vs. C | A vs. D | A vs. B | Accession | Gene | Strand | Protein |
|---------|---------|---------|-----------|-------|--------|---------|
| Up-regulated | 5.73216 | 6.01079649 | 2.987450315 | NM_002127 | HLA-G | + | Major histocompatibility complex, class I, G |
| 4.85154 | 1.062784137 | 1.958096274 | NM_001003681 | HMGXB4 | + | HMG box domain containing 4 |
| 4.631371 | 1.01712411 | 2.073246461 | NM_003156 | STIM1 | + | Stromal interaction molecule 1 |
| 1.245048 | 2.495066469 | 1.140838006 | NM_001004128 | QSOX1 | + | Quiescin Q6 sulfhydryl oxidase 1 |
| 3.57514 | 9.37050789 | 2.73985824 | NM_012215 | MGEA5 | - | Meningioma expressed antigen 5 (hyaluronidase) |
| 3.53793 | 9.655556342 | 1.820589859 | NM_005858 | AKAP8 | - | A kinase (PRKA) anchor protein 8 |
| 1.15697 | 3.411582277 | 0.877613084 | NM_001114133 | SYNO2L | - | Synaptopodin 2-like |
| 1.10173 | 2.908425097 | 1.198747287 | NM_003474 | ADAM12 | - | ADAM metallopeptidase domain 12 |
| 0.793904 | 2.295361844 | 0.664734368 | NM_000672 | ADH6 | - | Alcohol dehydrogenase 6 (class V) |
| Down-regulated | 0.4410479 | 0.49884761 | 0.29568434 | NM_004100 | EYA4 | + | Eyes absent homolog 4 (Drosophila) |
| 0.617984 | 1.086721628 | 0.481554083 | NM_001126240 | TP73 | + | Tumor protein p73 |
| 0.4626384 | 1.349994706 | 0.380171464 | NM_002632 | PGF | - | Placental growth factor |
| 0.616802 | 1.13796367 | 0.482993323 | NM_001145724 | HOMER3 | - | Homer homolog 3 (Drosophila) |

A = PPROM, B = FTB, C = PTB, D = PROM. doi:10.1371/journal.pone.0079897.t005
focusing on the lncRNAs regulation on the genes discussed here are necessary to illustrate their mechanism(s) of regulation. In summary, identification of thousands of differentially expressed lncRNAs from the human placentas of PPROM, FTB, PTB, and PROM provides evidence that lncRNAs may be participating in the physiological and pathogenic processes of human pregnancies relevant to reproductive conditions and disorders. Characterization of metabolic pathways further supports previous findings that infection and inflammatory response, ECM-receptor interactions, apoptosis and smooth muscle contraction are the major pathogenic mechanisms involved in the development of PPROM, along with PROM and PTB. Although the detailed function and pathogenesis how individual lncRNAs play their role(s) in the PPROM and PTB is still unknown, our findings have opened a new avenue for exploration.

Materials and Methods

Ethics statement

The Hospital Ethics Committee reviewed and approved the research project. Informed consent was obtained from all participants. All materials and data were previously encoded and kept anonymous to the authorship of this study.

Placentas

A total 40 placentas from age-matched (25–30 years old) were divided into four groups (10 placentas per group) of deliveries. These are: group A, PPROM ≤35 weeks of gestation; group B, FTB at 39–40 weeks of gestation without membrane rupture; group C, PTB at ≤35 weeks of gestation without membrane rupture; and group D, PROM (premature membrane rupture) at 39–40 weeks of gestation; group C, PTB at 39–40 weeks of gestation without membrane rupture; and group D, PROM (premature membrane rupture) at 39–40 weeks of gestation.

Microarray

The Arraystar Human LncRNA Array v2.0 was designed for researchers who were interested in profiling lncRNAs that are localized at UTRs and embedded in protein-coding regions in the human genome. 33,045 lncRNAs were collected from the authoritative data sources including RefSeq, UCSC Knowngenes, Ensembl and many related articles from the literature (www.arraystar.com).

