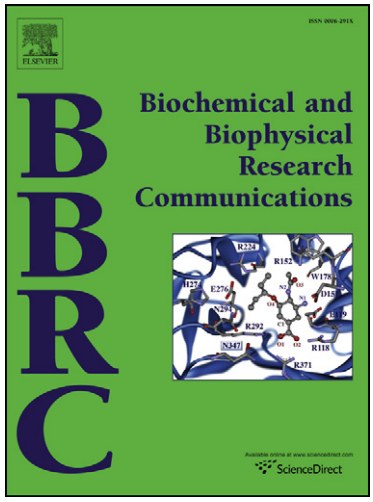

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Hydrogen gas treatment prolongs replicative lifespan of bone marrow multipotential stromal cells in vitro while preserving differentiation and paracrine potentials

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Abstract

Cell therapy with bone marrow multipotential stromal cells / mesenchymal stem cells (MSCs) represents a promising approach in the field of regenerative medicine. Low frequency of MSCs in adult bone marrow necessitates *ex vivo* expansion of MSCs after harvest; however, such a manipulation causes cellular senescence with loss of differentiation, proliferative, and therapeutic potentials of MSCs. Hydrogen molecules have been shown to exert organ protective effects through selective reduction of hydroxyl radicals. As oxidative stress is one of the key insults promoting cell senescence *in vivo* as well as *in vitro*, we hypothesized that hydrogen molecules prevent senescent process during MSC expansion. Addition of 3%hydrogen gas enhanced preservation of colony forming early progenitor cells within MSC preparation and prolonged the *in vitro* replicative lifespan of MSCs without losing differentiation potentials and paracrine capabilities. Interestingly, 3%hydrogen gas treatment did not decrease hydroxyl radical, protein carbonyl, and 8-hydroxydeoxyguanosine, suggesting that scavenging hydroxyl radical might not be responsible for these effects of hydrogen gas in this study.

Keywords

Mesenchymal stem cells; Hydrogen gas; Senescence; Differentiation; Paracrine; Hypoxia

Introduction

Adult bone marrow multipotential stromal cells / mesenchymal stem cells (MSCs) are multipotent cells with strong secretory activities of various growth factors. MSC-based cell therapy represents a promising approach to promote wound healing and tissue regeneration [1-9].

Sufficient number of MSCs retaining their multipotency and paracrine activity is needed for successful MSC-based therapeutics [10]. However, the frequency of MSCs declines age-dependently [11] so that these cells exist only one in 10^5-10^6 adult bone marrow mononuclear cells[5]. *Ex vivo* MSC expansion could compensate for low harvest of MSCs; however, such an expansion causes cellular senescence with loss of differentiation, proliferative, and therapeutic potentials of MSCs [12-14].

Hydrogen molecules have been shown to exert organ protective effects in the ischemia-reperfusion injuries and post-transplant rejections through selective reduction of hydroxyl radicals and possibly other unidentified mechanisms [15-18]. As oxidative stress is one of the key insults promoting cell senescence *in vivo* as well as *in vitro* [19], we hypothesized that hydrogen molecules prevent senescent process during *ex vivo* MSC expansion through anti-oxidative and cytoprotective effects. The aim of the present study was to test the hypothesis through addressing the effects of hydrogen molecules on replicative cell senescence, differentiation, and paracrine potentials of MSC.

Materials and Methods

Cell Culture

Cultured human primary bone marrow MSCs were from Lonza (Basal, Switzerland) for donor 1 and from Center for Gene Therapy, Tulane University (New Orleans, LA) for donor 2. Donor 1 was a 20 years old female and donor 2 was 19 years old male according to the product inserts. FBS was from Atlanta Biologicals (Lawrenceville, GA). MSCs were cultured in MEMα supplemented with 17% FBS, 2 mM L-glutamine, 1 mM pyruvate, and 100 μM nonessential amino acids. Cell culture media and supplements were all from Invitrogen (Carlsbad, CA) unless otherwise stated. All of the experiments were conducted at 37°C in a humidified air with 5% CO_2 unless otherwise specified.

