Oxygen regulation of macrophage migration inhibitory factor in human placenta

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Oxygen regulation of macrophage migration inhibitory factor in human placenta. Am J Physiol Endocrinol Metab 292: E272–E280, 2007. First published August 29, 2006. doi:10.1152/ajpendo.00086.2006.—Macrophage migration inhibitory factor (MIF) is an important proinflammatory cytokine involved in regulation of macrophage function. In addition, MIF may also play a role in murine and human reproduction. Although both first trimester trophoblast and decidua express MIF, the regulation and functional significance of this cytokine during human placental development remains unclear. We assessed MIF expression throughout normal human placental development, as well as in vivo (chorionic villous explants) and in vitro (high altitude placentae) models of human placental hypoxia. Dimethyloxalylglycine (DMOG), which stabilizes hypoxia inducible factor-1 under normoxic conditions, was also used to mimic the effects of hypoxia on MIF expression. Quantitative real-time PCR and Western blot analysis showed high MIF protein and mRNA expression at 7–10 wk and lower levels at 11–12 wk until term. Exposure of villous explants to 3% O2 resulted in increased MIF expression and secretion relative to standard conditions (20% O2). DMOG treatment under 20% O2 increased MIF expression. In situ hybridization and immunohistochemistry showed elevated MIF expression in low oxygen-induced extravillous trophoblast cells. Finally, a significant increase in MIF transcript was observed in placental tissues from high-altitude pregnancies. Hence, three experimental models of placental hypoxia (early gestation, DMOG treatment, and high altitude) converge in stimulating increased MIF expression. Supporting the conclusion that placental-derived MIF is an oxygen-responsive cytokine highly expressed in physiological low oxygen conditions, we (16) have reported that the early events of trophoblast proliferation and invasion are mediated by O2-sensitive transcriptional activity of the hypoxia inducible factor-1 (HIF-1) system and the subsequent expression of TGF-β3. Moreover, in vitro studies demonstrated that low oxygen tension, comparable with that of early placenta, can stimulate the expression of several proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) (9).

Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine first described as a T cell-derived factor capable of inhibiting random migration of macrophages (10, 17). Although the activity of MIF was originally described in T lymphocyte-conditioned media, MIF protein and transcript expression have been widely documented in numerous cell types (14, 1). We focused on MIF in this study because emerging evidence points to MIF as important to early pregnancy development. MIF is expressed during zygote and blastocyst formation in mice (40). MIF has been detected at the implantation site in maternal decidua, in chorionic villous trophoblast cells, and in extravillous trophoblast (EVT) cells within the anchoring villi of humans (5, 6). However, the biological significance of MIF during placental development and in particular its regulation remains to be elucidated.

Because we were struck by the apparent prominence of MIF in early gestational events, we tested the hypothesis that O2 tension contributes to the regulation of MIF expression and secretion. Herein, we demonstrate that MIF expression is spatially and temporally regulated during placental development. Furthermore, we show that low oxygen tension, both environmentally or pharmacologically induced, increases MIF expression and secretion in an in vitro villous explant system. Finally, we demonstrate that MIF expression is elevated in placentae from high-altitude pregnancy, an in vivo physiological model of chronic hypoxia.
**MATERIALS AND METHODS**

