

# Interaction of steroid receptor coactivators and estrogen receptors in the human placenta

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## Abstract

Female sex steroid hormones such as estrogen and progesterone have a pivotal role in maintaining pregnancy in human and animals. Especially, estrogen exerts specific effects on the cardiovascular system and angiogenesis, and thus affects significantly on placentation. Although the functions of estrogen have been emphasized during pregnancy, their signaling pathways in the placenta have not been fully understood. In this study, estrogen signaling was evaluated according to gestational age. Human placenta samples were collected and divided into early preterm ( $n=10$ ), late preterm ( $n=18$ ), and term ( $n=20$ ) groups. First, serum estrogen concentration and corticotropin-releasing hormone (*CRH*) mRNA expression, which is known as gestation clock gene, were increased following gestation age in our experimental condition, as we expected. Next, the expression of estrogen receptors (ERs) and steroid receptor coactivators (SRCs) in the placenta was evaluated. ER $\alpha$  (ESR1) and ER $\beta$  (ESR2) were expressed highly at term period compared with early preterm. In addition, SRC family including SRC1, SRC2, and SRC3 was expressed in the human placenta, and the levels of SRC1, SRC2, and SRC3 were increased in the placenta at the late stage of gestation. The interaction of ERs with SRCs was also examined, which was significantly enhanced at term period. In the immunostaining results, it was indicated that ERs and SRCs were all dominantly expressed in syncytiotrophoblast cells. These results suggested that SRC1, SRC2, and SRC3 were expressed and interact with ERs highly at the late stage of gestation, and may amplify the signaling of estrogen in the placenta to maintain pregnancy.

## Key Words

- ▶ placenta
- ▶ estrogen receptor
- ▶ steroid receptor coactivator
- ▶ gestation
- ▶ estrogen

*Journal of Molecular Endocrinology*  
(2016) **56**, 239–247

## Introduction

The placenta is a specialized organ during pregnancy, which in combination with fetal membranes and amniotic fluid supports normal growth and development of the fetus. The placenta has a hemochorial villus, whereby maternal blood comes into direct contact with placental trophoblast cells and allows an intimate relationship with the developing

embryo (Moore *et al.* 2015). The placental membrane is made of four layers, a maternal facing syncytiotrophoblast layer, a cytotrophoblast cell layer, connective tissue of the villus, and maternal endothelium lining the fetal capillaries (Gude *et al.* 2004). However, the cytotrophoblast cell layer of the villus becomes attenuated and disappears after approximately

20 weeks. Subsequently, in most chorionic villi, the membrane consists of three layers and becomes extremely thin in some areas such that the syncytiotrophoblast directly contacts the fetal capillary endothelium. Thus, in these positions, the maternal and fetal blood comes into very close proximity (as little as 2–4  $\mu\text{m}$ ) (Gude *et al.* 2004).

The main functions of the placenta can be categorized under the headings of transport and metabolism, protection, and endocrine functions. For transport, the placenta provides oxygen, water, carbohydrates, amino acids, lipids, vitamins, minerals, and other nutrients to the fetus, whereas removes carbon dioxide and other waste products (Gude *et al.* 2004). The placenta also metabolizes numerous substances and releases metabolic products into maternal and/or fetal circulations. It also helps to protect the fetus against certain toxic molecules, infections, and maternal diseases. For endocrine function, the placenta releases hormones into both the maternal and fetal circulations to affect pregnancy, metabolism, fetal growth, and parturition. The endocrine factors produced by the placenta include estrogen, progesterone, chorionic gonadotropin, placental lactogen, placental growth hormone, and many others (Gude *et al.* 2004, Lehrer *et al.* 1990).

During human pregnancy, the placenta produces large amounts of estrogen. Estrogen exerts a wide variety of effects on cellular growth, development, and differentiation. The physiological effects of estrogen include important regulatory functions within the reproductive systems of both females and males, which are related to mammary gland development and differentiation as well as regulation of the hypothalamic–gonadal axis (Pepe & Albrecht 1995). During pregnancy, estrogen regulates a sequence of events leading to initiation of labor (Gibb *et al.* 2006), biosynthesis of progesterone, and placental transfer mechanisms that modulate maturation of the fetal reproductive system (Pepe & Albrecht 1995). Furthermore, estrogen stimulates angiogenesis, which is a critical process for placental function, by regulating vascular endothelial growth factor (VEGF) and growth of endothelial cells in the placenta. Therefore, abnormal estrogen levels and angiogenesis in the placenta are associated with complications such as preeclampsia and preterm delivery (Albrecht & Pepe 2010).

