Effect of Pomegranate Extract in Mesenchymal Stem Cells by Modulation of microRNA-155, microRNA-21, microRNA-23b, microRNA-126a, and *PI3K/AKT1/NF-κB* Expression

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Today, mesenchymal stem cells (MSCs) are candidates for various autoimmune disease treatments due to immunomodulatory activity in these cells. Much research has recently been done to improve the immunomodulatory activity of MSCs. Genetic variation is one of these methods. microRNAs (miRNAs) are small noncoding RNAs that control most of the cell's biological activities. Recent studies have shown that miRNAs play a significant role in the regulation of MSC immunomodulatory activity. Pomegranate is a fruit that has antioxidant, anti-inflammatory, and anticancer properties and has been used for many years for therapeutic purposes. The objective of this research is to evaluate the immunoregulatory-related miRNAs level of adipose-derived MSCs (Ad-MSCs) obtained from adipose tissue in the presence or lack of pomegranate (*Punica granatum*) extract (PGE). Our results showed that miRNA-23 and miRNA-126 were upregulated by PGE treatment in MSCs, and in contrast, miRNA-21 and miRNA-155 were downregulated by PGE treatment in MSCs. In addition this research shows that PGE can downregulate the expression of *PI3K/AKT1/NF-κB* in Ad-MSCs. Our bioinformatics data have shown that the target of these four miRNAs and the signaling pathways, in which these targets are involved, can play an important role in regulating the immunomodulation function of stem cells. In conclusion, PGE can inhibit the expression of *PI3K/AKT1/NF-κB* genes involved in inflammatory pathways via miRNA-23 and miRNA-126 overexpression or miRNA-21 and miRNA-155 downregulation that plays a role in the pathways of immune modulation in Ad-MSCs. These results may provide insight into the mechanism underlying the regulation of the immunomodulatory activity of Ad-MSCs by PGE.

Keyword: mesenchymal stem cell, microRNA, immunoregulatory or immunomodulatory, pomegranate (*Punica granatum*)

Introduction

AUTOIMMUNE DISEASES ARE defined as immunological tolerance to self-antigens and immune-mediated destruction of self-tissues. It is estimated that 4–5% of the world population suffer from autoimmune diseases (Cooper *et al.*, 2009) such as systemic lupus erythematosus (Wang *et al.*, 2013), rheumatoid arthritis (Djouad *et al.*, 2005), type I diabetes (Bhonde *et al.*, 2014), multiple sclerosis, and amyotrophic lateral sclerosis (Phinney and Prockop, 2007; Morando *et al.*, 2012). Since the current treatment of these diseases with nonsteroidal anti-inflammatory drugs, such as ibuprofen (Motrin, Advil) and naproxen (Naprosyn), and

immune-suppressing drugs have many side effects such as increased risk of infection and even triggered the *de novo* development of autoimmune diseases; new approaches with higher therapeutic efficacy and less side effects are necessary to be designed for more effective treatment of these diseases (Liu and Han, 2008; Dorronsoro *et al.*, 2013; Castro-Manrreza and Montesinos, 2015; Li *et al.*, 2017).

Adipose-derived mesenchymal stem cells (Ad-MSCs) are multipotent nonhematopoietic adult stem cells that are isolated from various tissues such as bone marrow (BM) and white adipose tissue (Pittenger *et al.*, 1999; Jiang *et al.*, 2002). MSCs are used to treat several disorders such as cancer or degenerative disorder therapy (Kuo *et al.*, 2012;

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Ebrahimi and Rahim, 2014). In addition, MSCs show a powerful immunomodulatory ability and therapeutic potential for different inflammatory diseases such as severe acute graft-versus-host disease (Le Blanc *et al.*, 2004; Ren *et al.*, 2008; Shi *et al.*, 2010). The immunomodulation exerted by MSCs is due to a humoral impact mediated by cytokine secretion such as prostaglandin E2 (*PGE2*), indolamine 2,3-dioxygenase (*IDO*), interleukin 6 (*IL-6*), and nitric-oxide, transforming growth factor beta (*TGF-β*), interferon (*IFN-γ*) or interleukin-1 β , and cell–cell contact impact, including peripheral mononuclear blood cell regulation (William *et al.*, 2003; Aggarwal and Pittenger, 2005; Rasmusson *et al.*, 2005; Wang *et al.*, 2009; Shi *et al.*, 2011; English, 2013). In this study, we examine the Ad-MSCs, MSCs isolated from white adipose tissue because these cells are the most appropriate source of stem cells for clinical purposes owing to the option of intravenous transplantation of autologous Ad-MSCs without immune rejection, ethical issues, and tumorigenesis (Garcia-Olmo *et al.*, 2008, 2009). In addition, Ad-MSC identical to BM -derived MSCs have effective immunomodulatory activity (Elman *et al.*, 2014; Waldner *et al.*, 2018).