**Placental collection and processing.** All tissue samples were obtained after informed consent in accordance with participating institutions’ ethics guidelines. Tissue collection strictly adhered to the guidelines outlined in the Declaration of Helsinki. Placental tissues from first-trimester (5–10 wk of gestation, n = 19) and second-trimester (11–13 wk of gestation, n = 6) and 14–20 wk of gestation, n = 7) normal pregnancies, terminated for psychological reasons, were obtained in Toronto, Ontario, Canada, by dilatation and curettage. Gestational age was determined by the date of the last menstrual period and ultrasound measurement of crown-rump length. High-altitude placentae were collected in Leadville, CO [3,100 meters above sea level (masl)]. Moderate-altitude placentae were collected in Denver, CO (1,600 masl). Sea level placentae were collected from term deliveries at Mount Sinai Hospital in Toronto (~40 masl). All third-trimester specimens were obtained immediately after delivery from normal-looking cotyledons that were randomly collected. Areas with calcified, necrotic, or visually ischemic tissue were omitted from sampling. Subjects suffering from diabetes, essential hypertension, and pregnancies affected by preeclampsia and intrauterine growth restriction were excluded. All groups did not show clinical or pathological signs of preeclampsia, infections, or other maternal or placental diseases. Birth weight, gestational age, and laboratory values or clinical observations relevant to the health of the mother were abstracted from the clinical records. Term control placental tissues (n = 10) were obtained from women with normal pregnancies undergoing elective termination of pregnancy. Placentae were immediately rinsed in sterile cold phosphate-buffered saline (PBS) and undergoing elective termination of pregnancy. Placentae were immediately rinsed in sterile cold phosphate-buffered saline (PBS) and placed on and transferred to 24-well culture plates. The Millicel-CM culture dish inserts (Millipore, Bedford, MA) previously membranes were dissected out. Terminal villi were then placed on and 2 mM glutamine. Explants were maintained in standard condition immediately rinsed in sterile cold phosphate-buffered saline (PBS) and undergoing elective termination of pregnancy. Placentae were immediately rinsed in sterile cold phosphate-buffered saline (PBS) and placed on and transferred to 24-well culture plates. The Millicel-CM culture dish inserts (Millipore, Bedford, MA) previously membranes were dissected out. Terminal villi were then placed on and transferred to 24-well culture plates. The Millicel-CM culture dish inserts (Millipore, Bedford, MA) previously membranes were dissected out. Terminal villi were then placed on and transferred to 24-well culture plates. 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the microscope. The threshold of specific detection was automatically calibrated on control sections hybridized with the sense probe.

**Western blot analysis.** Placental tissues and villous explants were homogenized in ice-cold RIPA buffer [50 mM Tris·HCl, 150 mM NaCl, 1% (vol/vol) Triton X-100, 1% (wt/vol) Na deoxycholate, 0.1% (wt/vol) SDS, pH 7.5] supplemented with protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). After centrifugation at 15,000 g for 15 min at 4°C, the supernatant was assayed for total protein concentration by the Bradford method (12), and MIF was detected by Western blot analysis. Thirty micrograms of total protein were separated on 15% polyacrylamide gel in the presence of SDS. Proteins were then transferred to polyvinylidene difluoride membranes. Primary anti-human MIF monoclonal antibody (R&D Systems, Abingdon, UK) was used at dilution of 1:500. The membrane was then exposed to horseradish peroxidase-conjugated rabbit anti-mouse secondary antibody. The bands were detected using an enhanced chemiluminescence kit (PerkinElmer Life Sciences, Boston, MA) according to the manufacturer’s instructions. Equal loading of the proteins was confirmed by staining the blots with a 1% (vol/vol) Ponceau S solution (Sigma Chemical, St. Louis, MO) (31).

**Immunohistochemistry.** Immunohistochemical analysis was performed using an avidin-biotinylated horseradish peroxidase monoclonal antibody with normal murine immunoglobulins.

**MIF ELISA.** MIF release in supernatant of villous explant cultures maintained at 3 and 20% O2 was measured by a colorimetric sandwich ELISA. Briefly, 96-well plates were coated overnight at room temperature with anti-human MIF monoclonal antibody (2 µg/ml; R&D Systems). The plates were then washed with washing solution [10 mM PBS, pH 7.4, 0.05% (vol/vol) Tween 20], blocked by adding 300 µl of blocking solution [10 mM PBS, pH 7.4, 1% (wt/vol) bovine serum albumin (BSA), 0.5% (wt/vol) sucrose], and incubated at room temperature for 1.5 h. The samples, diluted in Tris-buffered saline-BSA [2.0 mM Tris·HCl, 150 mM NaCl, pH 7.3, 0.1% (wt/vol) BSA, 0.05% (vol/vol) Tween 20], were added in duplicate (100 µl/well) and incubated for 2 h at room temperature. The plates were then washed three times, and 100 µl of biotinylated goat anti-mouse secondary antibody (DAKO, Copenhagen, Denmark) diluted 1:300 for 1 h at room temperature. After being washed, slides were then incubated for 2 h at room temperature with avidin-biotin complex and then developed with 0.075% 3,3-diaminobenzidine (vol/vol) in PBS containing 0.002% hydrogen peroxide (vol/vol). Sections were then counterstained with hematoxylin and dehydrated. In each experiment, a negative control was used by replacing the primary antibody with normal murine immunoglobulins.