Estrogen acts by binding to its receptor, estrogen receptor (ER). ER exists in two main forms, ER $\alpha$  (ESR1) and ER $\beta$  (ESR2), which have distinct tissue-specific expression patterns (Mueller & Korach 2001). ER $\alpha$  and ER $\beta$  are nuclear steroid hormone receptors with similarities in terms of ligand-binding and nuclear DNA-binding regions (Arnal *et al.* 2007, Luksha & Kublickiene 2009). ER $\alpha$  and ER $\beta$  are encoded by separate genes, *ESR1* and *ESR2*, respectively,

located at different chromosomal locations, and numerous mRNA splice variants exist for both receptors in both diseased and normal tissues (Herynk & Fuqua 2004). In the ‘classical’ pathway, estrogen binds to ER to regulate the transcription of target genes in the nucleus by binding to estrogen response element (ERE) in the promoters of target genes and by recruiting coregulatory proteins such as coactivators or corepressors (McKenna & O’Malley 2002).

Several classes of coactivators have been identified and shown to be essential in estrogen responsiveness (McKenna & O’Malley 2002). The major coregulators involved in estrogen signaling are the p160 steroid receptor coactivator (SRC) family, consisting of SRC1/NCoA1, GRIP1/TIF2/SRC2, and pCIP/RAC3/ACTR/AIB1/TRAM1/SRC3, which enhance transcriptional activation and recruit other cofactors possessing histone acetyl transferase activity (Leo & Chen 2000). SRCs interact with ER and enhance estrogen signals. Coactivators such as SRCs are fundamental to the proper function of reproductive events such as fertility (Mukherjee *et al.* 2007), uterine growth, blastocyst implantation (Mukherjee *et al.* 2006), and mammary gland development (Xu *et al.* 2000). The pleiotropic actions of SRCs make them attractive candidates as biomarkers for a multitude of pathologies. In relation to cancer, SRC3 overexpression results in aggressive breast and lung cancers (Osborne *et al.* 2003, Wang *et al.* 2010), SRC2 mutations are associated with prostate cancer metastases (Taylor *et al.* 2010), and SRC1 overexpression leads to early resistance to breast cancer therapy (Redmond *et al.* 2009). Furthermore, the significance of SRC activity in shaping the metabolic landscape suggests that they may be etiological predictors for numerous metabolic diseases.

Despite their fundamental actions in reproductive organs, no study has examined the expression of SRCs in the human placenta. The objective of this study was to define the expression, localization, and possible function of SRCs in the human placenta according to gestational age. Interactions between SRCs and ERs were also explored to elucidate the actions of estrogen in the placenta during pregnancy.

## Materials and methods

### Tissue and plasma collection and processing

This study was approved by the Institutional Review Board of the Pusan National University Hospital Clinical Trials Center (H-1302-005-015) and all participants gave written informed consent. Placental tissue and plasma samples were obtained from pregnant women who met



anti-SRC1 mouse (Thermo Fisher Scientific diluted 1:500), anti-SRC2 mouse (Thermo Fisher Scientific, diluted 1:1000), and anti-SRC3 mouse antibodies (Santa Cruz Biotechnology, diluted 1:1000) for overnight, followed by HRP-conjugated anti-rabbit and anti-mouse secondary antibodies (diluted 1:2000) in 5% skim milk with PBS-T for 1 h. Luminol reagent (Bio-Rad) was used to visualize the antibody binding. Each blot was then stripped by incubation with 2% SDS and 100 mM mercaptoethanol in 62.5 mM Tris-HCl (pH 6.8) for 30 min at 50–60°C. The membranes were subsequently probed with antibody against actin (Santa Cruz Biotechnology, diluted 1:2000) as an internal control. The blots were scanned using Gel Doc 1000, version 1.5 (Bio-Rad), and band intensities were normalized to  $\beta$ -actin levels.