microRNA (miRNAs) are 18–25 nt long single-stranded RNAs that can suppress gene expression by binding primarily to 3'-untranslated regions of specific target mRNAs (Ambros, 2003; Baltimore *et al.*, 2008; Lindsay, 2008). miRNAs are important in immune response regulation (O'connell *et al.*, 2010). In this regard, it has been reported that miRNAs by modulation the targets can affect the immunoregulatory properties of MSCs in different manners such as positive effect on immunoregulatory activity of MSCs (positive regulator) for instance (mir-126 and mir-23) (Karachi *et al.*, 2015; Khosravi *et al.*, 2017; Wu *et al.*, 2017) or negative effect on immunoregulatory activity of MSCs (negative regulator) for instance (mir-155 and mir-21) (Xu *et al.*, 2013; Wu *et al.*, 2015; Onodera *et al.*, 2017). Therefore, changing the expression of miRNAs in MSCs can influence the immunoregulatory properties of MSCs (Chi *et al.*, 2016). In this study, we selected these four miRNAs based on previous studies as well as bioinformatics results (Fig. 4A). In addition, several components, for instance, natural products, can affect the miRNAs expression (Banerjee *et al.*, 2013).

Pomegranate (PG) (*Punica granatum*) is a fruit that is used in Asia and the Middle East for centuries to treat antiviral and gastrointestinal infections (Achraf *et al.*, 2018). PG has different impacts such as anticancer or anti-inflammatory effects by modulating the expression of miRNAs and this target gene, which plays a role in the inflammatory pathway (Banerjee *et al.*, 2012, 2013; Panth *et al.*, 2017). The present study was conducted to assess the impact of pomegranate extract (PGE) on the immunoregulatory-related miRNAs expression level of Ad-MSCs to improve its potential immunomodulatory properties.

Materials and Methods

Isolation and characterization of Ad-MSCs

Ad-MSCs were isolated from a human liposuction sample. Lipos aspirate was obtained from an individual who had undergone surgery for aesthetic purposes in Imam Reza Hospital (Birjand, Iran) (Institutional approval number for human studies: IR.BUMS.REC.1397.354) Osteogenic and adipogenic dif-

ferentiation and Immunophenotyping of isolated Ad-MSCs were performed as described previously (Khorashadizadeh *et al.*, 2015). Passage three of the cell culture to validate the differentiation and identity of MSCs. Cells were labeled with CD105-PE, CD90-PE, and CD45-FITC anti-human conjugate antibodies to confirm MSC immunophenotyping (eBiosciences, USA). All labeled cells have been analyzed using the Attune Acoustic Focusing Cytometer (Applied Biosystem, USA).

The isolated Ad-MSCs were cultured in Dulbecco's Medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin (all from Gibco, USA) in humidified and 5% CO₂ at 37°C. All the experiments were repeated three to seven times.

Preparation of PGE

In this study, two distinct pomegranates from different regions (Rudbar and Shahmirzad, Iran) were used. Pesticide was not used during the breeding stages of these pomegranates, and they are not kept in the refrigerator. After obtaining the desired fruits, the extracts were prepared, centrifuged at 8000 rpm for 20 min, and filtered with Whatman Grade 1 filter paper to remove suspended particles. The extracts were then freeze-dried and stock concentrations of 20 mg/mL were prepared, sterilized using a 0.22 μ m filter, and maintained at –80°C freezer until experiments.