MIF ELISA. MIF release in supernatant of villous explant cultures maintained at 3 and 20% O2 was measured by a colorimetric sandwich ELISA. Briefly, 96-well plates were coated overnight at room temperature with anti-human MIF monoclonal antibody (2 µg/ml; R&D Systems). The plates were then washed with washing solution [10 mM PBS, pH 7.4, 0.05% (vol/vol) Tween 20], blocked by adding 300 µl of blocking solution [10 mM PBS, pH 7.4, 1% (wt/vol) bovine serum albumin (BSA), 0.5% (wt/vol) sucrose], and incubated at room temperature for 1.5 h. The samples, diluted in Tris-buffered saline-BSA [2.0 mM Tris·HCl, 150 mM NaCl, pH 7.3, 0.1% (wt/vol) BSA, 0.05% (vol/vol) Tween 20], were added in duplicate (100 µl/well) and incubated for 2 h at room temperature. The plates were then washed three times, and 100 µl of biotinylated goat anti-mouse secondary antibody (DAKO, Copenhagen, Denmark) diluted 1:300 for 1 h at room temperature. After being washed, slides were then incubated for 2 h at room temperature with avidin-biotin complex and then developed with 0.075% 3,3-diaminobenzidine (vol/vol) in PBS containing 0.002% hydrogen peroxide (vol/vol). Sections were then counterstained with hematoxylin and dehydrated. In each experiment, a negative control was used by replacing the primary antibody with normal murine immunoglobulins.

**RESULTS**

**Expression of MIF during placental development.** We first investigated MIF mRNA expression in human placenta samples throughout gestation by qRT-PCR. Using specific primers and TaqMan probes, we found that MIF transcripts were present at all of the examined stages of gestation. However, the pattern of MIF mRNA expression was unique: it was significantly higher during early pregnancy (7–10 wk of gestation) and then declined in the late first trimester (11–12 wk of gestation) to levels that remained constant during the second trimester and at term (Fig. 1A).

A similar expression pattern was obtained at protein level using Western blot analysis. A specific 12-kDa band corresponding to the predicted molecular mass of MIF was expressed throughout gestation. Its intensity was higher in the early first trimester, decreased by week 10, and was similar at all time points tested thereafter (Fig. 1B, top). Protein densitometric analysis revealed that MIF was significantly increased between 7 and 10 wk of gestation compared with other gestational stages examined: late first trimester, second trimester, and term (7–10 wk vs. 11–12 wk fold increase = 1.85 ± 0.12, P < 0.01; 7–10 wk vs. 14–20 wk fold increase = 1.40 ± 0.15, P < 0.01; 7–10 wk vs. 39–40 wk fold increase = 1.68 ± 0.03, P < 0.01 P values; Fig. 1B, bottom). No differences in MIF mRNA and protein expression were found between tissues obtained from vaginal delivery and caesarean section (data not shown).

Immunostaining with anti-MIF antibody showed strong positive immunoreactivity in first-trimester villous cytotrophoblast and EVT cells within the invading columns (Fig. 1C). The syncytiotrophoblast exhibited no MIF staining. Low or positive immunoreactivity was observed in the stroma. By week 13, MIF staining was generally reduced. In stem chorionic cytotrophoblast cells MIF staining declined, and only a few cells in the distal part of the EVT were positive for MIF. No positive staining was observed in control sections, where nonimmune immunoglobulins were used in place of the MIF antibody in all tissues sections examined (Fig. 1C, right).

**Effect of oxygen on MIF expression in villous explants.** On the basis of the first set of experiments on the developmental expression of MIF, we hypothesized that changes in oxygen tension might regulate MIF mRNA and protein expression. To test this hypothesis, we cultured chorionic villous explants (5–10 wk of gestation, 14 separate sets; each experimental condition was repeated 3 times using 3 different explants from the same placenta) at 3 (physiological <10 wk) (36), 8 (physiological >10 wk) (22), and 20% PO2 (standard conditions) for 48 h. Quantitative analysis by qRT-PCR showed that MIF mRNA expression was increased at 8% relative to 20% PO2 and further increased by exposure to 3% PO2 (Fig. 2A). MIF recombinant MIF (R&D Systems) as standard. The sensitivity limit was 18 pg/ml. Intra- and interassay coefficients of variation were 3.86 (0.95) and 9.14 (0.47)% respectively.