### Histological analysis

The placental tissues were fixed with 10% formalin, embedded in paraffin wax, routinely processed, and then sectioned into 4  $\mu$ m thick slices. The tissue sections were then stained with hematoxylin & eosin (Sigma-Aldrich). For immunohistochemistry, the sections were deparaffinized and rehydrated through a graded series of alcohol using standard procedures. Endogenous peroxidase activity was quenched using a 10 min incubation step with 3% H<sub>2</sub>O<sub>2</sub> in MeOH. Nonspecific binding reactions were blocked by incubation with 10% bovine serum albumin and incubated with primary antibody solution overnight at 4°C. The tissue sections were then rinsed three times for 2 min, each in PBS while slowly warming to room temperature, followed by incubation with second antibody for 10 min. The primary antibody was detected using a Polink-2 Plus HRP DAB kit (GBI Labs, Mukilteo, WA, USA). The images of the tissue were captured at 40 $\times$  using a model BX50F-3 optical microscope (Olympus).

### Statistical analysis

The results are presented as the mean  $\pm$  standard deviation (s.d.). Data were analyzed using Sigma Plot 10.0 (Systat Software Inc, San Jose, CA, USA). The *P*-values <0.05 were considered to be statistically significant.

## Results

### Plasma estradiol concentration and *CRH* mRNA expression level during gestational age

For the initial experiment, plasma estradiol concentration during gestational period was measured. The plasma

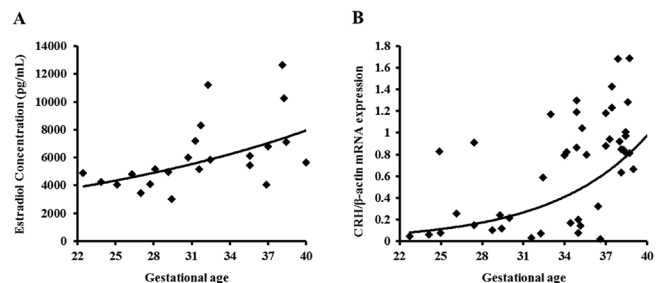
samples were obtained from 23 normal pregnant females, and estradiol concentration ranged between 2990 and 12,650 pg/mL with a median value of 6092 pg/mL. Estradiol plasma concentrations gradually increased with higher gestational age (Fig. 1A). The mean values of estradiol were 4279 pg/mL (early preterm), 6898 pg/mL (late preterm), and 7738 pg/mL (term). Next, we examined the expression of corticotrophin-releasing hormone (*CRH*), which is a well-known biomarker of gestational age in the human placenta. As expected, transcriptional levels of *CRH* increased according to the gestational age. The expression levels increased to 2.6- and 7.9-fold in the late preterm and term groups, respectively, compared with early preterm (Fig. 1B).

### Expression of ER $\alpha$ and ER $\beta$ in placenta during gestation

For the next experiment, we evaluated expression of both ER $\alpha$  and ER $\beta$  in placental tissues by Q-PCR (Fig. 2). As shown in Fig. 2A and B, mRNA levels of both ER $\alpha$  and ER $\beta$  were significantly elevated in the term placenta compared with early preterm placenta by 2.3- and 1.8-fold, respectively. The protein levels of ERs were also analyzed by western blot assay, and their photographic figures are represented in Fig. 2C. The results indicate that ER $\alpha$  and ER $\beta$  protein levels in the term gestational placenta were dramatically greater than those in early preterm (Fig. 2D and E), which was consistent with the results obtained for mRNA levels. The uniformity of the  $\beta$ -actin (internal standard) bands in these western blots further confirms that equal amounts of protein were loaded onto the gels.

