# Expression of Nephrin in the human placenta and membranes

Bo Hyon Yun

Department of Medicine

The Graduate School, Yonsei University

# Expression of Nephrin in the human placenta and membranes

Directed by Professor Ja Young Kwon

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Bo Hyon Yun

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This certifies that the Master's Thesis of
Bo Hyon Yun is approved.

권자영

Thesis Committee Member#1: Ja Young Kwon

임범진

Thesis Committee Member#2: Beom Jin Lim

이승미

Thesis Committee Member#3: Seung Mi Lee

The Graduate School
Yonsei University

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ABSTRACT

## Expression of Nephrin in the human placenta and membranes

Bo Hyon Yun

*Department of Medicine*
*The Graduate School, Yonsei University*

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Nephrin, the signature molecule in the podocyte of the glomerulus, which forms the renal slit diaphragm, the main functional unit of the glomerulus. This study focused on the expression of nephrin in the human placenta, which may also play a role in filtration and maintaining homeostasis. We prospectively studied 9 placentas from normal, healthy pregnant women in term. Real-time quantitative polymerase chain reaction, western blotting, and immunofluorescence were performed. The expression of the nephrin mRNA was relatively stronger in the chorion than in villi and amnion. The nephrin gene was detected in villous cytotrophoblast and the endothelium of intravillous vessels. It was also present in chorionic and amniotic membranous lining, with its distribution being particularly dense in the amniocytes. The discovery of nephrin in the human placenta, especially at the maternal–fetal interface, gives a new insight into the molecular basis of placental permselectivity and barrier, which needs further elucidation.

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Key words : term, placenta, nephrin, podocyte

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## I. INTRODUCTION

The podocyte is the key component of the glomerular slit diaphragm. The podocyte and related proteins form the diaphragm, and these cells have an intimate interaction with capillary endothelial cells, which play an important role in selective filtration and act as barriers. Recently, several researchers have identified podocyte-related proteins as the main factors in nephropathy showing glomerular basement membrane alteration, such as diabetic nephropathy. Interestingly, the blood–brain barrier of the central nervous system, another representative homeostatic barrier system in the human body, has astrocytes, which are similar to podocyte in structure and function[1,2].

Nephrin is one of podocyte-related proteins, which is a constituent of the slit diaphragm of the glomerulus, which seems to play a critical role in maintaining glomerular filtration. And inactivation of nephrin gene in mouse embryo demonstrated a detrimental loss of ultrafiltering function of the kidney[3].

As kidney, placenta is a major organ of the fetus which filtrates and exchanges fetal and maternal blood so placental cotyledon and glomerulus may exhibit common

homeostatic functions. And interestingly, the expression of the nephrin gene has been reported in the fetal membranes and placenta of pregnant Sprague-Dawley rats[4]. This led us to hypothesize that nephrin may be involved in regulating homeostasis at the maternal–fetal interface. However, thus far, evidence of nephrin expression in the human placenta is lacking. Therefore, the objective of this study was to evaluate the presence and localization of nephrin, a signature molecule of podocytes[5], in the human placenta.

## II. MATERIALS AND METHODS

### *Participants*

Women with a singleton pregnancy who delivered a normal infant between 37 and 40 weeks of gestation by Cesarean section at Severance hospital, Yonsei University Health System (YUHS), between January 2011 and July 2012 were enrolled in this study. We excluded patients with a history of active labor prior to the surgery, multiple pregnancies, prior or current diagnosis of any medical illness (diabetes, gestational diabetes, hypertensive disease, thyroid disease, or infectious disease), placenta previa, fetal anomaly, oligohydramnios, hydramnios, fetal aneuploidy, preterm labor, or premature rupture of the membranes during the present pregnancy. This study was approved by the Institutional Review Board of YUHS, and all patients consented to participate.

### *Sample collection*

When the placenta was delivered, the amnion and chorion were dissected under aseptic conditions. Dissected membranes were sampled at a size of 1.0 × 1.0 × 1.0 $cm^3$. Villous tissue measuring 1.0 × 1.0 × 1.0 $cm^3$ was sampled at the site near the cord insertion. After the tissue sampling, the amnion, chorion, and villous tissue were refrigerated in liquid nitrate and stored for further analysis.

