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- A.M. Nuzzo,
- D. Giuffrida,
- C. Zenerino,
- A. Piazzese,
- E. Olearo,
- T. Todros,
- A. Rolfo’ ’

- Dept. of Surgical Sciences, University of Turin, Turin, Italy

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Highlights

- We characterized AP-1 expression in normal and preeclamptic PDMSCs.
- JunB was significantly increased in PE vs control PDMSCs.
- Cyclin-D1 was significantly down-regulated in PE vs control PDMSCs.
- JunB siRNA was accompanied by increased Cyclin-D1 in normal PDMSCs.
- We demonstrated that JunB over-expression in PE-PDMSCs affects Cyclin-D1 regulation.

Abstract

Introduction

In the present study, we characterized the expression of Activating Protein 1 (AP-1) factors, key cell cycle regulators, in primary placental mesenchymal stromal cells (PDMSCs) derived from normal and preeclamptic (PE) pregnancies with fetal-placental compromise.

Methods

PDMSCs were isolated from control (n = 20) and preeclamptic (n = 24) placentae. AP-1 expression was determined by semi-quantitative RT-PCR (sqRT-PCR), Real Time PCR and Western Blot assay. PDMSCs were plated and JunB siRNA was performed. JunB and Cyclin-D1 expression were assessed by Real Time and Western Blot analyses.

Results

JunB expression was significantly increased while Cyclin-D1 expression was significantly down-regulated in PE relative to control PDMSCs. JunB siRNA was accompanied by JunB down-regulation and increased Cyclin-D1 in normal PDMSCs.

Conclusions

We described, for the first time, AP-1 expression in PDMSCs derived from physiological and PE placentae. Importantly, we demonstrated that JunB over-expression in PE-PDMSCs affects Cyclin-D1 regulation. Our data suggest a possible contribution of these pathological placental cells to the altered cell cycle regulation typical of preeclamptic placentae.

Keywords

- Preeclampsia;
- Placental mesenchymal stromal cells;
- Placenta;
- Activating protein 1;
- JunB;
- Cyclin D1

Abbreviations

- PE, preeclampsia;
- FGR, fetal growth restriction;
- PDMSCs, placenta-derived mesenchymal stromal cells;
- AP-1, activating protein 1

1. Introduction

Preeclampsia (PE) is a severe pregnancy-related syndrome affecting about 5–8% of all women worldwide, and is thus a leading cause of fetal–maternal mortality and morbidity [1] Preeclamptic pregnancies are characterized by abnormal placenta development with immature hyper-proliferative trophoblast phenotype and shallow invasion of maternal spiral arteries [2], [3] and [4]. These defects entail placental hypoperfusion that, in the most severe cases, it is associated to fetal growth restriction (FGR) [5] and [6].

During the last years, the investigation of the complex PE pathogenesis has been mainly focused on the trophoblast, considered the main site of those placental aberrations responsible for PE onset. Nevertheless, we recently demonstrated the central role of placental derived mesenchymal stromal cells (PDMSC) in placental physiopathology. We reported aberrant release of pro-inflammatory cytokines by PDMSCs derived from preeclamptic placental villi (PE-PDMSCS) and their ability to induce a PE-like phenotype in term physiological villous explants [7]. Moreover, mesenchymal stromal cells are the most abundant placental cellular component and they represent the structural support for the forming primary villi during placental development, and they may drive capillary network establishment [8] and [9]. We described a slow PE-PDMSCs proliferation rate accompanied by increased senescence [7], thus indicating the contribution of PE-PDMSCs to the aberrant villous architecture typical of PE. Indeed, PDMSCs could directly cause or contribute to the placental anomalies typical of PE and FGR.

Activating Protein-1 (AP-1) early response proto-oncogenes are key cell cycle modulators pivotal for appropriate placentation. The inducible AP-1 transcription factors are dimeric complexes that contains members of the Jun and Fos protein families. While Fos proteins (c-Fos, FosB, Fra1, Fra2) hetero-dimerize only with members of the Jun family, Jun proteins (c-Jun, JunB, JunD) both homo- and heterodimerize with Fos members to form transcriptionally active complexes. Our group recently demonstrated an aberrant AP-1 expression pattern in the PE trophoblast [10].

In the present study, we investigated the expression of AP-1 family members in PDMSCs derived from normal and PE placentae with fetal-placental compromise in order to determine whether AP-1 dysregulation could also be present in pathological placental mesenchymal cells as we previously demonstrated for trophoblast [10].

2. Materials and methods

2.1. Ethics statement

The study was approved by the Institutional Ethical Committee of O.I.R.M. S.Anna Hospital and "Ordine Mauriziano di Torino" (n.209; protocol 39226/C.27.1 04/08/09) (Turin, Italy). All patients provided written informed consent for samples collection and subsequent analysis.