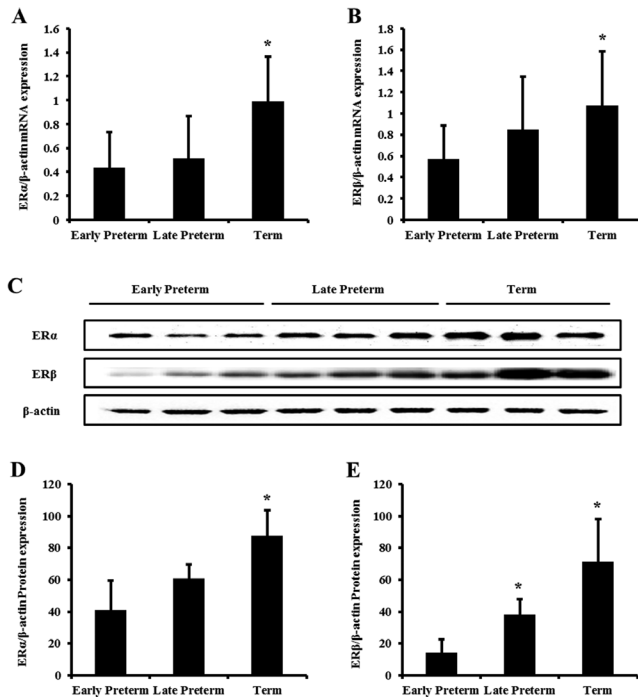
### Expression of SRCs in placenta during gestation

Expression levels of SRC mRNA in the placenta were examined by Q-PCR. *SRC1* and *SRC2* mRNA levels were



**Figure 1**

Plasma estradiol concentration and mRNA levels of *CRH* in human placenta according to gestational age. Plasma estradiol concentration (pg/mL) (A) and mRNA levels of *CRH* (B) were measured and represented. Data are expressed as the mean  $\pm$  s.d. \**P*<0.05 compared with the early preterm group.



**Figure 2**  
The mRNA and protein levels of *ERα* and *ERβ* in human placenta according to gestational age. Total mRNA and proteins were harvested from the human placenta after onset of labor. Transcriptional levels of *ERα* (A) and *ERβ* (B) were analyzed by Q-PCR depending on gestational age. Proteins from at least six different samples were examined and quantified for graphs, and the representative samples were shown in (C). The values for *ERα* (D) and *ERβ* (E) were represented as schematic graphs. Total mRNA and protein expression levels were normalized to that of  $\beta$ -actin. Data are expressed as the mean  $\pm$  s.d. \* $P < 0.05$  compared with the early preterm group.

elevated in the term placenta compared with early preterm placenta up to 2- and 2.8-fold, respectively (Fig. 3A and B). The expression levels of *SRC3* mRNA slightly increased, although the change was not significant (Fig. 3C). Protein expression of *SRC1*, *SRC2*, and *SRC3* was also examined by western blot assay (Fig. 3D) and represented as schematic graphs (Fig. 3E, F, and G). Both *SRC1* and *SRC2* proteins were upregulated in the term placenta, which was similar to the mRNA levels. The protein levels of *SRC3* showed similar pattern for *SRC1* and *SRC2* by increasing at term placenta 2.8- and 4.5-fold compared with late preterm and early preterm.

### Correlation between ERs and SRCs in placenta according to gestational age

To confirm the interaction between ER and SRCs proteins, we performed co-immunoprecipitation experiments. We immunoprecipitated total proteins from early preterm