Hydrogen gas treatment

Hydrogen gas treatment was made by culturing cells in premixed gas (3%H_2, 21%O_2, 5%CO_2, balance N_2) (Praxair, Danbury, CT) in a hypoxia chamber (Stemcell Technologies, Vancouver, Canada). In brief, cell culture dishes or multiwell plates were placed in the chamber equipped with airtight seal. Then, the chamber was flushed for 5 min or more with premixed gas (20 L/min) according to the manufacture's instruction. The chamber was flushed with premixed gas every 3 days or less to ensure the composition of mixed gas.

Hypoxia treatment

Hypoxic treatment was made by culturing cells in premixed gas (1%O_2, 5%CO_2, balance N_2) (Praxair, Danbury, CT) in a hypoxia chamber (Stemcell Technologies, Vancouver, Canada) in a similar way to hydrogen gas treatment described previously.

Colony formation assay

Total of 500 cells was seeded in 100 mm culture dish and culture in 14 days as indicated. To exclude the possibility that hydrogen gas exposure affects MSC colony formation through altering adherence efficiency of MSC to plastic, MSCs were allowed to adhere to plastic after seeding for 24 h before initiation of hydrogen gas treatment. Upon completion of the indicated treatment, the formed colonies were rinsed with PBS twice to dislodge the dead cells and debris, then fixed and stained by 5% crystal violet in methanol. The number of colony formation (> 2 mm diameter) was enumerated manually by three independent researchers. The results were given as a colony count per 1000 seeded cells.

Osteogenic differentiation

Osteogenic differentiation was induced as previously described [3]. In brief, MSCs were seeded at 3×10^4 cells per 6-well and cultured in Osteogenic differentiation medium containing dexamethasone, ascorbate, and β-glycerophosphate (Lonza, Basal, Switzerland) for 7 days. Osteogenic differentiation was evaluated by the induction of alkaline phosphatase expression, an early stage of osteogenic differentiation of MSC [20]. Intracellular alkaline phosphatase enzyme activity was detected by using quantitative colorimetric assay kit (BioAssay Systems, Hayward, CA) as well as by histochemical staining with StemTAG™ Alkaline Phosphatase Staining kit (Cell Biolab, San Diego, CA), according to manufactures' protocols.

Adipogenic differentiation

Adipogenic differentiation was induced as previously described after slight modifications [3]. In brief, MSCs were seeded at 1×10^6 cells per 6-well and cultured in Adipogenic differentiation induction medium for 2-3 days followed by Adipogenic differentiation maintenance medium for 2 days in 1-2 cycles, as manufacture's instruction (Lonza, Basal, Switzerland). Adipogenic differentiation was evaluated by staining intracellular lipid droplets by Oil Red O and by induction of adipogenic marker peroxisome proliferation-activated receptor γ (PPARγ) transcript [3; 5].

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

The level of mRNA transcripts was assessed by two-step qRT-PCR. In brief, after isolation of total RNA with TRIzol plus (Invitrogen, Carlsbad, CA), cDNA was synthesized with High Capacity RNA-to-DNA kit (Applied Biosystems, Foster City, CA). Then, cDNA was mixed with Taqman® Universal Master Mix and each Taqman® gene-specific probe/primers (Applied Biosystems) and subject to the Taqman® PCR universal thermal cycling conditions defined by Applied Biosystems: 95°C for 10 min and then, 40 cycles of two-temperature PCR at 95°C for 15 s for denaturing, 60°C for 1 min for annealing and extension. 7900MT (Applied Biosystem) was used as a quantitative real-time PCR thermal cycler.

Taqman® probe/primers were used for $p16^{INK4A}$ or cyclin-dependent kinase inhibitor 2A (Hs00923894_m1), PPARγ (Hs01115513_m1), indoleamine 2,3-dioxygenase-1 (IDO)(Hs00984148_m1), and human ribosomal protein, large, P0 (RPLPO) (housekeeping gene) (4333761F)(Applied Biosystems). All PCR primers were designed to span intron(s) to discriminate cDNA amplicons from genomic amplicons. Comparative Ct method was utilized to assess the levels of each mRNA transcript relative to that level of RPLPO mRNA transcript.