### *Quantification of mature mRNA levels using real-time quantitative polymerase chain reaction*

We first isolated RNA fractions from the tissue samples by using the RNA extraction kit (iNtRON Biotechnology, Gyeonggi, Korea). We measured RNA

concentrations using absorbance at 260/280 nm. Total RNA (1 μg) was converted to cDNA using oligo-dT as the primer and reverse transcriptase (Invitrogen, Carlsbad, CA). The mixture was incubated at 65℃ for 5 min, 50℃ for 50 min, and 85℃ for 5 min. Quantitative real-time quantitative polymerase chain reaction (RT-PCR) was performed with Applied Biosystems 7500 Real-Time PCR system using the Taqman® Gene Expression Assays. A total of 20 μL of the qRT-PCR reaction mixture contains 2 μL of RT-PCR products, 10 μL Taqman® 2X Universal PCR Master Mix, and 1 μL 20X Taqman® Gene Expression Assay mix (qRT-PCR primers). Nuclease-free water was used to adjust the final volume to 20 μL. The reactions were incubated in a 96-well optical plate at 95℃ for 10 min, followed by 40 cycles of 95℃ for 15 s and 60℃ for 60 s. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an endogenous reference gene for normalizing the results. The relative abundance of each mRNA was calculated using the comparative cycle threshold 2(-ΔΔCt) method. Individual samples were assayed in triplicate, with three independent biological replicates. The assay numbers for the mRNA endogenous control (*GAPDH*) and target mRNAs are depicted in Table 1.

Table 1. Sequence of primers of *GAPD* and the human nephrin gene

| **Gene** | | **Primer sequence (5′→ 3′)** | **Size (bp)** |
|---|---|---|---|
| ***GAPDH*** | Sense | AGGCCAACCGCGAGAAGATGACC | 320 |
| | Antisense | GAAGTCCAGGGCGACGTAGCAC | |
| **Human** | Sense | CCAACATCGTTTTCACTTGG | 349 |
| **nephrin** | Antisense | GGGTGGTACGACATCCACAT | |

***Western blotting***

Nephrin expression was examined by western blotting analysis of the tissue homogenate samples. After isolating each protein by using the cell lysis buffer (Cell Signaling Technology, Inc.), 30 μg of total protein loading was used to run 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (PVDF; Millipore, Etten-Leur, the Netherlands). The blots membranes were blocked with 5% skim milk in Tris-buffered saline–Tween 20 (TBS–T: 10 mM Tris, pH 8.0, 150 mM NaCl, 0.0025% Tween 20) at room temperature for 1 h. Then, the membrane was incubated overnight at 4℃ with specific antibodies against anti-nephrin (polyclonal rabbit anti-human, 1:1000; ProSci, Portway, CA) and anti-beta actin (monoclonal mouse anti-human, 1:2000; Sigma, St Louis, MO) antibodies. The membrane was then washed six times with TBS–T for 5 minutes. Thereafter, the membrane was incubated with anti-rabbit antibody (Ig G, Abcam, Cambridge, MA) and anti-mouse antibody conjugated with horseradish peroxidase (Ig G, 1:5000; Abcam, Cambridge, MA) for 1 h at room temperature. The membrane was washed eight times with TBS–T for 5 minutes at room temperature. The protein was visualized on LAS-4000 by enhanced chemiluminescence (Amersham, Arlington Heights, IL). Relative densities for nephrin expression were normalized using tubulin expression in each sample.