**Data analysis.** All data are presented as means ± SE of ≥4–6 separate experiments carried out in triplicate. Statistical significance was determined by one-way ANOVA followed by Student-Newman-Keuls post hoc testing. Significance was defined as P < 0.05. qRT-PCR statistical analysis was performed using the relative expression software tool, a pair-wise fixed reallocation randomization test (34).

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concentration in villous explant conditioned media (measured by ELISA) showed that the amount of soluble MIF was significantly higher in cultures kept at 3% relative to those kept at 20% PO2 (fold increase = 1.75; Fig. 2B).

We next tested the possibility for involvement of the HIF-1α transcription factor in the modulation of MIF expression. Villous cultures were maintained at 3% of oxygen overnight to induce HIF-1α expression, and subsequently, some of them were transferred to 20% oxygen in the presence or absence of DMOG, a general competitive inhibitor of the oxygen-sensing enzymes HIF-α prolyl hydroxylases, which target HIF-1α for degradation (20, 21). MIF transcript was significantly increased in DMOG-treated cultures in 20% O2 compared with control cultures, and the increase in MIF transcript was similar to that observed in untreated cultures maintained at 3% oxygen (Fig. 2C). MIF protein expression as assessed by Western blot analyses in explants in 5 vs. 10 wk of pregnancy showed that MIF induction under low oxygen tension and DMOG treatment was more prominent at 5 wk (Fig. 2D, left), consistent with the greater expression of HIF-1α in placenta tissue early on in gestation (16). Densitometric analysis showed a 1.4-fold increase in MIF protein levels in the 3% oxygen-treated explants (P < 0.05) and a 1.88-fold increase in the DMOG-treated (P < 0.05) cultures (Fig. 2D, right).

Although villous explant sections for in situ and immunohistochemistry (n = 3 separate sets of placental explants) analyses were prepared from placenta at 5–7 wk of gestation, when MIF is present, exposure of explants to 3% oxygen induced strong MIF mRNA (Fig. 3A) and protein (Fig. 3B) expression within EVT cells forming outgrowths...
and in cytotrophoblast cells. Interestingly, immunohistochemical analysis revealed intense nuclear staining in EVT and cytotrophoblast cells of explants kept at 3% and in cytotrophoblast cells in those maintained at 20% O2. Low or absent immunoreactivity for MIF was observed mainly in the syncytiotrophoblast of the 3% O2-treated cultures (Fig. 3B). No immunoreactivity was observed in control sections in which primary MIF antibody was omitted (Fig. 3B, right). 

**MIF expression in high-altitude placental tissues.** To further investigate the effect of low oxygen tension, we performed a quantitative analysis of MIF transcript and protein in normal-term placentae from women living at high (3,100 masl), moderate (1,600 masl), and sea level altitudes. MIF mRNA expression increased 70-fold ($P < 0.004$) and 26-fold ($P < 0.001$) in high- and moderate-altitude vs. sea level placentae, respectively (Fig. 4A). MIF Protein expression was parallel: densitometric analyses revealed a twofold increase in MIF at 3,100 masl vs. sea level ($P < 0.05$) and a 1.5-fold increase at 1,600 masl vs. sea level ($P < 0.05$; Fig. 4B). No difference in MIF mRNA and protein expression levels were noted between vaginal delivery and caesarean section (data not shown).