Cell viability assay

The Ad-MSCs were seeded at 2×10^3 cells per well in a 96-well plate and kept in complete medium for one day to attach. The day after, culture media were replaced with 200 μ L medium containing PGE in various concentrations (0, 0.25, 0.5, 1, and 2 mg/mL) for 24 and 48 h. On the next day, 20 μ L 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole (MTT) reagent (5 mg/mL) was added to each well and incubated at 37°C for 4 h. Then, the media were discarded and 100 μ L of dimethyl sulfoxide was added to dissolve the formazan crystals. The absorbance of the solubilized formazan was read at 570 and 630 nm using Epoch Microplate Spectrophotometer (BioTek Instrument, USA). Cells incubated in control medium were considered as 100% viable (Vega-Avila and Pugsley, 2011).

Total miRNA and RNA extraction and reverse transcription polymerase chain reaction analysis

The Ad-MSCs were seeded at 50×10^3 cells per well in a six-well plate and kept in complete medium for one day to attach. After treating Ad-MSCs with (1 mg/mL) doses of PGE for 24 h, total RNAs were extracted using RNX-Plus reagent (SinaClon, Tehran, Iran). Next, cDNAs for mir were synthesized using miRNAs first-strand cDNA synthesis kit (Zist Royesh, Tehran, Iran). U6 miRNAs was used as an endogenous control. Comparative real-time PCR using SYBR Green Supermix (Zist Royesh, Tehran, Iran) and Equal amount (1 μ g) of mRNAs was converted to complementary DNA using a reverse transcription kit (Parstus, Mashhad, Iran). Real-time PCR reactions were performed using 2 μ L of complementary DNA using SYBR Green Supermix (Amplicon). Real-time reactions have been performed using special primers with ABI step one, Carlsbad, CA. B-act was used as an endogenous control.

The relative abundance of detected RNAs was calculated using the $2^{-\Delta\Delta C_t}$ method. The sequence of primers was designed using Primer3Plus®.

Prediction of miRNAs target genes

We used miRDB (version 5.0) and TargetScan (Release 7.0) for predicting target genes for specific immunoregulatory-related selected miRNAs. Since the prediction programs often undergo a high ratio of false-positive results, only the target genes predicted by both programs were taken.

Venny analysis

A Venn diagram analysis from Venny was performed to determine the overlapping target gene sets, which were defined as common predicted targets in both miRDB and TargetScan tools. The overlapping lists were determined as the final target genes for each responsive miRNA.

Kyoto Encyclopedia of Genes and Genomes pathway analysis of target genes

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was done to compare the specific miRNA targets with the whole reference genes background using Database for Annotation, Visualization, and Integrated Discovery (DAVID; version 6.7). The count number >2 and p -value <0.05 were selected as cutoff criteria. Also, Cytoscape 3.1.1 was used to create the possible functional network of the chosen miRNAs.

EnrichR cluster gram

Analysis of gene set EnrichR Clustergram was performed by EnrichR API (Chen *et al.*, 2013a), using the complete list of gene targets of the immunoregulatory-related miRNAs.

Statistical analysis

All values were analyzed using the SPSS ver. 16.0 software (SPSS, Inc., Chicago, IL, USA). The results for each experiment are presented as mean $\pm$ standard deviation for each condition, at least for three independent experiments. Next, group differences were analyzed by one-way analysis of variance and Tukey's test. Real-time PCR results were evaluated by Rest 2009 software (T -test). Statistical significance differences were considered at $p < 0.05$.

Results

Ad-MSCs isolation and differentiation

Ad-MSCs were isolated and cultured from human adipose aspirates. Immunophenotyping has been verified for the purity of isolated Ad-MSCs. Upper surface marker standard flow cytometry analysis showed that isolated Ad-MSCs were positive for CD105 and CD90 and negative for CD45 (Fig. 1A). The cells had a typical spindle shape (Fig. 1B, upper left) and were capable to differentiate into osteogenic (Fig. 1B, upper right) and adipogenic lineages (Fig. 1B, lower left). The degree of differentiation into osteocyte and adipocyte was evaluated by alizarin red and oil red staining, respectively.

PGEs had no effect on Ad-MSCs cell viability

The MTT results revealed no statistically significant differences in cell viability between PGE-treated and control groups. Moreover, Ad-MSCs treated with PGE did not show any cytotoxic responses (Fig. 2).