2.2. Patients

The study population included 24 singleton pregnancies complicated by severe preeclampsia fetal-placental compromise and 20 physiological term control pregnancies (Table 1). PE diagnosis was made according to the following criteria [1]: hypertension (systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mmHg) and proteinuria (>300 mg/24 h) after twenty week of gestational age in previously normotensive women. FGR was defined as birth weight below the fifth centile according to the Italian growth curves normalized for gestational age and sex [11] and [12] accompanied by pathological umbilical artery Doppler waveforms (absent or reverse end diastolic flow – A/REDF) and increased resistance to flow in maternal uterine arteries (early

diastolic notch or pulsatility index – PI – more than 0.58). Control patients were healthy women with singleton term physiological normotensive pregnancies and no signs of preeclampsia or FGR. Exclusion criteria were: congenital malformation, chromosomal abnormalities (in number and/or structure), maternal and/or intrauterine infections, cardiovascular diseases and metabolic syndrome.

Table 1.

Clinical features of the study population.

	Controls (*n* = 20)	**Preeclampsia (*n* = 24)**	***P* value**
Nulliparae (%)	30	41.6	n.s.
Gestational age at delivery (weeks)	39.5 ± 1.08 (37–41)	33.6 ± 3.30 (28–41)	$p < 0.01$
Maternal age at delivery (years)	33.1 ± 4.54 (24–41)	33.4 ± 5.63 (20–41)	n.s
Ethnicity (%)			
Caucasian	100	100	
Prenatal medications (%)			
Albumin	–	12.5	
Antibiotics	15	16.6	
Antidepressant	–	4.1	
Diuretics	–	12.5	
Eutirox	5	–	
Folin	10	4.1	
Heparin	–	4.1	
Iron	5	4.1	
Proton Pump Inhibitors (PPIs)	5	16.6	
Ventolin	5	4.1	
Smokers (%)	10	–	
Alcohol (%)	15	–	
Previous prenatal admission (%)	10	33.3	
Systolic Blood pressure (mm Hg)	114.7 ± 14.18	150 ± 19.41	<0.01
Diastolic Blood pressure (mm Hg)	73 ± 10	95 ± 12.18	<0.01
Proteinuria (g/24 h)	Absent	2.92 ± 4.57	<0.01
A/REDF (%)	0	58.3	<0.01
Pathological Uterine Doppler (%)	0	75	<0.01
Labor (%)	60	25	$p = 0.03$
Antibiotics in labor (%)	25	100	<0.01
Delivery to processing (range in hours)	0–3	0–3	
Caesarean section (%)	55	87.5	$p = 0.02$
Anesthesia (%)	80	91.6	n.s.
Local	37.5	–	$p = 0.01$
Spinal	62.5	86.36	

	Controls (n = 20)	Preeclampsia (n = 24)	P value
Epidural	–	9.1	
General	–	4.54	
Maternal oxygen given at delivery? (%)	–	25	n.s.
Birth weight (g)	3530.5 ± 379.54	AGA (n = 8):2490 ± 845.3 FGR (n = 16):1245 ± 473.9	p = 0.017 <0.01
Placental weight (g)	598 ± 95.27	349.9 ± 123.1	<0.01
Fetal sex (%)			n.s.
Male	65	45.8	
Female	35	54.2	
Magnesium sulfate (%)	–	50	<0.01

Values are expressed as mean ± SD and percentage. *p < 0.05. A.G.A, appropriate for gestational age; FGR, fetal growth restriction; n.s. not significant.

Table options

2.3. Placenta-derived mesenchymal stromal cells (PDMSCs) isolation and characterization

PDMSCs were isolated by enzymatic digestion and gradient as previously described [7]. Briefly, the decidua was peeled off from the basal plate and removed in order to avoid maternal cell contamination. Next, membranes were removed and 30 g of villous tissue were sampled from the chorionic plate. Placental tissue was washed several times with Hank's Buffered Salt Solution (HBSS, Gibco, Life Technologies, Italy) in order to remove the excess of blood and next it was mechanically minced and digested for 3 h with 100 U/ml collagenase type I (Gibco, Life Technologies, Italy) plus 5 μg/ml DNAse I (Gibco, Life Technologies, Italy). Finally, cells were separated by gradient using 1.073 Ficoll Paque Premium (GE Healthcare Europe, Italy). The mononuclear cells ring was collected, washed and PDMSCs were resuspended in Dulbecco's modified Minimum Essential Medium (DMEM, Gibo, Life Technologies, Italy) supplemented with 10% Fetal Bovine Serum (FBS Australian origin, Italy) and maintained at 37 °C and 5% CO2. After passage five, physiological and PE PDMSCs were characterized by flow cytometry for the expression of the following antigens: HLA-I, HLA-DR, CD105, C166, CD90, CD34, CD73, CD133, CD20, CD326, CD31, CD45 and CD14 (Miltenyi Biotech, Italy). Normal and PE-PDMSCs were analyzed by semi-quantitative PCR to assess gene expression levels of stem cell markers Oct-4 and Nanog. Primers were designed as previously described [7]. Moreover, at the same passage, control and PE-PDMSCs were plated in 6 well plates at a density of 1×10^5 cells/ml in DMEM LG without FBS. After 72 h of culture, cells were collected and processed for mRNA and protein isolation.