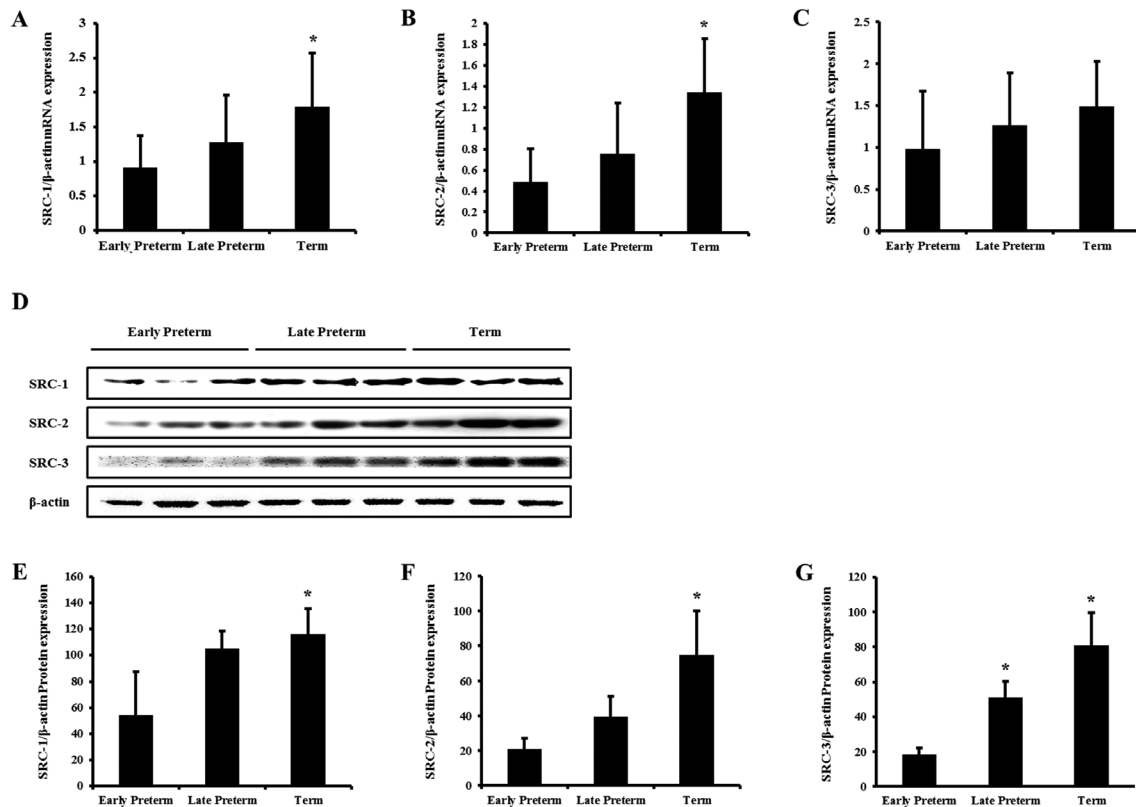
and term placenta using antibodies for *SRC1*, *SRC2*, and *SRC3*, and performed western blots for *ERα* and *ERβ*. First, both *ERα* and *ERβ* interacted with *SRC1* more strongly at term period compared with early preterm period (Fig. 4A). The basal interactions of *SRC1* with *ERα* and *ERβ* were not significantly different in early preterm. However, interaction of *SRC1* with *ERα* was drastically stronger than that with *ERβ* at term period, suggesting that *SRC1* was more closely associated with *ERα* than *ERβ* during this period. Next, interaction of ERs with *SRC2* and *SRC3* was evaluated (Fig. 4B and C). *SRC2* and *SRC3* strongly interacted with both *ERα* and *ERβ* at term compared with early preterm. In contrast to *SRC1*, interactions of *SRC2* and *SRC3* with *ERα* and *ERβ* at term period were not significantly altered.

### Immunohistochemical detection of ERs and SRCs in the placenta

To further explore the expression patterns of ERs and SRCs in placental tissues, formalin-fixed tissues were subjected to immunohistochemical analysis using antibodies specific for ERs and SRCs as described in the Materials and methods section. The morphology of placental tissue was first confirmed by H&E staining (Fig. 5A). The cytotrophoblast section was surrounded with a layer of syncytiotrophoblasts, which is a general morphological feature of the placenta. As shown in Fig. 5B, tissue was stained only with secondary antibody in order to test the specificity of the immunoreaction as a negative control. *ERα* and *ERβ* were localized to both cyto- and syncytiotrophoblast cells. However, signals were more dominant in the nuclei of syncytiotrophoblast cells (Fig. 5C and D). The expression patterns of *SRC-2* and *-3* proteins were similar with those of ERs by localizing more at syncytiotrophoblast (Fig. 5F and G). The signal of *SRC1* was strongly shown in the cytotrophoblast cell than any other antibody (Fig. 5E).