Expression status of the miRNAs detected by quantitative reverse transcription PCR

As mentioned earlier, four immunoregulatory-related miRNAs (i.e., -21, -155, -126, and -23) were chosen for this study. The expression status of these miRNAs was then evaluated in the PGE-treated and nontreated Ad-MSCs. Among these miRNAs, mir-23b and mir-126 were significantly upregulated in the PGE-treated Ad-MSCs ($p < 0.05$), whereas mir-21 and mir-155 were downregulated compared to nontreated group ($p < 0.05$) (Fig. 3 and Supplementary Figures S1–S10).

Expression status of the RNAs detected by quantitative reverse transcription PCR

The *PI3K/Akt/NF- κ B* expression status was evaluated in the PGE-treated and untreated Ad-MSCs. In both cases, PGE treatment substantially decreased *PI3K/Akt/NF- κ B* expression (Fig. 3C).

The relationship between the expression of mir and *PI3K/Akt/NF- κ B* in Ad-MSCs

The findings showed that mir-23b and mir-126 overexpression inhibited *PI3K/Akt/NF- κ B* expression, and *PI3K/Akt/NF- κ B* expression was significantly inhibited following inhibition of mir-21 and mir-155 expression (Fig. 3). Clearly, there was a negative link among the expression of mir-23b, mir-126, and *PI3K/Akt/NF- κ B*. On the contrary, there was a positive correlation among the expression of mir-21, mir-155, and *PI3K/Akt/NF- κ B*.

Analysis of EnrichR cluster gram

The most enriched pathways related to the immunoregulatory properties of Ad-MSCs were mitogen-activated protein kinase (*MAPK*). The response to *MAPK* and signaling pathway regulation of stem cells has the most intense interaction. Figure 4B shows gene clusters corresponding to the top enriched biological pathways.

Analysis of involved pathways on the interaction between four miRNAs

The target genes for mir-21, -23, -126, and -155 compared with the whole reference gene background are shown in Figure 5. To recognize possible pathways involved in immunoregulatory activity, Cytoscape software V3.5.1 was then applied to read the KEGG pathway and explain their biological functions. The predicted target genes of the four selected miRNAs were involved in 27 pathways with p -value <0.05.

Discussion

Autoimmune diseases are a global concern because such that 4–5% of the worldwide population suffers from autoimmune diseases (Vyse and Todd, 1996). Despite several approaches proposed for the treatment of autoimmune diseases,

FIG. 1. Isolation of Ad-MSCs from lipoaspirate of normal adults; **(A)** Immunofluorescent staining and flow cytometry analysis of cell surface markers showed that hA-MSCs were positive for CD105 and CD90, but completely negative for CD45. **(B)** After 1 week, the isolated Ad-MSCs were uniform and showed a fibroblastoid morphology (*upper left*). Osteogenic differentiation was evidenced by the formation of a mineralized matrix, revealed by alizarin red staining (*upper right*) Microscopic image showed the typical morphology of adipocytes, characterized by lipid droplet accumulation (*lower left*). Ad-MSC, adipose driven mesenchymal stem cell; hA-MSCs, human amniotic mesenchymal stromal cells; MSC, mesenchymal stem cell. Color images are available online.

FIG. 2. Two groups of Ad-MSCs were treated by (0, 0.25, 0.5, 1, 2, and 4 mg/mL) pomegranate extract for 24 and 48 h, respectively. Cell viability was determined by MTT assay as described in Materials and Methods section. The values given are the mean $\pm$ SD of five independent experiments. * $p < 0.05$ compared to mock control. MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole); SD, standard deviation.

FIG. 3. Pomegranate extract treatment impact on miRNAs expression in Ad-MSC: (A) pomegranate extract-1 (PGE1) or (PG1) impact on miRNAs expression in Ad-MSC; (B) PGE2 or (PG2) impact on miRNAs expression in Ad-MSC; (C) PGE1,2 or (PG1,2) impact on *PI3K/Akt/NF-κB* expression in Ad-MSC; and (D) Fold changes and p -values of miRNAs. (E) Fold changes of *PI3K/Akt/NF-κB* expression. All experiments were performed as described in materials and methods, and results are expressed as mean $\pm$ standard error. *Indicates significant changes at ($p < 0.05$). (*significant, NS, nonsignificant). miRNA, microRNA; PGE, pomegranate extract.