**DISCUSSION**

The present findings demonstrate for the first time that MIF expression in human placenta is upregulated by low oxygen tension both in vivo and in vitro placenta hypoxia. In this study, we found that MIF mRNA and protein in placental tissues peak at 7–10 wk of gestation when oxygen is low, whereas the levels decrease at 11–12 wk and thereafter when the blood flow is fully established and oxygen tension increases. In vitro experiments confirmed that reduced PO2 is a powerful inducer of MIF expression from early first-trimester trophoblast cells. Explants maintained under low (3%) oxygen tension exhibited significantly higher levels of MIF transcript and protein as well as a higher MIF secretion into conditioned medium with respect to standard (20%) oxygen conditions. Finally, we showed that, under conditions of chronically reduced oxygen tension in vivo, at the physiological equivalent of 18% inspired O2 at 1,600 m and 15% at 3,100 m, MIF expression in placental tissues paralleled that of our in vitro experiments at 8 and 3% O2, respectively. This remarkable gradation of MIF response in relation to relatively subtle changes in oxygen suggests exquisite sensitivity of MIF regulation to oxygen tension. The present findings are consistent
with our recent data showing a pattern of global gene expression that is similar in placentae from first-trimester and high-altitude pregnancies as well as first-trimester villous explants maintained at 20 and 3% O₂ (39). Our results suggest that MIF is one of the genes accounting for similarity between in vivo and in vitro models of placental hypoxia.

MIF expression, normally constitutive at low levels, can be induced by increased glucose levels in β-cells of the pancreatic islet and adipocytes (46, 37), mitogens in T cells (7), corticotrophin-releasing factor in the anterior pituitary cells, and lipopolysaccharide in monocytes/macrophages (45, 15). More recently, it was shown that human chorionic gonadotrophin increases MIF secretion in granulosa cell cultures (44). Consistent with our experiments testing the influence of HIF-1α on MIF expression, new studies on tumor cell lines and cardiac myocytes indicate that MIF is one of the hypoxia-induced genes characterizing tumor phenotypes and that upregulation of MIF mRNA and protein occurs under hypoxic conditions (41, 25, 8).

The presence of MIF has been widely demonstrated in tissues and fluids during pregnancy. MIF transcript and protein are expressed in first-trimester trophoblast, and evidence of an MIF-like protein was shown in term placenta (5, 49). MIF is also expressed by term extraembryonic membranes, and high concentrations of MIF were detected in amniotic fluid and maternal serum (19). Although MIF is ubiquitous, increasing or decreasing concentrations of this cytokine have been correlated with various physiological and pathological events during pregnancy. A recent report by Yamada et al. (47) showed decreased MIF plasma levels during early gestation in women with recurrent miscarriages. We (19) previously reported that MIF levels in amniotic fluid are higher at term than in midgestation and higher in laboring than in nonlaboring women and that this is possibly due to the local secretion by fetal membranes. More recently, we (42) found that maternal serum MIF is significantly higher in patients affected by severe preeclampsia, a clinical condition known to be associated with reduced uteroplacental perfusion and placental hypoxia (39), suggesting that there is increased placental MIF production and secretion into the maternal circulation in response to placental ischemia.

Whether oxygen has a direct effect on MIF induction remains to be elucidated. We (16) have previously reported that, in the early phases of placental development, low oxygen tension is associated with increased expression and transcriptional activity of HIF-1α, a basic helix-loop-helix pre arnt sim domain transcription factor. HIF-1α mediates the transcriptional response to oxygen by binding to hypoxia response elements in the promoter region of TGF-β3, the molecule that mediates the biological effects of a low-oxygen environment.

Fig. 3. MIF localization in first-trimester villous placental explants. A: in situ hybridization for MIF mRNA in paraffin sections of placental explants maintained at 20 and 3% O₂. Bright-field photomicrograph with 35S-labeled anti-sense MIF RNA probe in 20 and 3% O₂ (left). Dark-field photomicrograph with 35S-labeled anti-sense MIF RNA probe in 20 and 3% O₂ (right). Strong reactivity (depicted as white bright dots) was observed in the villous cytotrophoblast and EVT of 3% O₂ culture explants. A reduced reactivity was depicted in the 20% O₂ explant cultures. B: immunohistochemical localization of MIF in villous explants cultured under 20 and 3% O₂. Positive immunoreactivity (depicted as brownish staining) was observed as intranuclear immunostaining in the villous cytotrophoblast (arrows) and EVT cells in both 20 and 3% O₂ explant cultures. Syncytiotrophoblast was negative (arrowheads). Negative control was obtained by replacing the primary antibody with normal murine immunoglobulins; ×40 original magnification.
DMOG, an inhibitor of HIF-1α, strongly inhibited MIF expression levels in placental tissues from HA (3,100 meters above sea level (masl), n = 4 separate experiments), moderate-altitude (MA; 1,600 masl, n = 4 ), and sea level (SL, n = 4 ). Significantly higher level of MIF expression was found in HA (*P < 0.005) and MA (**P < 0.05) placental tissues compared with SL placentae. Results are expressed as fold increase. *P < 0.005 vs. SL; **P < 0.05 vs. SL.