***Immunofluorescence (IF)***

Cells were fixed with acetone for 15 minutes and washed three times with PBS-T (PBS/0.1% Tween 20 [v/v]). After blocking with 5% goat serum for 1 hour, the cells were incubated with anti-nephrin antibody (polyclonal rabbit anti-human, 1:1000; ProSci, Portway, CA) overnight at 4℃. Negative controls consisted of cells incubated with rabbit IgG (1:200, ab27478, Abcam, Cambridge, MA) at the same concentration

as the antibody against nephrin. The cells were washed three times with PBS-T and incubated with goat polyclonal secondary antibody to rabbit IgG (1:200, DyLight® 594, ab96901, Abcam, Cambridge, MA) for 1 hour. The cells were washed three times in PBS-T and mounted with a fluorescent mounting medium (Vector Laboratories, Inc., Burlingame, CA). Fluorescent microscopy was performed using LSM700 (Carl Zeiss; OBerkochen, Germany).

***Statistical analysis***

Statistical analysis was performed using the SPSS software package, version 18.0 (IBM Inc., Armonk, NY). The mRNA expression level of the nephrin gene was expressed as mean ± SD. We used descriptive statistics for the baseline characteristics and the linear mixed model for comparing the mRNA expression of the nephrin gene in the villi, chorion, and amnion. P-value under 0.05 was considered statistically significant.

III. RESULTS

In all, 9 healthy pregnant women were enrolled in this study. The basal characteristics of the patients are shown in Table 1. Because of the small sample size, data was expressed as median and range. One participant had a history of previous cesarean section and concurrent myomectomy.

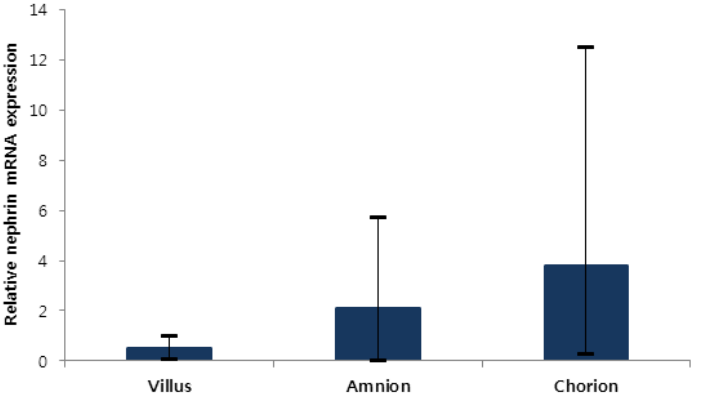
Table 2. Clinical characteristics of pregnancies included in the study

| **Variables** | | |
|---|---|---|
| Maternal age (years) | 34 (30–42) | |
| Primigravida (n) | 2 | |
| Nulliparity (n) | 4 | |
| Gestational days | $38^{+3}$ ($37^{+3}$–$39^{+1}$) | |
| Cesarean section indication (n) | Prior cesarean section | 6 |
| | Prior myomectomy | 1 |
| | Advanced maternal age | 1 |
| | Cephalopelvic disproportion | 2 |

Data were expressed as median and range

### *1. Gene and protein expressions in placenta*

The expression of nephrin gene and protein in the uteroplacental unit were confirmed by real-time PCR and Western blotting, respectively (Figure 1). The gene expression was relatively higher in the chorion when compared to the villus and amnion (mean, $2^{-\Delta\Delta Ct}$ 3.79 ± 4.49 vs. 0.50 ± 0.45 and 2.12 ± 2.41), but the difference was not statistically significant ($p = 0.37$).