2.4. RNA isolation and Real Time PCR

Total RNA was isolated from physiological and PE-PDMSCs using TRIzol reagent (Life Technologies, Italy) according to manufacturer's instructions. Genomic DNA contamination was removed by DNAse I digestion before RT-PCR. cDNA was generated from 5 μg of total RNA

using a random hexamers approach and RevertAid H Minus First Strand cDNA Synthesis kit (Fermentas, Italy).

mRNA expression levels of JunB, JunD, c-Jun, FosB, c-Fos, Fra-1 and Fra-2 were first assessed by sqRT-PCR in order to identify possible splicing variants. Human 18S rRNA was used as housekeeping gene to normalize the data. Primers sequences, annealing temperatures and expected amplicon size were designed as previously published [10].

Gene expressions levels of JunB, Cyclin D1 and sex determining region gene of the Y chromosome (SRY) were determined by Real Time PCR using specific TaqMan primers and probes (Life Technologies, Italy) following the manufacturer's TaqMan protocol. Gene expression levels were normalized using endogenous 18s as internal reference (Life Technologies, Italy). Relative expression and fold change were calculated according to Livak and Schmittgen [13].

2.5. Western blot analyses

Total proteins were isolated from PDMSCs using 1× Radio Immuno-precipitation Assay (RIPA) buffer. Thirty μg of total protein from PDMSCs were processed by SDS-page on 4–20% and 7.5% polyacrylamide pre-cast gradient gels (Bio-Rad Laboratories S.r.l., Italy). Next, proteins were transferred on PVDF membranes and probed at room temperature with primary antibodies using the SnapID system (Merck-Millipore, Italy) following the manufacturer's instructions. Primary antibodies were: rabbit polyclonal anti-human JunB (07-1333, 1:2000 dilution, Merk-Millipore, Italy), rabbit polyclonal anti-human Fra-1 (sc-183, 1:500 dilution, Santa Cruz Biotechnology, USA), rabbit polyclonal anti-human Fra-2 (sc-604, 1:500 dilution, Santa Cruz Biotechnology, USA), mouse monoclonal anti-human Cyclin D1 (2926s, 1:1500 dilution, Cell Signalling, USA), Biotinylated secondary antibodies were goat anti-mouse for Cyclin D1, (1:1000 dilution, Vector Laboratories, UK), donkey anti-rabbit for JunB, Fra-1, Fra-2 (1:1000 dilution, Vector Laboratories, UK). Protein expression levels were normalized to β-actin by blotting with mouse monoclonal anti-human β-actin antibody (1:1000, Sigma–Aldrich, Italy) after stripping with 10× ReBlot Plus Strong Antibody Stripping Solution (Merck Millipore, Italy). Protein expression levels were assessed by chemo-luminescence using LuminataTM Classico Western HRP reagent (Merck Millipore, Italy) followed by densitometry.

2.6. Immunoprecipitation

Fra-1, Fra-2 and JunB association levels in normal and PE-PDMSCs were determined by immunoprecipitation. Briefly, one hundred μg of cell lysates were incubated with rabbit polyclonal anti-human Fra-1 (1:1000 dilution, Santa Cruz Biotechnology, USA) or rabbit polyclonal anti-human Fra-2 antibodies (1:1000 dilution, Santa Cruz Biotechnology, USA) for 2 h at 4 °C on a rotator and next incubated with protein A-Sepharose beads (SantaCruz Biotechnology, USA) at 4 °C overnight. Next, immunocomplexes were precipitated by centrifugation, separated from protein A-sepharose beads and denaturated by heating at 95 °C for 5 min. Finally, immunocomplexes were subjected to SDS-PAGE and blotted with anti-JunB antibody as described above.