RNA labeling and array hybridization

Sample labeling and array hybridization were performed according to the Agilent One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technology, Santa Clara, CA) with minor modifications. Briefly, mRNA was purified from total RNA after removal of rRNA with mRNA-ONLY™ Eukaryotic mRNA Isolation Kit (Epitome, Omaha, NE). Then, each sample was amplified and transcribed into fluorescent cRNA along the entire length of the transcripts without 3’ bias utilizing a random priming method. The labeled cRNAs were purified by RNeasy Mini Kit (Qiagen, Valencia, CA). The concentration and specific activity of the labeled cRNAs (pmol Cy3/μg cRNA) were measured by NanoDrop ND-1000. 1 μg of each labeled cRNA was fragmented by adding 5 μl 10× Blocking Agent and 1 μl of 25× Fragmentation Buffer, then heated the mixture at 65°C for 30 min, finally 25 μl 2× GE Hybridization buffer was added to dilute the labeled cRNA. 50 μl of hybridization solution was dispensed into the gasket slide and assembled to the LncRNA expression microarray slide. The slides were incubated for 17 hours at 65°C in an Agilent Hybridization Oven. The hybridized arrays were washed, fixed and scanned with using the Agilent DNA Microarray Scanner (Agilent Technology, Santa Clara, CA).

Data analysis

Agilent Feature Extraction software (version 11.0.1.1) was used to analyze acquired array images. Quantile normalization and subsequent data processing were performed with using the GeneSpring GX v12.1 software package (Agilent Technologies, Santa Clara, CA). After normalization of the raw data, lncRNAs and mRNAs that have flags (“All Targets Value”) were chosen for further data analysis. Differentially expressed lncRNAs and mRNAs with statistical significance between the two groups were identified through Volcano Plot filtering. Hierarchical Clustering was performed using the Agilent GeneSpring GX software (Version 12.1). Both “GO analysis” and “Pathway analysis” were performed in the standard enrichment computation method.

Quantitative real-time PCR analysis

Total RNA was extracted from placentas, and cDNA was synthesized. The expression level of lncRNAs were determined by qPCR, and the primer sequences were listed in Table 2. qPCR reactions were performed by the ABI7900 system (Applied Biosystems) and SYBR green dye PCR master mix (SuperArray). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control, and lncRNAs’ values were normalized to GAPDH. For each lncRNA, the result was finally reported as relative expression value by setting the expression value in PTB (group C) at 1 and the expression value in PPROM (group C) was calculated relative to this control. All data were given in terms of relative expression of mean ± S.E. (N = 5). The data were subjected to one-way analysis of variance (one-way ANOVA) followed by an unpaired, two-tailed t-test. Differences were considered significant at P<0.05 (labeled as “*” in Figure 2) and extremely significant at P<0.01 (labeled as “**” in Figure 2).

Acknowledgments

The authors thank the pregnant women and their families for participating in this study. We also thank KangChen Bio-tech (www.kangchen.com.cn) for their technical support in performing the microarray analyses. We appreciate everyone who helped in this research project.

Author Contributions

Conceptualized and designed the experiments: NZ. Performed the experiments: XL, QS Yang Gu JP MH ML ZD MZ Ying Gu. Analyzed the data: XL ML ZD JZ NZ XZ. Contributed reagents/materials/analysis tools: QS Yang Gu JP. Wrote the paper: ML LW JZ ECJ WTB NZ.
References

1. Romero R, Friel LA, Velez Edwards DR, Kusanovic JP, Hassan SS, et al. (2010) A genetic association study of maternal and fetal candidate genes that predispose to preterm prelabor rupture of membranes. PLoS Med 7: e1000230. doi:10.1371/journal.pmed.1000230

2. Lawn J, Graevel M, Nunes T, Rubens C, Stanton C (2010) Global report on preterm birth and stillbirth. 1 (of 7): definitions, description of the burden and opportunities to improve data. BMC Pregnancy Childbirth 10: S1. DOI: 10.1186/1471-2393-10-S1.

3. Srivastava S, Macones GA (2005) Preterm premature rupture of the fetal membranes: current concepts. Minerva ginecologica 57: 309.

4. DeFranco E, Teramoto K, Muglia L (2007) Genetic influences on preterm birth. Best Pract & Res Clin Obstet Gynaecol 21: 467.

5. Menon R, Fortunato SJ (2007) Infection and the role of inflammation in preterm premature rupture of the membranes: Best Practice & Res Clin Obstet Gynaecol 21: 467.

6.روماني، ر.، فرثان، س.، مدن، ر. (2007) التأثيرات الجينية على مولودات المبكر. دولل. ريبورت ريبورت 187: 1159–1162.

8. Menon R, Fortunato SJ (2007) Preterm birth: causes, consequences, and prevention. National Academy Press.

9. Menon R, Fortunato SJ (2004) Preterm premature rupture of the membrane: a novel role of MMPs and apoptosis. J Reprod Med 49: 427–437.

10. Plunkett J, Dongier S, Oribona G, Morgan T, Haataja R, et al. (2011) An evolutionary genomic approach to identify genes involved in human birth timing. PLOS Genet. 7: e1001365.