Relative nephrin mRNA expression
14
12
10
8
6
4
2
0
Villus
Amnion
Chorion

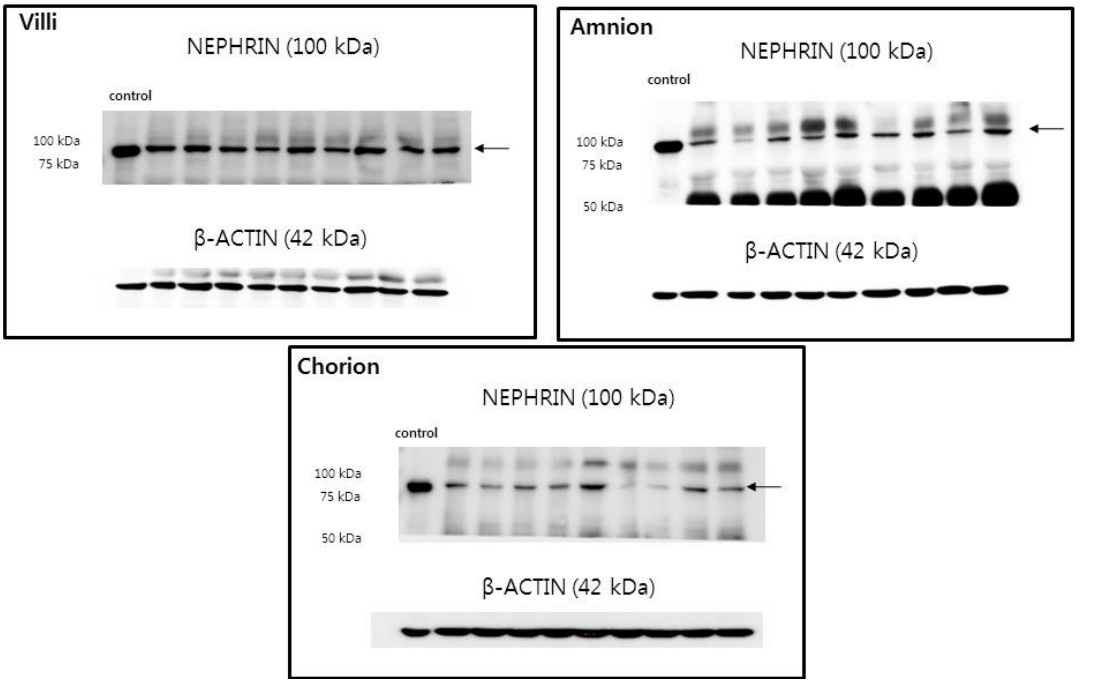
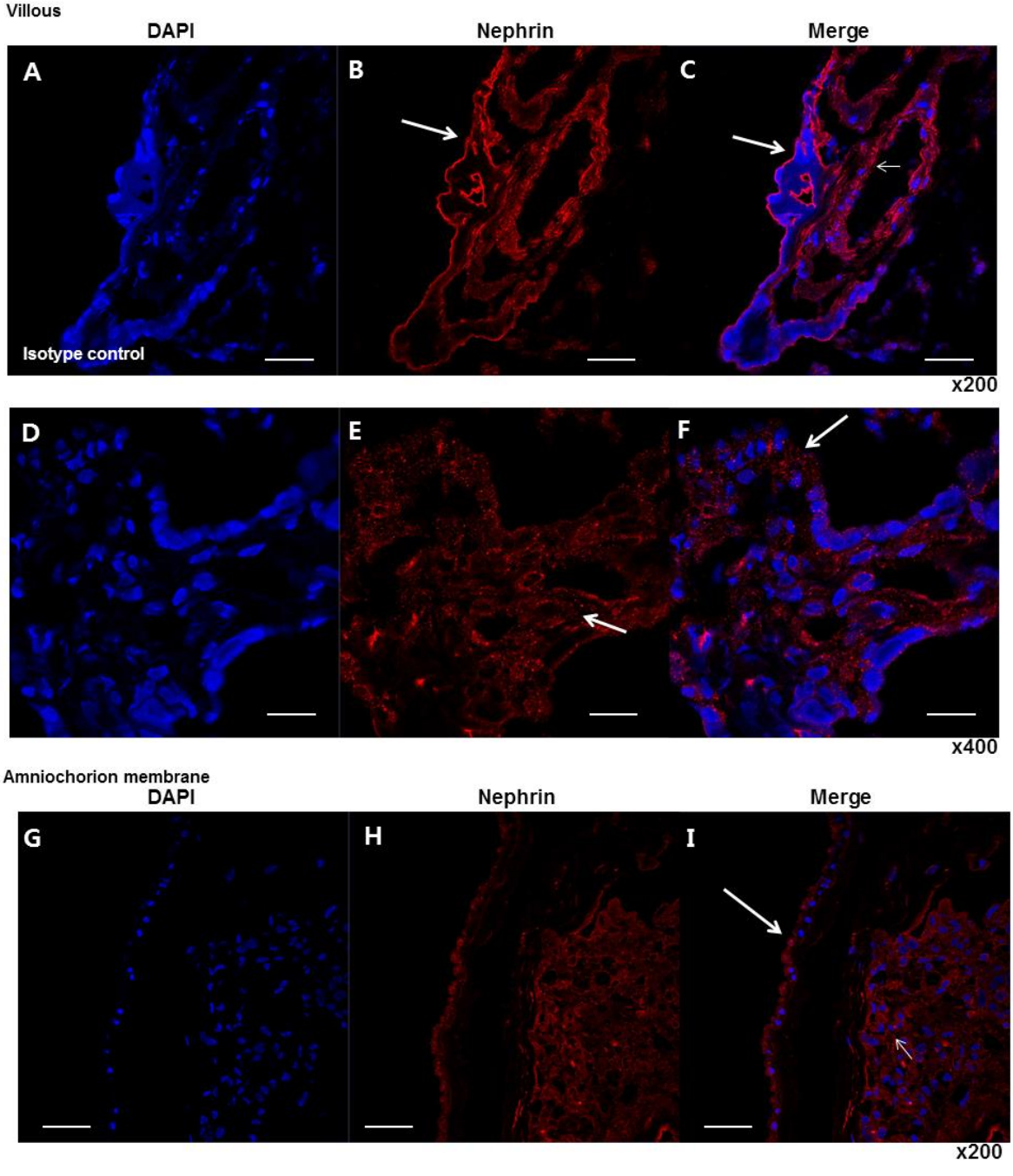