2.7. JunB silencing in placenta derived mesenchymal stromal cells

Control PDMSC were plated at a density of 3×10^4 cells/well in 24 well plates and cultured in DMEM LG without antibiotics at standard culture conditions (5% CO2, 37 °C). When cells reached 70% confluency, they were transfected with 400 nM of StealthTM RNAi directed against human JunB gene (Invitrogen, Life Technologies, Italy) using LipofectamineTM 2000 (Invitrogen, Life

Technologies, Italy) following manufacturer's protocol. PDMSCs transfected with 400 nM StealthTM RNAi Negative Control (scrambled sequences – SS) (Invitrogen, Life Technologies, Italy) were used as a control. Cells were processed for mRNA and protein isolation.

2.8. Statistical analysis

All data are represented as mean ± SE. For comparison of data between multiple groups we used one-way analysis of variance (ANOVA) with posthoc Dunnett's test. For comparison between 2 groups we used paired and unpaired Student's *t*-test as appropriate. Categorical variables are presented as frequencies (percentages) and the comparison among different groups was done with $\chi 2$ by means of a 2 × 2 or 2 × 3 contingency table; Fisher's exact test was used for small sample sizes. Statistical test were carried out using SPSS Version 18 statistical software and significance was accepted at $P < 0.05$.

3. Results

3.1. Study population

The clinical features of the study population were reported in Table 1. Control ($n = 20$) and PE ($n = 24$) pregnancies were comparable for maternal age, while gestational age ($p < 0.01$, 1.17 Fold Decrease), neonatal weight (CTRL vs PE-AGA $p = 0.01$, 1.41 Fold Decrease, CTRL vs PE-FGR $p < 0.01$, 2.83 Fold Decrease) and placental weight ($p < 0.01$, 1.7 Fold Decrease) at delivery were, as expected, significantly lower in PE group vs controls. All PE pregnancies presented fetal-placental compromise with abnormal umbilical artery (58.3%, $p < 0.01$) and/or uterine artery (75%, $p < 0.01$) abnormal Doppler velocimetries associated in 66.6% of the cases with FGR ($p < 0.01$).

3.2. Characterization of placenta-derived MSCs

All PDMSCs cell lines ($n = 44$) displayed the proper mesenchymal stromal phenotype as assessed by flow cytometry. Cells were positive for CD105, CD166, CD90, CD73 and negative for HLA-II, for hematopoietic surface markers CD34 and CD45 and endothelial progenitor markers CD133 and CD31 (Supplementary Fig. 1Sa). Control and PE PDMSCs were also negative for B cell, neutrophil and macrophage markers CD20 and CD14 and for trophoblast and epithelial markers CD326 (Supplementary Fig. 1Sa), thus excluding any significant contamination. RT-PCR detected the expression of typical stemness markers Oct-4 and Nanog, in all PDMSCs cell lines ($n = 44$) (Supplementary Fig. 1Sb). Finally, to characterize control and PE PDMSCs genotype and to confirm absence of maternal cells contamination, PDMSCs isolated from placentae of male fetuses were tested for SRY expression by Real Time PCR. All our PDMSCs lines derived from placentae of male fetus ($n = 24$) resulted positive for SRY mRNA expression indicating the absence of significant maternal cell contamination that, if present, would have become prevalent (Supplementary Fig. 1Sc).

3.3. AP-1 family members expression in physiological and PE-PDMSCs

We previously demonstrated aberrant expression of AP-1 factors in preeclamptic placental tissue relative to controls [10]. Therefore, we first investigated the expression of AP-1 family members in MSCs derived from both physiological ($n = 20$) and preeclamptic ($n = 24$) placentae by semi-quantitative RT-PCR in order to identify possible splicing variants. We showed that all AP-1 members were expressed in PDMSCs except for FosB, not present in both control and PE cells (Fig. 1a). Moreover, PE-PDMSCs expressed significantly higher JunB mRNA levels relative to

controls ($p < 0.01$, 1.66 Fold Increase) (Fig. 1a). JunB gene expression levels were confirmed by quantitative Real Time PCR showing significantly increased mRNA expression levels in PE-PDMSCs relative to controls ($p = 0.02$, 1.86 Fold Increase) (Fig. 1b left panel). Western Blot analyses showed increased JunB protein expression levels in PE-PDMSCs compared to control cells ($p < 0.01$, 2.00 Fold-Increase), confirming data obtained at the mRNA level (Fig. 1b right panel). Finally, to rule out the possibility that JunB expression could be a cell culturing artefact, we tested its expression in freshly isolated, unpassaged, normal and PE PDMSCs (passage 0 – p0). JunB expression was confirmed in both control and PE unpassaged PDMSCs, thus excluding the presence of cell culturing artefacts. Importantly, Real Time PCR and Western Blot analyses confirmed increased JunB gene (6.87 Fold Increase) and protein (1.47 Fold Increase) levels in p0 PE-PDMSCs compared to p0 controls (Fig. 2S a and b) as we reported for cells at passage 5.