### Discussion

During pregnancy, estrogen synthesized by the uteroplacental unit using steroid precursors from maternal and fetal adrenal glands plays a crucial role in maintaining pregnancy (Albrecht & Pepe 1990, Magness 1998). Therefore, the plasma levels of estrogen are potentially useful for understanding the physiological cause of clinical complications. In this study, we measured the concentration of plasma estrogen during an experimental gestational period (22–40 weeks of gestation). Many studies have reported that serum estrogen levels increase

**Figure 3**

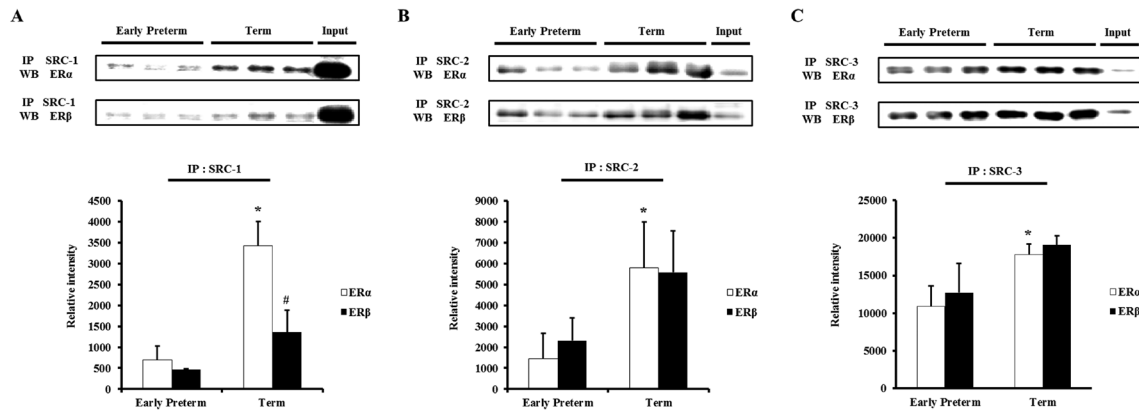
The mRNA and protein levels of SRCs in human placenta according to gestational age. Total mRNA and proteins were harvested from the human placenta after onset of labor. Transcriptional levels of SRC1 (A), SRC2 (B), and SRC3 (C) were analyzed by Q-PCR depending on gestational age. Proteins from at least six different samples were examined and quantified for graphs, and the representative samples were shown in (D). The values for SRC1 (E), SRC2 (F), and SRC3 (G) were represented as schematic graphs. Total mRNA and protein expression levels were normalized to that of  $\beta$ -actin. Data are expressed as the mean  $\pm$  s.d. \* $P < 0.05$  compared with the early preterm group.

progressively from 22 week of gestation to regulate placental growth (Mesiano 2001, You *et al.* 2006).

In this study, the experimental groups were separated by early preterm, late preterm, and term period. Depending on the period of gestation, estrogen may exert different functions. For example, during preterm, it is closely involved in the angiogenesis and growth of placenta, which is necessary for the growth of fetus. However, in term period, estrogen is associated with the preparation of delivery by regulating uterine contraction (Mesiano *et al.* 2002). To confirm this, our placenta samples were arranged according to gestational age, we examined the expression of *CRH*, which is proposed to regulate the placental clock and controls a cascade of physiological events leading to parturition (Smith *et al.* 2002). *CRH* is synthesized primarily in the paraventricular nucleus of the hypothalamus, whereas it is produced by the placenta during pregnancy. Placental *CRH* production increases dramatically over the course of normal

human gestation (McLean *et al.* 1995), reaching levels at term observed only in the hypothalamic portal system (median eminence) during physiological stress (Lowry 1993). Abnormally accelerated rates or excessive levels of placental *CRH* are the significant risk factors for earlier onset of spontaneous birth (Holzman *et al.* 2001, Ng *et al.* 2002, Wadhwa *et al.* 2004). In our results, *CRH* expression progressively increased depending on gestation age, as expected, suggesting that our placental samples reflected proper gestational age.