Villi
NEPHRIN (100 kDa)
control
100 kDa
75 kDa
β-ACTIN (42 kDa)
Amnion
NEPHRIN (100 kDa)
control
100 kDa
75 kDa
50 kDa
β-ACTIN (42 kDa)
Chorion
NEPHRIN (100 kDa)
control
100 kDa
75 kDa
50 kDa
β-ACTIN (42 kDa)

Figure 1. Expression of the nephrin gene in placental villi and fetal membranes. The bar graph shows relative mRNA expression by qRT-PCR (n = 9). Data are the means ± SD and are expressed as relative ratios of the expression levels of mRNA of the nephrin gene to those of *GAPDH*. The lower panel shows western blot for nephrin expression in the villi and fetal membranes (n = 9).

***2. Imunolocalization of nephrin***

A strong positive staining for nephrin was observed in the villi, chorion, and amnion on immunofluorescence studies. Nephrin expression was strongly localized to the syncytiotrophoblast and endothelial cells of the arteries and veins of the chorionic plate, where the labeling was pronounced at the apical membrane of the syncytiotrophoblast. In the fetal membranes, intense immunoreactivity was localized to the cuboidal amniotic epithelium, especially at the apical membrane and in the stromal cells (Figure 2).


Villous
DAPI
Nephrin
Merge
A
B
C
Isotype control
x200
D
E
F
x400
Amniochorion membrane
DAPI
Nephrin
Merge
G
H
I
x200