3.4. JunB/Fra1 and JunB/Fra2 association

The dimerization of AP-1 family members is a crucial events to form the active transcription complex [14]. We first investigated the expression of Fra-1 and Fra-2, the main JunB dimerization partners, in control ($n = 20$) and PE-PDMSCs ($n = 24$). Immunoblotting analyses revealed no differences in both Fra-1 and Fra-2 protein expression levels between physiological and PE-PDMSCs ($p > 0.05$) (Fig. 2a).

To determine differences in JunB/Fra-1 and JunB/Fra-2 association, we separately immunoprecipitated Fra-1 and Fra-2 proteins and immunoblotted for JunB. No differences were found JunB/Fra-1 hetero-dimerization between control and PE-PDMSC ($p > 0.05$) (Fig. 2b, left panel). In contrast, we found a significant increase in JunB/Fra-2 heterodimer formation in PE-PDMSC relative to controls ($p = 0.03$, 1.7 Fold Increase) (Fig. 2b, right panel).

3.5. Cyclin D1 expression and JunB siRNA in control PDMSCs

JunB is a key cell cycle regulator able to arrest the G1/S phase transition through transcriptional inhibition of Cyclin D1 [15]. Therefore, we investigated whether JunB over-expression in PE-PDMSCs was accompanied by aberrant Cyclin D1 expression. We found that Cyclin D1 mRNA ($p = 0.001$, 1.53 Fold Decrease) and protein ($p = 0.016$, 5.55 Fold Decrease) expression levels were significantly down-regulated in PE-PDMSCs relative to controls (Fig. 3a and b).

In order to determine the functional significance of JunB-mediated Cyclin D1 regulation in our experimental model, we silenced JunB gene expression by siRNA technology. We optimized siRNA protocol by initially using three different JunB siRNA duplexes (D1, D2 and D3). Real Time PCR showed that JunB gene expression was efficiently silenced by D1 ($p = 0.03$, 3.1 Fold Decrease) and D3 ($p = 0.02$, 4.16 Fold Decrease) siRNA duplexes relative to control SS siRNA treated cells (Fig. 4a). Decreased JunB levels were associated to increased Cyclin D1 mRNA expression in PDMSCs treated by D1 ($p = 0.03$, 3.23 Fold Increase) and D3 (2.63 Fold Increase) siRNA duplexes compared to SS siRNA treated cells (Fig. 4a and b, left panel). Western Blot analyses (Fig. 4a and b, right panel) showed decreased JunB (D1: 1.78 Fold Decrease; D3: 1.96 Fold Decrease) and increased Cyclin D1 (D1: 1.2 Fold Increase; D3: 1.12 Fold Increase) protein expression levels in JunB siRNA PDMSCs compared to SS siRNA control cells, thus confirming data obtained at mRNA level.

4. Discussion

In the present study we demonstrated, for the first time, a differential AP-1 expression pattern in preeclamptic relative to physiological chorionic mesenchymal stromal cells. In particular, we

reported JunB over-expression accompanied by increased JunB/Fra2 dimerization and Cyclin D1 downregulation in PE-PDMSCs. These data suggest augmented JunB pathway activation in PE-PDMSCs and its possible involvement in the transcriptional regulation of the key cell cycle mediator Cyclin D1, which is a proto-oncogene regulator of G1/S phase progression. Therefore, PDMSCs defects could contribute to the aberrant villous architecture typical of PE pregnancies with fetal-placental compromise.

It was previously demonstrated that AP-1 transcription factors are critical for normal development and neoplastic transformation of different cell types such as hepatocytes, keratinocytes and lymphocytes [17], [18], [19], [20] and [21]. Our group recently showed decreased c-Fos and Fra-2 expression in PE relative to physiological trophoblast cells [10]. These findings are indicative of altered placental AP-1 expression pattern that might contribute to the impaired trophoblast development typical of preeclampsia.

Caplan et al. showed that mesenchymal stromal cells, beside possessing well renowned stem cells-like features like high proliferative and self-renewal potentials [22], are able to control proliferation and apoptosis in neighbouring cells through secretion of trophic mediators [23]. We recently reported aberrant release of pro-inflammatory cytokines and anti-angiogenic molecules by PDMSCs derived from PE placentae, indicating a direct contribution of this specific cell population to the anomalies typical of PE [7].

Herein, we found that all AP-1 molecules were expressed in placental-derived MSC except for FosB, not present in both normal and PE-PDMSCs. Previous studies have shown that FosB−/− mice have a physiological birth rate, they are fertile and do not present evident phenotypic or histological anomalies [24]. Moreover FosB-deficient embryonic stem cells and mouse embryonic fibroblast normally enter the S phase of cell cycle [25]. These data suggest that FosB might not be crucial for cell cycle regulation and embryonic development. Otherwise, lack of FosB, usually necessary for the osteogenic differentiation of mesenchymal cells [26], may be fundamental for maintaining the basal undifferentiated state of MSCs.