Proliferation Assay

MSCs were seeded in 6-well plate at the density of 50 cells/cm^2 (480 cells/well) in regular medium containing 17%FBS. Cell culture medium was changed every 3-4 days. Live cell counts were determined by manual enumeration of cells excluding trypan blue in the indicated timing, and the 480 live cells were seeded for the next round of experiment. The same process was repeated 3 times during 56 or 58-day experiments (Figs. 1C and 1D). Population doubling was calculated based upon those cell counts.

Quantitation of secreted growth factors, cytokine, and bioactive mediator

After rinsing with PBS, MSCs were cultured in 0.1%BSA-containing serum free medium for 24 h during the indicated treatments. The conditioned media were collected and the secreted VEGF, hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), stromal cell-derived factor-1 (SDF-1), prostaglandin E_2 (PGE_2), and interleukin-6 (IL-6) were measured by quantitative ELISA kits according to each manufactures' instructions (R&D Systems, Minneapolis, MN)(Cayman Chemical, Ann Arbor, MI). The readouts were adjusted per 1×10^6 cells.

β-galactosidase staining

Senescence-associated β-galactosidase (SA-β-gal) activity was detected histochemically by using Senescence β-Galactosidase Staining Kit according to the product insert (Cell Signaling Technology, Danvers, MA). SA-β-gal positive cells were counted manually under microscope and expressed per 100 total cells.

Determination of intracellular reactive oxygen species (ROS)

The levels of intracellular ROS production were determined using 3-(p-hydroxyphenyl) fluorescein (HPF) (Invitrogen, Carlsbad, CA). Briefly, cell suspension (5×10^5 cells/ml) in PBS with 5%FBS was made after indicated treatment, and incubated with 5 μM HPF for 30 min at 37°C. All buffers and reagents were pre-equilibrated with the same mixed gas used in the treatment to prevent ROS production during preparation of cell suspension and incubation with HPF. The fluorochrome-loaded cells were excited using a 488 nm argon-ion laser and the fluorescent emission from HPF was recorded at 515-540 nm. Data were collected using BD FACSCalibur flowcytometry (BD Bioscience, Franklin Lakes, NJ) from at least 10000 cells, and average signal strength was calculated per cell.

Determination of oxidative damages

Oxidative DNA damage was assessed through quantitation of 8-hydroxydeoxyguanosine (8-OHdG) within cellular DNA by using OxiSelect™ Oxidative DNA Damage ELISA kit (Cell Biolab). Protein carbonylation, the irreversible and unrepairable oxidative damage of protein [21], was evaluated through quantitation of protein carbonyls within protein lysates in Laemmli buffer by using OxiSelect™ Protein Carbonyl ELISA kit (Cell Biolab).

Statistical Analysis

All experiments were performed in duplicate or triplicate. Data were analyzed using student *t-tests*. Significance was set at $P < 0.05$ or more stringent as noted in the text and figure legends.

The data of multiple observations were provided as mean ± SEM for at least three separate experiments unless stated otherwise.

Results

Hydrogen gas treatment preserves colony forming early progenitors within MSCs population and prolongs replicative lifespan of MSCs *in vitro*

One of the prominent characteristics of MSC is their ability to produce colonies after seeded at low density [6; 22]. MSC populations are shown to be heterogeneous *in vitro*, containing both colony forming early progenitors or rapidly self-renewing (RS) cells and mature/senescent cells [12; 23; 24]. Preservation of the colony forming early progenitors is critical during *ex vivo* MSC expansion for successful MSC-based therapeutics, which can be assessed easily by colony forming assay[12; 22; 25]. Hydrogen gas treatment increased colony formation of MSCs, suggesting that early progenitor fraction of MSCs is better preserved with hydrogen gas treatment (Figs. 1A and B).

Prolongation of replicative lifespan is another important mechanism to promote *ex vivo* MSC expansion. Oxidative stress is regarded one of the main mechanisms limiting replicative lifespan through accelerating senescent process[19]. Hydrogen gas treatment prolonged replicative lifespan of culture MSCs obtained from two independent donors (Figs. 1C and D).

SA-β-gal is a well-known marker widely used to identify senescent cells [26]. $p16^{INK4A}$ expression has been shown to elevate in senescent MSCs [27]. Hydrogen gas delayed cellular senescence process, as it lowered the number of MSCs expressing SA-β-gal and the overall transcript level of $p16^{INK4A}$ at day 58 of culture (Figs. 1E-G).