11. Kalish RB, Vardhana S, Normand NJ, Gupta M, Perni SC, Chasen ST, et al. (2004) A maternal CD14 -159 gene polymorphism with preterm premature rupture of membranes and spontaneous preterm birth in multi-fetal pregnancies. J Reprod Immunol 68: 109–117.

12. Valdez-Velazquez LL, Quintero-Ramos A, Perez SA, Mendoza-Carrera F, Kalish RB, Vardhana S, Normand NJ, Gupta M, Witkin SS (2006) Association of a maternal CD14 –159 gene polymorphism with preterm premature rupture of membranes and spontaneous preterm birth in multi-fetal pregnancies. J Obstet Gynaecol 169: 805–816.

13. Shore AN, Kabotyanski EB, Roarty K, Smith MA, Zhang Y, et al. (2012) The J Renin-Angiotensin-Aldosterone System 8: 160–168.

14. Dermitzakis ET, Reymond A, Antonarakis SE (2005) Conserved non-genic sequences—a unexpected feature of mammalian genomes. Nat Rev Genet. 6: 151–157.

15. Kapranos P, Cheng J, Dike S, Nis DA, Duttagupta R, et al. (2007) RNA maps reveal new RNA classes and a possible function for pervasive transcription. Science 316: 1484–1488.

16. Talt RJ, Paun KC, Mercer TR, Dinger M, Mattick JS (2010) Non-coding RNAs: regulators of disease. J Pathol. 226: 139–149.

17. Willers JE, Sunwoo H, Spector DI (2009) Long noncoding RNAs: functional surprises from the RNA world. Genes Dev. 23: 1494–1504.

18. Mercer TR, Dinger ME, Mattick JS (2009) Long non-coding RNAs: insights into functions. Nat Rev Genet. 10: 155–159.

19. Gibbs EA, Brown CJ, Lam WL (2011) The functional role of long non-coding RNA in human carcinomas. Mol Cancer. 10: 33.

20. Koerner MV, Pauler FM, Huang R, Barlow DP (2009) The function of non-coding RNAs in genomic imprinting. Development 136: 1717–1783.

21. Tripathi V, Ellis JD, Shen Z, Song DY, Pan Q, et al. (2010) The nuclear-retained noncoding RNA MALAT1 regulates alternative splicing by modulating SR splicing factor phosphorylation. Mol Cell 39: 925–938.

22. Fung, J. B. C., Clark BS, Mady R, Shaw P, et al. (2006) The ES-2 noncoding RNA is transcribed from the Dsc-3/6 ultraconserved region and functions as a Dsc-2 transcriptional coactivator. Genes Dev. 20: 1470–1484.

23. Willingham A, Orth A, Batalov S, Peters E, Wen B, et al. (2005) A strategy for probing the function of noncoding RNAs finds a repressor of NFAT. Science Signaling 309: 1570.

24. Clemson CM, Hutchinson JN, Sara SA, Ensminger AW, Fox AH, et al. (2009) An Architectural Role for a Nuclear Noncoding RNA: RNA Is essential for the structure of paraspeckles. Mol Cell 33: 717–726.

25. Wapinski O, Chang HY (2011) Long noncoding RNAs and human disease. Trends Cell Biol. 21: 534–544.

26. Wilusz JE, Sunwoo H, Spector DL (2009) Long noncoding RNAs: functional surprises from the RNA world. Genes Dev. 23: 1494–1504.

27. Mercer TR, Dinger ME, Mattick JS (2009) Long non-coding RNAs: insights into functions. Nat Rev Genet. 10: 155–159.

28. Willingham A, Orth A, Batalov S, Peters E, Wen B, et al. (2005) A strategy for probing the function of noncoding RNAs finds a repressor of NFAT. Science Signaling 309: 1570.

29. Clemson CM, Hutchinson JN, Sara SA, Ensminger AW, Fox AH, et al. (2009) An Architectural Role for a Nuclear Noncoding RNA: RNA Is essential for the structure of paraspeckles. Mol Cell 33: 717–726.

30. Mercer TR, Dinger ME, Mattick JS (2009) Long non-coding RNAs: insights into functions. Nat Rev Genet. 10: 155–159.

31. Feng, J. B. C., Clark BS, Mady R, Shaw P, et al. (2006) The ES-2 noncoding RNA is transcribed from the Dsc-3/6 ultraconserved region and functions as a Dsc-2 transcriptional coactivator. Genes Dev. 20: 1470–1484.

32. Willingham A, Orth A, Batalov S, Peters E, Wen B, et al. (2005) A strategy for probing the function of noncoding RNAs finds a repressor of NFAT. Science Signaling 309: 1570.