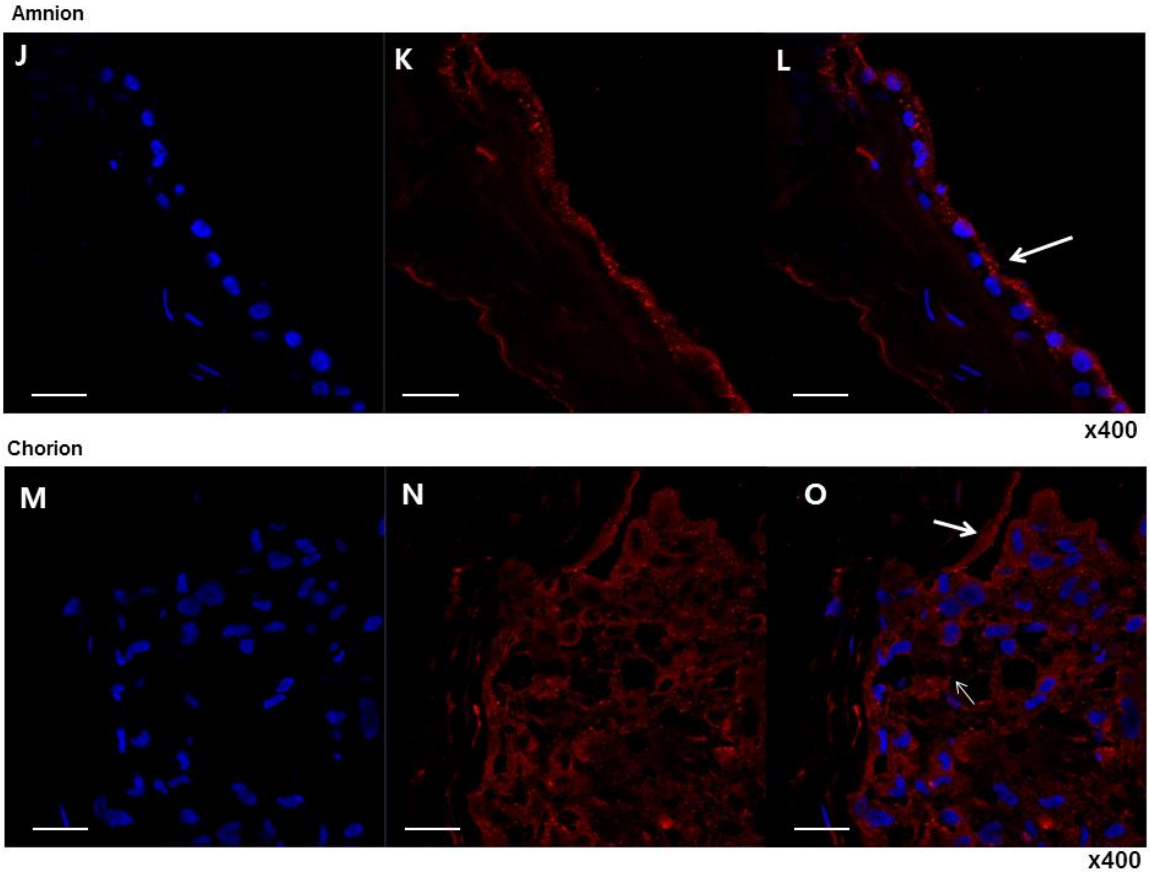



Figure 2. Immunolocalization of nephrin in the placenta by immunofluorescence. A and D, isotype control with 4′,6-diamidino-2-phenylindole (DAPI) in villous tissue. Magnification, ×200 (A), ×400 (D). B, C, E, and F. Immunofluorescence staining of nephrin in the villi. Note that immunoreactive staining is distributed over almost all of villous cytotrophoblasts (Arrow in B, E, and F) and endothelium of intravillous vessels (Small arrow in E). Magnification, ×200 (B, C), ×400 (E, F). G, J and M, isotype control with DAPI in fetal membranes. Magnification, ×200 (G), ×400(J, M). I, L, and O. Immunofluorescence using nephrin in fetal membranes revealed not only a specific distribution at membranous lining (Arrow in I, L and O), but also in

fibroblasts in the chorion (Small arrow in I and O). Note that specifically in the amnion, the outer lining of amniocytes was strongly positive (arrow in I and L). Magnification, ×200(H, I), ×400 (K, L, N, and O). Bar = 50 μm.

## IV. DISCUSSION

The main role of the placenta is the separation of maternal and fetal circulation, while also facilitating the transport of nutrients and substances from the mother to the fetus. Since the placenta has extensive contact between the mother and the fetus at levels such as the villus, amnion–chorion, and chorion–decidua, it is speculated that a filtering, barrier-like structure exists within the placenta at such interfaces. As hypothesized, we found evidence of the presence of nephrin in cells at the maternal–fetal interface, and immunoreactivity to nephrin was most prominent at the apical membrane of the syncytiotrophoblasts and amniotic epithelium and in the stromal cells of the chorion. These finding suggests the existence of a barrier-like structure formed by nephrin in the placenta similar to that of glomerulus.