Hydrogen gas treatment preserves differentiation potentials of MSCs

Multidifferentiation potential of MSC is another indispensible feature for MSC-based therapeutics and tissue engineering, and it should be preserved during *ex vivo* MSC expansion.

Osteogenic and adipogenic differentiations are two major differentiation fates for MSC [3-5]. Hydrogen gas treatment did not induce either osteogenic or adipogenic differentiations, and it did not alter both differentiations induced by osteogenic and adipogenic differentiation media respectively (Fig 2).

Hydrogen gas treatment preserves paracrine potentials of MSCs

MSC transplantation was shown to promote angiogenesis and tissue regeneration through its strong paracrine capability of various growth factors such as VEGF, bFGF, or HGF[7; 28]. MSCs were also shown to exert anti-inflammatory or immunomodulatory effects through paracrine mechanisms [29; 30]. Thus, it is critically important to evaluate the effects of hydrogen gas treatment on MSC paracrine profile.

Hydrogen gas treatment increased the secretion of bFGF and HGF, whereas it decreased VEGF secretion (Figs. 3A-C). SDF-1 is a chemokine which attracts stem/progenitor cells to regenerating tissues [31; 32], and SDF-1-mediated recruitment of stem/progenitor cells is believed to be one of the mechanisms in MSC-mediated angiogenesis [33]. SDF-1 secretion from MSCs was unaltered by hydrogen gas treatment (Fig 3D). IL-6 is a pro-inflammatory cytokine, but at the same time, it was also shown to play a key role in self-renewal of undifferentiated MSCs *in vitro* [34]. IL-6 secretion was decreased by hydrogen gas treatment (Fig 3E).

PGE_2 and IDO are the ones of the main molecules mediating anti-immunologic and anti-inflammatory effects of MSCs [30]. Interferon-γ (IFN-γ) was shown to enhance immunosuppressive property of MSC through up-regulation of IDO [35; 36]. Although hydrogen gas treatment decreased the PGE_2 secretion, it increased IDO expression in MSCs; however, such

an effect was not apparent in the presence of IFN-γ, presumably due to extremely strong induction of IDO by IFN-γ (Figs. 3F-I).

Hydrogen gas might exert these effects without scavenging hydroxyl radicals

Hydrogen molecule was initially shown to exert anti-oxidant effect through selectively reducing hydroxyl radical, the most powerful ROS in the body [18]; however, the involvements of other unknown mechanisms have been suggested for hydrogen-associated cytoprotective effects [37].

We chose 3% as a concentration of hydrogen gas because of serious and realistic concerns about flammability of hydrogen gas over 4.6%; however, 3% is much lower than the one used in the initial study describing hydroxyl radical scavenging effects by hydrogen molecule[18]. Interestingly, 3%hydrogen gas did not reduce the fluorescent intensity of HPF, whereas 1%O_2 decreased it (Fig. 4A). Moreover, it did not decrease the levels of protein carbonyls and 8-OHdG, whereas 1%O_2 decreased both of them (Figs. 4B and 4C). These data suggested that anti-oxidant effect might not be responsible for these hydrogen gas-mediated effects in this study.

Discussion

Oxidative stress is one of the major insults accelerating cell senescence *in vivo* as well as *in vitro* [19]. Reduction of oxidative stress by lowering oxygen tension or adding antioxidant such as vitamin C or N-acetylcysteine has been shown to prolong replicative lifespan of human cells including MSCs *in vitro*[38-41]. Hydrogen molecule was shown to exert anti-oxidant effect through selectively reducing hydroxyl radical, the most powerful ROS in the body [18], and indeed, hydrogen gas prolonged the *in vitro* replicative lifespan of MSCs without losing differentiation potentials and paracrine capabilities. Hydrogen gas treatment also enhanced preservation of colony-forming early progenitor cells within MSC preparation.

However, 3%hydrogen gas treatment did not decrease hydroxyl radical, protein oxidative carbonylation and 8-OHdG (Fig 4). The apparent mechanism is unclear at this moment, but it indicates that 3% hydrogen gas might be too low to scavenge hydroxyl radical, but still adequate to enhance MSC expansion *ex vivo*. Indeed, the involvement of the unknown mechanisms has previously been suggested[37], which is consistent with our data.