In contrast, we reported JunB over-expression in PE-PDMSCs. AP-1 factors are induced by cytokines, oxidative stress and growth factors [27]. Therefore, JunB over-expression in PE-PDMSCs could be an intrinsic feature and/or result from anomalous cytokine-mediated autocrine stimulation. Moreover, JunB over-activation could be further elevated in utero by hypoxia/oxidative stress, which is typical of PE placentae [28].

AP-1 activity is tightly regulated at the protein level by homo- and hetero-dimerization of Fos and Jun proteins. Jun/Fos dimers are required to bind DNA and promote transcription of different target genes. In bone marrow dendritic cells, Fra-2 was described as the main JunB dimerization partner [29]. However, other studies performed on endothelial cells derived from sheep fetal-placental arteries reported Fra-1 as the most suitable factor to dimerize with JunB [30]. Furthermore, lack of Fra-1 or JunB in mouse placentation processes could give rise to very similar phenotypes such as a non-vascularized placental labyrinth, defects in the yolk sac and embryos severely growth retarded. These strikingly similar phenotypes suggest that Fra1 and JunB might have overlapping functions during extra-embryonic development, an idea supported by the observations that the temporal and spatial expression patterns of Jun-B and Fra1 during embryogenesis are partly overlapping [31] and [32]. Herein, we didn't find differences in Fra-1 and Fra-2 protein levels or in the formation of Fra-1-JunB heterodimers between PE and control cells. In stark contrast, we demonstrated a significant increase in JunB/Fra-2 heterodimer formation in PE-PDMSCs relative to control cells. These data suggest that in our model the mechanism leading to the aberration of cellular processes typical of PE placentae such as an impaired vascularization is not due to

JunB/Fra-1 heterodimers, and thus is different to the mouse model. Instead, Fra-2 could be the highest affinity partner of JunB in PE and the JunB/Fra-2 heterodimer could be responsible of the aberrant cellular processes controlled by JunB such as proliferation and vascular development.

AP-1 family members regulate expression and function of cell-cycle regulators Cyclin D1, Cyclin A, Cyclin E, p53, $p21^{Cip1}$, $p16^{INK4A}$ and $p19^{ARF}$[20] and [33]. In particular, JunB could act as inhibitor of cell division [34] and [35], senescence inducer [35] and tumor suppressor [36], [37] and [38]. JunB knock-in strategy and transgenic complementation approach demonstrated abrogation of proliferation by aberrant regulation of Cyclin D1, p53 and $p21^{Cip1}$ in fibroblast [39]. Moreover, a negative correlation between JunB and Cyclin D1 levels, necessary for the rapid transition from G1 to S phase of the cell cycle, was observed in primary MEFs [15].

The role of JunB and Cyclin D1 in chorionic PDMSCs has never been addressed. Therefore, we determined whether JunB over-expression negatively influence Cyclin D1 causing and/or contributing to the decreased proliferation rate previously described in PE-PDMSCs [7]. First, we demonstrated Cyclin D1 down-regulation in PE-PDMSCs characterized by JunB over-expression. Next, we reported that JunB siRNA in physiological PDMSCs leaded to increased Cyclin D1 gene expression, confirming JunB-mediated Cyclin D1 regulation in our model. Indeed, aberrant JunB/Cyclin D1 balance could affect G1/S cell cycle transition in PE-PDMSCs inducing reduced cell proliferation and increased senescence [7]. A recent study demonstrated that fibroblasts derived from JunB over-expressing mice display reduced proliferation, due to an extended G1 phase and undergo to premature senescence [35]. Furthermore, it was described that JunB absence in the myeloid lineage of transgenic mice leaded to increased proliferation of myeloid progenitors [36]. Our data are in line with these studies, thus supporting our hypothesis. A significant reductions in PDMSCs number could in turn alter their function as a structural support for primary villi formation and modify their paracrine activity on trophoblast cells. Importantly the PE-PDMSCs described in the present study were all derived from pregnancies with fetal and/or placental compromise. Indeed, the JunB/Cyclin-D1 imbalance that we reported in preeclamptic mesenchymal cells may not apply to PE pregnancies characterized by normal placentation.