Although the present study did not investigate the function of nephrin in the placenta, it is likely that in the placenta, the protein may play a role similar to that observed in other organs[6-11]. Its presence in astrocytes that interact with the capillaries in the blood-brain barrier of the CNS suggested similar role as in the glomerular slit membrane[11]. The role for neprhin as a barrier system has also been suggested in the testis by demonstrating nephrin in the Sertoli cells colocalized with zona occludens-1 (ZO-1) along the basement membrane of seminiferous tubule in the testis[7]. However, recent studies demonstrated that nephrin is expressed in the radial glial cells which are involved in the directional migration of neurons and development of glial cell lineages[12], and that it binds with glutamate receptors and scaffolding molecule of primary neuronal cells[3]. Furthermore, active role in the vesicle and actin interaction involved in the insulin release process was described in the pancreatic beta islet cells[9,10]. These findings suggest that nephrin not only functions as a barrier but also exhibits functional role such as cell maturation and development, cell-to-cell interaction, or signaling[13-17].

It can be hypothesized that nephrin expressed in villi and the membranes holds

such functions as well. Nephrin may be involved in the seclusion of fetal materials from the maternal circulation and thereby provide an immunologically anergic environment, it may be crucial to the exchange of nutrients between the mother and the fetus, or it may take role in the placental development. And if so, impairment of placental nephrin may result in adverse pregnancy outcome such as preeclampsia, restricted fetal growth, or abnormal placental growth. As serum from a preeclampsia patient has been shown to damage the podocyte and shed nephrin via endothelin-mediated endothelin-1[18], theoretically, nephrin in the syncytiotrophoblasts or the chorioamniotic membranes in close contact with the maternal serum can also be affected or disrupted via a similar mechanism. And this may partly contribute to the pathophysiologic mechanism of adverse fetal outcomes.

## V. CONCLUSION

The present study is the first to provide evidence of the presence of nephrin in the human placenta and placental membranes. The discovery of podocyte-related nephrin in the placenta, especially at the maternal–fetal interface, gives new insight into the molecular basis of placental permselectivity and barrier, which needs further elucidation. Our findings highlight the need for further investigation of the placental expression of podocyte-related proteins in complicated pregnancies and their specific roles in the human placenta.

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ABSTRACT (IN KOREAN)

# 인간 태반에서의 족세포 연관 단백질 발현

<지도교수 권자영>

연세대학교 대학원 의학과

윤 보 현

신장 사구체의 족세포에서 가장 대표적인 단백질인 네프린은, 사구체 여과 장벽의 기능적 구조인 세극막을 구성한다. 이 연구는, 사구체와 같이 여과와 항상성 유지 기능을 임신 중 태아와 산모 사이에서 매개하는 인간 태반에서, 사구체와 동일하게 네프린 발현이 관여할 가능성에 초점을 맞추어 계획되었다. 본 연구에서는 건강에 이상이 없는 정상 만삭 산모 9명을 포함시켜 연구를 진행하였다. 산모의 태반 조직으로 네프린 단백에 대한 실시간 정량 PCR, Western blotting, 면역화학염색을 시행하였다. 네프린 mRNA의 발현은 융모와 양막에 비해 융모막에서 상대적으로 더 강하게 나타났다. 네프린 유전자는 융모의 세포영양아세포와 융모간 혈관의 내피세포에서 발견되었다. 네프린 유전자는 또한 융모막과 양막의 막 표면을 따라 나타났고, 특히 양막세포에서 그 분포밀도가 높은 것을 관찰하였다. 인간 태반에서의 네프린 존재의 발견은, 특히 모체-태아 경계에서, 태반의 투과 장벽으로서의 역할에 대한 새로운 분자생물학적

기초를 제공하는 바이며, 추후 이와 관련된 심도 있는 연구들이 필요하다.

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핵심되는 말: 임신, 태반, 네프린, 족세포