Estrogen exerts its function via binding to its specific receptors,  $ER\alpha$  and  $ER\beta$ , which play important roles in maintaining uteroplacental and systemic circulations during pregnancy (Byers *et al.* 2005). In a previous report, reduced  $ER\alpha$  expression caused by a single-nucleotide polymorphism was implicated in the pathogenesis of spontaneous abortion (Lehrer *et al.* 1990). Furthermore, a woman with prior preeclampsia also showed reduced risk of breast cancer, which is consistent with reduced  $ER$

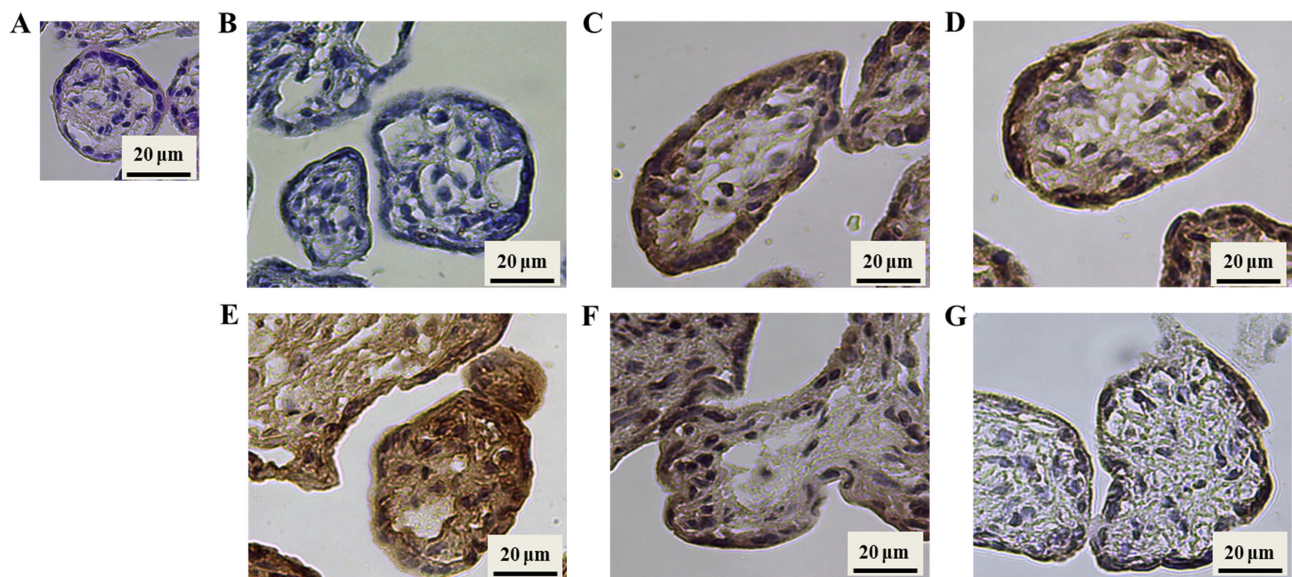
**Figure 4**

Interactions between ER and SRC proteins in human placenta according to gestational age. Proteins in the placenta were immunoprecipitated using anti-SRC1 (A), anti-SRC2, and anti-SRC3 (B) antibodies. The immunoprecipitates were then probed with anti-ER $\alpha$  and anti-ER $\beta$  antibodies, and values are represented as graphs. \* $P < 0.05$  compared with the control group; #significant compared ER $\alpha$ .

expression (Forman *et al.* 2005). ER $\alpha$  and ER $\beta$  are necessary for estrogen-mediated protection against vascular injury (Karas *et al.* 2001, Pare *et al.* 2002). In this study, transcriptional and translational levels of ER $\alpha$  and ER $\beta$  in the placenta increased at the late stage of gestation. Although many studies have focused on the concentration of estrogen, only one study examined ER expression in the human placenta. Specifically, only mRNA levels of ER $\alpha$  and ER $\beta$  in the placenta were tested from the first trimester to term (Fujimoto *et al.* 2005). The mRNA levels of both ER $\alpha$  and ER $\beta$  increased from the first to second

trimester and then suddenly decreased until normal term delivery, which differs from our results. They also observed that the mRNA expression of estrogen-related receptors (ERRs), ERR $\alpha$ , ERR $\beta$ , and ERR $\gamma$ , all gradually increased until term delivery. On the other hand, placental ER expression in rats has been shown to gradually decrease during gestation (Al-Bader 2006).