In conclusion, our data suggest that JunB/Cyclin-D1 inbalance in PE-PDMSCs could contribute to the aberrant villous architecture typical of preeclamptic pregnancies with fetal-placental compromise. Indeed, PDMSCs could represents a future therapeutic tool or target for human placenta-related disorders as preeclampsia.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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[1] ACOG practice bulletin. Diagnosis and management of preeclampsia and eclampsia. Number 33, January 2002 Obstet Gynecol, 99 (1) (2002), pp. 159–167

○

[2] R. Pijnenborg, L. Vercruysse, L. Verbist, F.A. Van Assche Interaction of interstitial trophoblast with placental bed capillaries and venules of normotensive and pre-eclamptic pregnancies Placenta, 19 (8) (1998), pp. 569–575

[3] B. Huppertz, M. Kadyrov, J.C. Kingdom Apoptosis and its role in the trophoblast. Am J Obstet Gynecol, 195 (1) (2006), pp. 29–39

- [4] A.D. Allaire, K.A. Ballenger, S.R. Wells, M.J. McMahon, B.A. Lessey. Placental apoptosis in preeclampsia. Obstet Gynecol, 96 (2) (2000), pp. 271–276
- [5] N. Ishihara, H. Matsuo, H. Murakoshi, J.B. Laoag-Fernandez, T. Samoto, T. Maruo Increased apoptosis in the syncytiotrophoblast in human term placentas complicated by either preeclampsia or intrauterine growth retardation. Am J Obstet Gynecol, 186 (1) (2002), pp. 158–166
- [6] I.P. Crocker, S. Cooper, S.C. Ong, P.N. Baker. Differences in apoptotic susceptibility of cytotrophoblasts and syncytiotrophoblasts in normal pregnancy to those complicated with preeclampsia and intrauterine growth restriction. Am J Pathol, 162 (2) (2003), pp. 637–643
- [7] Rolfo A, Giuffrida D, Nuzzo AM, Pierobon D, Cardaropoli S, Piccoli E, et al. Pro-inflammatory profile of preeclamptic placental mesenchymal stromal cells: new insights into the etiopathogenesis of preeclampsia. PLoS One 8(3):e59403.
- [8] Klein JD and Fauza DO. Amniotic and placental mesenchymal stem cell isolation and culture. Methods Mol Biol 698:75–88.
- [9] R. Demir, P. Kaufmann, M. Castellucci, T. Erbengi, A. Kotowski. Fetal vasculogenesis and angiogenesis in human placental villi. Acta Anat (Basel), 136 (3) (1989), pp. 190–203
- [10] D. Marzioni, T. Todros, S. Cardaropoli, A. Rolfo, T. Lorenzi, P. Ciarmela, *et al.* Activating protein-1 family of transcription factors in the human placenta complicated by preeclampsia with and without fetal growth restriction. Placenta, 10 (2010), pp. 919–927
- [11] E. Bertino, E. Di Battista, A. Bossi, M. Pagliano, C. Fabris, G. Aicardi, *et al.* Fetal growth velocity: kinetic, clinical, and biological aspects. Arch Dis Child Fetal Neonatal Ed, 74 (1) (1996), pp. F10–F15
- [12] F. Parazzini, I. Cortinovis, R. Bortolus, L. Fedele. Standards of birth weight in Italy Ann Ostet Ginecol Med Perinat, 112 (4) (1991), pp. 203–246

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- [13] K.J. Livak, T.D. Schmittgen. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods, 25 (4) (2001), pp. 402–408
- [14] C.E. Malnou, T. Salem, F. Brockly, H. Wodrich, M. Piechaczyk, I. Jariel-Encontre Heterodimerization with Jun family members regulates c-Fos nucleocytoplasmic traffic J Biol Chem, 282 (42) (2007), pp. 31046–31059

- [15] S. Andrecht, A. Kolbus, B. Hartenstein, P. Angel, M. Schorpp-Kistner. Cell cycle promoting activity of JunB through cyclin A activation. J Biol Chem, 277 (39) (2002), pp. 35961–35968
- [17] B.W. Ozanne, L. McGarry, H.J. Spence, I. Johnston, J. Winnie, L. Meagher, *et al.* Transcriptional regulation of cell invasion: AP-1 regulation of a multigenic invasion programme Eur J Cancer, 36 (13 Spec No) (2000), pp. 1640–1648
- [18] E. Tulchinsky. Fos family members: regulation, structure and role in oncogenic transformation. Histol Histopathol, 15 (3) (2000), pp. 921–928
- [19] W. Jochum, E. Passegue, E.F. Wagner. AP-1 in mouse development and tumorigenesis Oncogene, 20 (19) (2001), pp. 2401–2412
- [20] E. Shaulian, M. Karin. AP-1 in cell proliferation and survival. Oncogene, 20 (19) (2001), pp. 2390–2400
- [21] J. Hess, P. Angel, M. Schorpp-Kistner. AP-1 subunits: quarrel and harmony among siblings. J Cell Sci, 117 (Pt 25) (2004), pp. 5965–5973