FIG. 4. (A) Model of selected miRNAs and their confirmed targets of varying molecular mechanisms leading to downstream effects on the immunoregulatory activity of MSCs. (B) EnrichR Clustergram of the most significant comparisons; clusters of 40 gene targets of miRNAs to the 10 top enriched biological pathway terms are shown.

FIG. 5. The network between the specific immunoregulatory-related miRNAs and their predicted targets in this study.

there are no efficient solutions for these diseases. Therefore, new therapies with less side effects and more effectiveness are needed to be designed (Liu and Han, 2008; Dorronsoro *et al.*, 2013; Castro-Manrreza and Montesinos, 2015). MSCs have gained extensive recognition and international attention because of their immunomodulatory properties and use of this capacity for the treatment of autoimmune and degenerative diseases (Kuo *et al.*, 2012; Willis *et al.*, 2018). In addition, miRNAs can influence the immune-regulatory activities of MSCs through targeting various modulatory genes, as a positive regulator or negative regulator (Xu *et al.*, 2013; Karachi *et al.*, 2015; Wu *et al.*, 2015; Khosravi *et al.*, 2017; Onodera *et al.*, 2017; Wu *et al.*, 2017). In addition, studies have shown that PG has different properties such as anti-inflammatory effects by modulating some miRNAs expression and this target gene, which plays a role in the inflammatory pathway (Banerjee *et al.*, 2012, 2013). So, in this study, we assess the impact of PGEs on immunoregulatory-related miRNAs expression in Ad-MSCs for the first time.

First, we evaluated the probable toxicity effects of three different PGEs on Ad-MSCs. Our results did not show any changes in Ad-MSCs cell viability even when 2 mg/mL of PGE was used up to 48 h (Fig. 2). Then, we studied the effects of PGEs treatment on some miRNAs expression in Ad-MSCs. The results showed that all four studied miRNAs are modulated by two different PGEs. Through this process, mir-126 was upregulated when Ad-MSCs were treated with PG1, but PG2 treatment had no significant effect compared to the control group. This finding complies with prior studies. Banerjee *et al.* (2013) indicated that therapeutic potentials of PG in colon tumorigenesis were due, in part, to increase the level of mir-126, significantly suppressed nuclear factor-kappa B (NF- κ B) mRNA and protein expression. Moreover, Khosravi *et al.* (2017) showed that miR-126 has a positive effect on the immunoregulatory activity of Ad-MSCs. Therefore, increasing the expression of miR-126 in Ad-MSCs through PGEs treatment may also improve the immunoregulatory properties of Ad-MSCs.

mir-23 significantly was increased in Ad-MSCs treated with PG1 therapy. Moreover, it was observed that mir-126, PG2 treatment had no significant effect on mir-23 expression compared to the control group. In this line, Wu *et al.* (2017) showed that miR-23b overexpression inhibited activation of the NF- κ B pathway, so mir-23 may have a positive regulatory effect on the immunoregulatory property of MSCs (Karachi *et al.*, 2015; Wu *et al.*, 2017). According to our results, it can be expected that the upregulation of mir-23 by PGEs treatment can improve the immunoregulatory properties of Ad-MSCs.

Although PG1 had a significant effect on the level of mir-126 and mir-23, there was no significant change in mir-155 expression compared to the control group in which Ad-MSCs was treated with PG1. Moreover, although PG2 had no significant effect on mir-126 and mir-23, mir-155 was downregulated by PG2 treatment. In addition, in another study, Banerjee *et al.* (2012) investigated the roles of miR-155-SHIP-1-PI3K in the anti-inflammatory and cytotoxic activity of PGE and showed that PGE may reduce mir-155 expression. Recent studies have shown that mir-155 has negative effects on the immunoregulatory properties of MSCs (Xu *et al.*, 2013; Hou *et al.*, 2016; Onodera *et al.*, 2017). Therefore, decreasing the level of mir-155 expression may improve the immunoregulatory process in Ad-MSCs.