Hydrogen gas treatment did not diminish paracrine activity of MSCs; still, it altered the paracrine profiles (Fig. 3). For example, hydrogen gas treatment increased both bFGF and HGF secretions whereas it decreased VEGF secretion. Hydrogen gas treatment increased the IDO expression, whereas it decreased PGE_2. The net effects of hydrogen gas treatment on the MSC-mediated angiogenic and anti-inflammatory/immunomodulatory actions are uncertain based on these data alone. The effects of hydrogen gas treatment appeared reversible, though some residual effects of hydrogen gas was still observed 5 days after it was discontinued (Fig. 3), suggesting that pretreatment of MSCs with hydrogen gas might still be effective after MSC transplantation *in vivo*.

In conclusion, we demonstrate that hydrogen gas treatment enhances preservation of colony forming early progenitor cells within MSC preparation and prolongs the *in vitro* replicative lifespan of MSCs without losing differentiation potentials and paracrine capabilities. Hydroxyl radical scavenging effects might not be responsible for these effects of 3%hydrogen gas.

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Figure legends

Fig. 1. (A and B) Effects of H_2 exposure on MSC colony formation. Five hundred cells were seeded in 10 cm dish and the number of formed colonies was counted in day 14. [*$p<0.05$ to control (Ctrl) condition in (B)]. In (A), shown are representative images of MSC colony formation in the presence and absence of H2. (C and D) Effects of H_2 exposure on the replicative lifespan of MSC from donor 1 (C) and donor 2 (D). [**$p<1 \times 10^5$, *$p< 1 \times 10^3$ to control (Ctrl) condition in (C) and (D), respectively]. (E-G) Effects of H_2 exposure on the induction of $p16^{INK4A}$ transcript by quantitative RT-PCR in (E) and SA-β-gal activity in (F) in donor 1.[*$p<0.005$ to control (Ctrl) in (E) and (F)]. In (G), shown are representative images of SA-β-gal staining of MSCs at day 58. Each photograph is 550 μm square.

Fig. 2. Effects of H_2 exposure on the osteogenic and adipogenic differentiation of MSCs. (A, C, and E) Effects of H_2 exposure on osteogenic differentiation of MSCs. Cells were cultured in regular medium (Reg) or osteogenic medium (Osteo) in the presence and absence of H_2 for 7 days, and an early phase of osteogenic differentiation was evaluated by histochemical staining of alkaline phosphatase macroscopically in (A) and microscopically in (C) and biochemical quantitation of alkaline phosphatase activity in (E). [*$p<1 \times 10^6$, #$p<1 \times 10^6$ to regular medium control of each respective condition in (E)]. (B, D, and F) Effects of H_2 exposure on adipogenic differentiation of MSCs. (B and D) Effects of H2 exposure on adipogenic differentiation of MSCs. Cells were cultured in regular medium (Reg) or 2 cycles of adipogenic stimulations (Adipo) in the presence and absence of H_2, and an adipogenic differentiation was evaluated with Oil Red O staining of intracellular lipid droplets macroscopically (B) and microscopically (D) and with mRNA transcript levels of PPARγ by quantitative RT-PCR (F). [*$p<0.005$, #$p<0.01$ to

regular medium control of each respective condition in (F)]. Each photograph is 550 μm square in (E) and (F).

Fig. 3. Effects of H_2 exposure on the secretion of bFGF (A), HGF (B), VEGF (C), SDF-1 (D), IL-6 (E), PGE_2 (F and G), and IDO (H and I) from MSCs. For H_2(+) group, cells were incubated with H_2 for 4 days before collection of conditioned media. For H_2 pretreatment (preTx) group, cells were incubated with H_2 for 4 days, and then cultured for additional 4 days without H_2 before collection of conditioned media. The levels of growth factors and cytokines in the serum-starved conditioned media were determined by ELISA. The levels of IDO mRNA transcript relative to RPLPO transcripts (housekeeping gene) were by quantitative RT-PCR (H and I). The levels of PGE_2 and IDO were determined in the presence (G and I) and absence (F and H) of 20 ng/mL interferon-γ (INF-γ). P-values were given in each figure.