[22] K. Igura, X. Zhang, K. Takahashi, A. Mitsuru, S. Yamaguchi, T.A. Takashi. Isolation and characterization of mesenchymal progenitor cells from chorionic villi of human placenta. Cytotherapy, 6 (6) (2004), pp. 543–553

- [23] A.I. Caplan, J.E. Dennis. Mesenchymal stem cells as trophic mediators. J Cell Biochem, 98 (5) (2006), pp. 1076–1084
- [24] J.R. Brown, H. Ye, R.T. Bronson, P. Dikkes, M.E. Greenberg. A defect in nurturing in mice lacking the immediate early gene fosB. Cell, 86 (2) (1996), pp. 297–309
- [25] M.C. Gruda, J. van Amsterdam, C.A. Rizzo, S.K. Durham, S. Lira, R. Bravo Expression of FosB during mouse development: normal development of FosB knockout mice. Oncogene, 12 (10) (1996), pp. 2177–2185
- [26] C. Haasper, M. Jagodzinski, M. Drescher, R. Meller, M. Wehmeier, C. Krettek, *et al.* Cyclic strain induces FosB and initiates osteogenic differentiation of mesenchymal cells Exp Toxicol Pathol, 59 (6) (2008), pp. 355–363
- [27] M. Karin. The regulation of AP-1 activity by mitogen-activated protein kinases J Biol Chem, 270 (28) (1995), pp. 16483–16486
- [28] K.R. Laderoute The interaction between HIF-1 and AP-1 transcription factors in response to low oxygen Semin Cell Dev Biol, 16 (4–5) (2005), pp. 502–513
- [29]

- Gomard T, Michaud HA, Tempe D, Thiolon K, Pelegrin M and Piechaczyk M. An NF-kappaB-dependent role for JunB in the induction of proinflammatory cytokines in LPS-activated bone marrow-derived dendritic cells. PLoS One 5(3):e9585.
- [30]
- Mata-Greenwood E, Liao WX, Wang W, Zheng J and Chen DB. Activation of AP-1 transcription factors differentiates FGF2 and vascular endothelial growth factor regulation of endothelial nitric-oxide synthase expression in placental artery endothelial cells. J Biol Chem 285(23):17348–58.
- [31]
- M. Schorpp-Kistner, Z.Q. Wang, P. Angel, E.F. Wagner. JunB is essential for mammalian placentation. EMBO J, 18 (4) (1999), pp. 934–948
- [32] M. Schreiber, Z.Q. Wang, W. Jochum, I. Fetka, C. Elliott, E.F. Wagner. Placental vascularisation requires the AP-1 component fra1. Development, 127 (22) (2000), pp. 4937–4948
- [33]. E. Shaulian, M. Karin. AP-1 as a regulator of cell life and death. Nat Cell Biol, 4 (5) (2002), pp. E131–E136
- [34] L. Bakiri, D. Lallemand, E. Bossy-Wetzel, M. Yaniv. Cell cycle-dependent variations in c-Jun and JunB phosphorylation: a role in the control of cyclin D1 expression. EMBO J, 19 (9) (2000), pp. 2056–2068
- [35] E. Passegue, E.F. Wagner JunB suppresses cell proliferation by transcriptional activation of p16(INK4a) expression EMBO J, 19 (12) (2000), pp. 2969–2979
- [36] E. Passegue, W. Jochum, M. Schorpp-Kistner, U. Mohle-Steinlein, E.F. Wagner
- Chronic myeloid leukemia with increased granulocyte progenitors in mice lacking junB expression in the myeloid lineage Cell, 104 (1) (2001), pp. 21–32
- [37] E. Passegue, E.F. Wagner, I.L. Weissman JunB deficiency leads to a myeloproliferative disorder arising from hematopoietic stem cells Cell, 119 (3) (2004), pp. 431–443
- [38]
- U. Steidl, F. Rosenbauer, R.G. Verhaak, X. Gu, A. Ebralidze, H.H. Otu, *et al.* Essential role of Jun family transcription factors in PU.1 knockdown-induced leukemic stem cells Nat Genet, 38 (11) (2006), pp. 1269–1277
- [39] E. Passegue, W. Jochum, A. Behrens, R. Ricci, E.F. Wagner. JunB can substitute for Jun in mouse development and cell proliferation. Nat Genet, 30 (2) (2002), pp. 158–166

Corresponding author. Dept. of Surgical Sciences, University of Turin, c/o O.I.R.M. Sant'Anna Hospital, Via Ventimiglia 3, 10126 Turin, Italy. Tel.: +39 0113134433; fax: +39 0113134450.