Our research on mir-21 level changes in the treatment of Ad-MSCs with two different PGEs shows that mir-21 has increased through PG1 treatment. In contrast, PG2 treatment has a significant downregulating effect. A previous study aimed at examining the antimetastatic effects of cellular and molecular mechanisms of PGE on prostate cancer cells revealed that PG can reduce the mir-21 expression (Wang *et al.*, 2011). Moreover, the miR-21 modulates the immunoregulatory function of MSC, as a negative regulator (Wu *et al.*, 2015). The other study by Fafián-Labora *et al.* (2017) shows that inhibition of miR-21 disrupts *TLR4* through the pathway of *AKT/mTOR* and immune response because *mTOR* and *pAKT* have been downregulated as well as *IL-6* and *IL-1 β* gene expressions. As a result, a reduction in the expression level of mir-21 may improve the immune regulation properties of Ad-MSCs by affecting their own targets.

Several studies have been conducted to improve the immunoregulatory activity of MSCs via different manners and mechanisms (Chen *et al.*, 2019; Yousefi *et al.*, 2019). For instance, MSCs treatment with *IFN- γ* was found to improve the immune-modulating activity of MSC's (García *et al.*, 2019). In addition, another study showed that the molecular mechanism underlying the effect of *IFN- γ* on MSCs has changed the MSCs miRNAs profile (Chi *et al.*, 2016). Overall, it can be expected that the immune-regulating function of MSCs could be improved by distinct substances by changing the expression of miRNAs.

Based on the results of the present study, all three PGEs may lead to the improved immunoregulatory activity of Ad-MSCs, but the mechanism pattern of these three PGEs varied. The difference in the pattern of the mechanism can be caused by the difference and variety of the components of the extract. Since they were from three different geographical regions, they differed in terms of weather and cultivation conditions and species. Therefore, it can be stated that geographical factors affect the variations between the PG compounds, leading to different mechanisms of its action.

In addition, we showed that PGE can downregulate the expression of *PI3K/AKT/NF- κ B* in Ad-MSCs (Fig. 3C). This finding is consistent with previous studies (Banerjee *et al.*, 2012, 2013). Moreover, bioinformatics analysis predicts that the target genes of four miRNAs selected will participate in MAPK pathways. In addition, there is an important interaction among mediators of the *MAPK*, *PI3K* networks, and *NF- κ B* pathway. Among these pathways, *PI3K/Akt/NF- κ B* (Karachi *et al.*, 2015; Wu *et al.*, 2017), and *TGF- β* (Wu *et al.*, 2015) signaling pathways were found to be associated with the immunoregulatory activity of Ad-MSCs. According to the obtained results, Ad-MSC treated with PGEs by changing the expression of miRNAs causes a change in their target expression. As a result, it would play a significant role in important signaling pathways controlling the immunoregulatory properties of Ad-MSCs (Figs. 4A, B and 5).

Conclusion

The results showed that PGEs treatments might improve the immunoregulatory property of Ad-MSCs through miRNA-23 and miRNA-126 overexpression or miRNA-21 and miRNA-155 downregulation that plays a role in the pathways of immune modulation in Ad-MSCs. In addition, PGE can inhibit the expression of *PI3K/Akt/NF- κ B* genes

involved in inflammatory pathways. It can be speculated that this effect improves the immune-modulating activity of Ad-MSCs at the functional level. In addition, our bioinformatics data have shown that the target of these four miRNAs and the signaling pathways can play an important role in regulating the immunomodulation function of stem cells. These results could be a step toward improving the immunoregulatory properties of Ad-MSCs for the treatment of many diseases.

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Ethics Approval and Consent to Participate

Ad-MSCs were isolated from a human liposuction sample. Lipoaspirate was obtained from an individual who had undergone surgery for aesthetic purposes in Imam Reza Hospital (Birjand, Iran) (Institutional approval number for human studies: IR.BUMS.REC.1397.354) from the Ethics Committee of Birjand University of Medical Science.

Disclosure Statement

No competing financial interests exist.

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Supplementary Material

Supplementary Figure S1
Supplementary Figure S2
Supplementary Figure S3
Supplementary Figure S4
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Supplementary Figure S9
Supplementary Figure S10

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