To understand estrogen-related signaling, interactions of ERs with their coregulators should be elucidated, because coregulators such as SRCs critically modulate initiation of ER/ERE transcription. Although the expression

**Figure 5**

Immunostaining of ERs and SRCs in human placental tissue. Placental tissue was stained with H&E to confirm placental morphology (A, 40 $\times$  magnification). The section was stained only with secondary antibody as a negative control (B). To examine localization of each protein, tissues were stained with specific antibodies for ER $\alpha$  (C), ER $\beta$  (D), SRC1 (E), SRC2 (F), and SRC3 (G). A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-15-0248>.

and regulation of SRC family proteins have been well established in other tissues such as the uterus and mammalian glands, they have not been explored in the placenta. We thus measured the mRNA levels of SRCs in the placenta. First, *SRC1*, *SRC2*, and *SRC3* were all present in the human placenta. Specifically, mRNA expression levels of *SRC1*, *SRC2*, and *SRC3* were enhanced in the term placenta compared with early preterm placenta, although the result of *SRC3* was not significant, but the protein levels of SRC1, SRC2, and SRC3 were upregulated in term placenta compared with early preterm placenta. These results indicate that SRC1, SRC2, and SRC3 may regulate estrogen signaling in the placenta and play pivotal roles during pregnancy.

We also evaluated the interactions between ERs and SRCs in the human placenta by immunoprecipitation assays. In the results, the signal of input was different among IP samples. The intensity of input in IP sample for SRC1 was stronger than SRC2 and SRC3. It may be due to the basal interaction of SRC1 with ERs, which was weaker than those of SRC2 and SRC3. The interactions of ERs with SRC1, SRC2, and SRC3 were significantly augmented in the term placenta. Interestingly, SRC1 interacted with ER $\alpha$  more than ER $\beta$  at term, whereas SRC2 and SRC3 did not show any change depending on the isoforms of ER. Numerous studies have shown that SRC and ER interactions are closely involved in the estrogen-mediated pathway. In breast cancer cells, knockdown of *SRC1* abolished estrogen-related signaling (Klinge 2000). Furthermore, another previous study showed that the fertility of both sexes is impaired in *Src2*-knockout mice. Specifically, male hypofertility was found to be due to defects in spermatogenesis and testicular degeneration, while that of female was because of placental hypoplasia, suggesting the possible critical function of SRC2 in the placenta (Gehin *et al.* 2002).

A recent report has shown that ER $\alpha$  and SRC1 proteins are expressed in distinct subsets of cells within the epithelium of the estrogen-responsive rat mammary gland (Shim *et al.* 1999). Results from *Src1*-null mice revealed decreased mammary responsiveness to estrogen (Xu *et al.* 1998), suggesting that ER $\alpha$  and SRC1 have distinct patterns of expression and function within the mammary gland. In contrast, ER $\alpha$  and SRC1 were colocalized to the rat uterine epithelium (Shim *et al.* 1999). Taken together, these results indicate that SRC1 plays a cell type-specific role in ER actions. To determine whether or not ERs are colocalized with SRCs, we performed immunohistochemistry analysis. ERs and SRCs were detected mainly in syncytiotrophoblasts but weakly in cytotrophoblasts, suggesting that ERs and SRCs may be associated.

In summary, our results demonstrate that estrogen signaling may be amplified by several pathways in the placenta during gestation. High levels of plasma estrogen that increased the expression of ERs and SRCs and stimulated the interactions between ERs and SRCs in the placenta are involved in this signaling. The amplified signaling of estrogen may stimulate the function and growth of placenta in the late stage of gestation to maintain pregnancy.

#### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

#### Author contribution

B.S. An, and S.C. Kim initiated and designed the study and played a role in recruitment of patients and sample collection. M.N. Park contributed towards acquisition of data. B.S. An, M.N. Park, S.C. Kim, Y.J. Lee, and J.K. Joo were involved in analysis and interpretation of the data. M.N. Park drafted the manuscript. All authors contributed to the revision of the manuscript and approval of the final draft.

#### Acknowledgments

This study was supported by a Biomedical Research Institute Grant (2013–09), Pusan National University Hospital.

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Received in final form 4 December 2015

Accepted 4 January 2016

Accepted Preprint published online 5 January 2016