Fig. 4. Effects of H_2 or hypoxia (1%O_2) on the generation of intracellular reactive oxygen species (ROS) (A), 8-OHdG (B), and protein carbonyls (C). MSCs were cultured in the presence of H_2 or under hypoxic condition for 10 days prior to assays. Intracellular ROS levels were determined using fluorescent intensity of HPF by flowcytometry. The levels of 8-OHdG and protein carbonyls were determined by ELISA. [*$p<0.005$ (A), *$p<0.05$ (B and C) to 20%O2 condition in each graph.].

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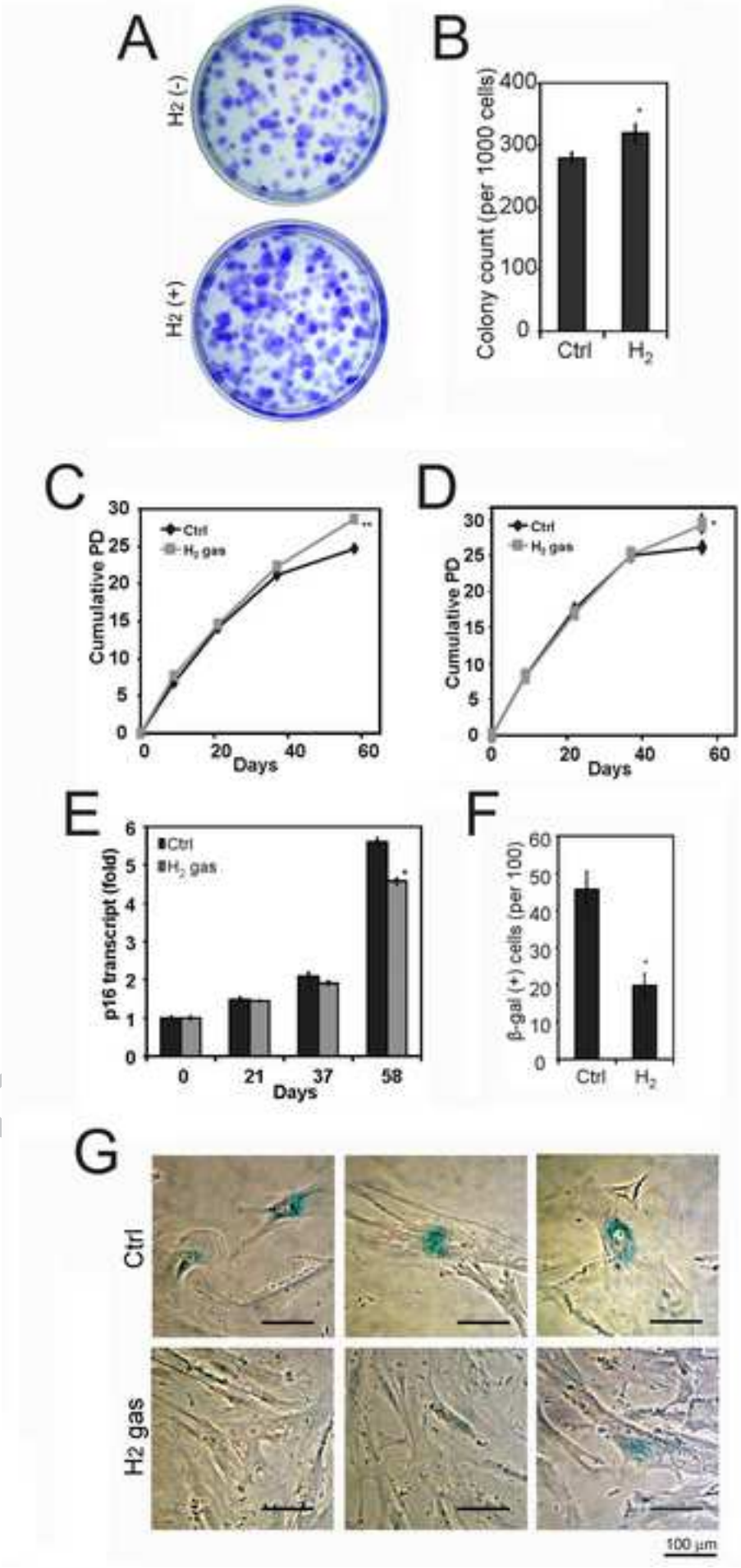