33. Clemson CM, Hutchinson JN, Sara SA, Ensminger AW, Fox AH, et al. (2009) An Architectural Role for a Nuclear Noncoding RNA: RNA Is essential for the structure of paraspeckles. Mol Cell 33: 717–726.
52. Annilo T, Kepp K, Laan M (2009) Natural antisense transcript of natriuretic peptide precursor A (NPPA): structural organization and modulation of NPPA expression. BMC Mol Biol. 10: 81.

53. Kino T, Hurt DE, Ichijo T, Nader N, Chrousos GP (2010) Non-coding RNA gas5 is a growth arrest-and starvation-associated repressor of the glucocorticoid receptor. Science Signalling 3: ra11.

54. Khachane AN, Harrison PM (2010) Mining mammalian transcript data for functional long non-coding RNAs. PLOS One 5: e10316.

55. Ota T, Suzuki Y, Nishikawa T, Ooski T, Sugiyama T, et al. (2003) Complete sequencing and characterization of 21,243 full-length human cDNAs. Nat Genet 36: 40–45.

56. Okazaki Y, Furuno M, Kasukawa T, Adachi J, Bono H, et al. (2002) Analysis of the mouse transcriptome based on functional annotation of 60,770 full-length cDNAs. Nature 420: 563–573.

57. Tupy JL, Bailey AM, Dailey G, Evans-Holm M, Siebel CW, et al. (2005) Identification of putative non-coding polyadenylated transcripts in Drosophila melanogaster. PNAS USA. 102: 5495–5500.

58. Wang KC, Chang HY (2011) Molecular mechanisms of long noncoding RNAs. Mol Cell 43: 904–914.

59. Ponning CP, Oliver PL, Reik W (2009) Evolution and functions of long noncoding RNAs. Cell 136: 629–641.

60. Erez O, Romero R, Tarca AL, Chaiworapongsa T, Kim YM, et al. (2009) Differential expression pattern of genes encoding for anti-microbial peptides in the fetal membranes of patients with spontaneous preterm labor and intact membranes and those with preterm PROM. J Matern-Fetal Neonat Med. 22: 1103–1115 (doi: 10.3109/14767050902994796).

61. Gomez-Lopez N, Laregotti-Servije E, Olson DM, Estrada-Gutierrez G, Vazillo-Ortega F (2010) The Role of Chemokines in Term and Premature Rupture of the Fetal Membranes: A Review. Biol Reprod. 82: 809–814.

62. Menon R, Fortunato SJ (2007) Infection and the role of inflammation in preterm premature rupture of the membranes. Best Pract Res Clin Obstet Gynaecol. 21: 467–478.

63. Hanada T, Yoshimura A (2002) Regulation of cytokine signaling and inflammation. Cyto Growth Fact Rev. 13: 413.

64. Menon R, Fortunato SJ (2004) The role of matrix degrading enzymes and apoptosis in rupture of membranes. J Soc Gynecol Invest 11: 427–437.

65. Kamada S, Kikkawa U, Tsujimoto Y, Hunter T (2005) A-kinase-anchoring protein 95 functions as a potential carrier for the nuclear translocation of active caspase 3 through an enzyme-substrate-like association. Mol Cell Biol 25:9469–9477.

66. Morel C, Adami P, Mustafi JF, Duval D, Radom J, et al. (2007) Involvement of sulphydryl oxidase QSOX1 in the protection of cells against oxidative stress-induced apoptosis. Exp Cell Res. 313:3971–3982.

67. Yoshikawa H, Nagashima M, Khan MA, McMinnamin MG, Hagihara K, et al. (1999) Mutational analysis of p73 and p53 in human cancer cell lines. Oncogene 18:3415–3421.

68. Kugai M, Bonnet H, Yang A, Creancier L, Biscan JC, et al. (1997) Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. Cell 90: 809–819.

69. Lorcher F, Gilpin BJ, Engvall E, Albrechtsen R, Wewer UM (1998) Human ADAM 12 (meltrin alpha) is an active metalloprotease. JBC. 273:16993–16997.

70. Kveiborg M, Albrechtsen R, Couchman JR, Wewer UM (2008) Cellular roles of ADAM12 in health and disease. Int J Biochem Cell Biol. 40:1685–1702. doi: 10.1016/j.biocel.2008.01.025.

71. Maglione D, Guerriero V, Vighetto G, Delli-Bovi P, Persico MG (1991) Isolation of a human placenta cDNA coding for a protein related to the vascular permeability factor. PNAS USA. 88: 9267–9271.