The specific role of MIF during placentation remains unclear. MIF is an important regulator of cell proliferation and differentiation (48, 26). Normal placental development is dependent upon proliferation of the cytotrophoblast and further differentiation of the various trophoblast cell populations: the syncytiotrophoblast that forms the epithelial covering of the villous tree and is the main endocrine component of the placenta, the villous cytotrophoblast that represents the trophoblast stem cell population that proliferates throughout pregnancy and fuses to generate the syncytiotrophoblast layer, and the extravillous trophoblast cells that anchor the villi to the maternal uterus. It is well established that, during early placental development, a low-oxygen environment supports the early events of trophoblast differentiation, including proliferation of the undifferentiated villous cytotrophoblast (18, 16) and of the extravillous trophoblast cells forming the proximal column of the anchoring villi (16). Higher MIF expression in the early stages of placentation and its localization to the villous cytotrophoblast suggests that MIF might contribute to sustain the trophoblast proliferative phenotype, typical of a low-oxygen environment. Consistent with our previous reports (5), we showed that immunostaining for MIF in the proliferative villous cytotrophoblast and extravillous trophoblast was drastically reduced by the 13th week of gestation. First-trimester explants also showed MIF protein and transcript expression in the proliferative cytotrophoblast and in the extravillous trophoblast with prominent staining in the cellular outgrowth of cultures maintained in 3% O2. Intracellular MIF immunoreactivity observed in trophoblast cells, particularly in 3% O2 explants, seems to be in agreement with reports (35, 23) on other cell types possessing a high proliferation index, e.g., pituitary adenoma and lung adenocarcinoma cells. The proliferative response to MIF stimulation in fibroblasts is associated with phosphorylation and activation of p44/p42 ERK [extracellular signal-regulated kinase (ERK)1/2] (30). In human placenta, the expression of ERK1 and ERK2 is localized in the proliferative cytotrophoblast, and, interestingly, expression of the active forms of ERK1/2 is much higher in first-trimester placental tissues compared with second trimester (24). The primary action of MIF is on macrophages, where it inhibits their migration and stimulates their scavenger activity at the site of inflammation (10). Macrophages constitute the majority of immune cell types populating the endometrium and decidua, and their number increases during early pregnancy (29). Decidual macrophages help to remove cellular debris, thereby facilitating the invasion of trophoblast into the maternal tissues (2). We showed that in vitro explants release high levels of MIF under reduced oxygenation. Hence, MIF could be a paracrine mediator in early gestation, determining macrophage accumulation and activation. Alternatively, MIF could also suppress activity of decidual natural killer (NK) cells, an important population in the maternal decidua, particularly at the implantation site (43). Indeed, MIF inhibits NK cell-mediated cytolysis of both neoplastic and normal target cells (4). Although the functions of decidual NK cells must be clarified, inhibition of their cytolytic activity could be a critical mechanism of immunotolerance at the feto-maternal interface.

Although these studies support a central role of MIF in human pregnancy, MIF−/− mice failed to show reduced fertility (11). However, from the relevant report, litter sizes, pup weight, and other indicators of functional pregnancy outcome were not examined (11). Additionally, MIF has complex interactions with other cytokines, i.e., TNF-α, IFN-γ, and IL-1β (13). Hence, it can be hypothesized that other cytokines compensate for the deficiency of MIF.

In view of the multifunctional aspects of MIF, our results in the human placenta, showing a higher expression in the earlier phases of pregnancy and MIF upregulation by low-oxygen conditions, suggest a major role of MIF in the control of
trophoblast growth and in the modulation of maternal immune tolerance. Of clinical importance, reduced placental perfusion leading to placental hypoxia/ischemia might induce the synthesis of placental MIF. This might account for the high MIF serum levels in patients affected by severe preeclampsia (42).

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