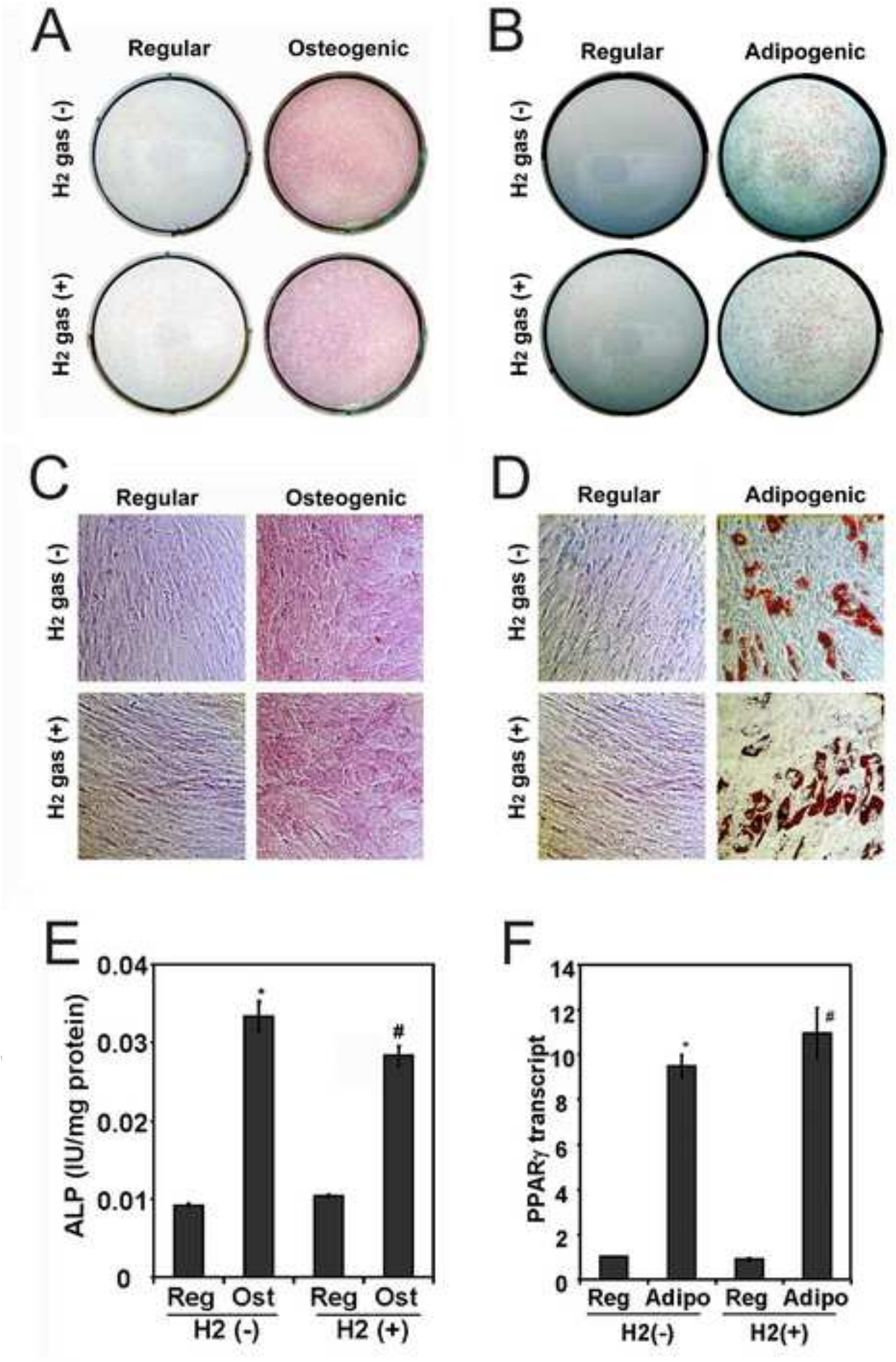
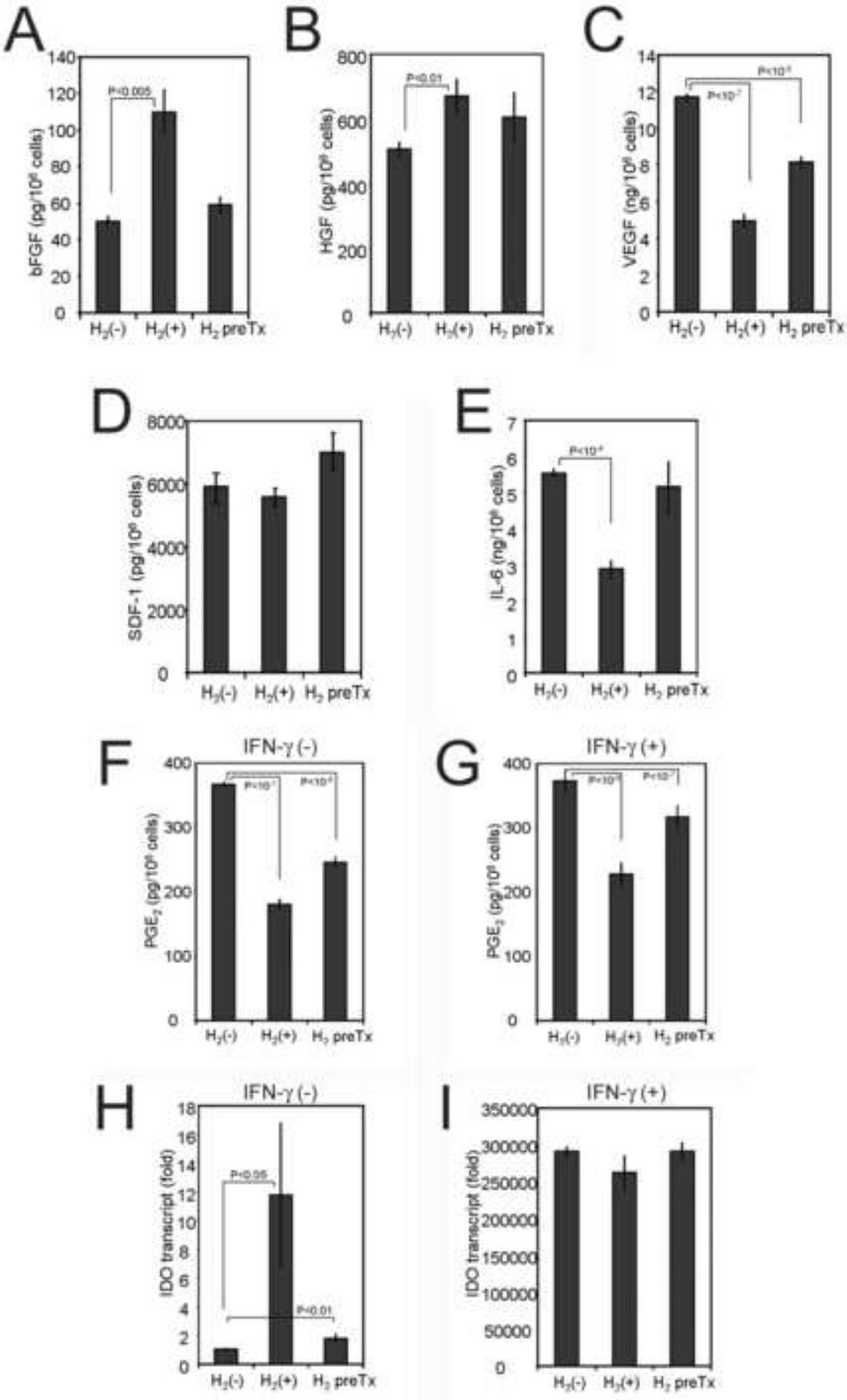



A
Regular
Osteogenic
H2 gas (-)
H2 gas (+)
B
Regular
Adipogenic
H2 gas (-)
H2 gas (+)
C
Regular
Osteogenic
H2 gas (-)
H2 gas (+)
D
Regular
Adipogenic
H2 gas (-)
H2 gas (+)
E
ALP (IU/mg protein)
0.04
0.03
0.02
0.01
0
Reg Ost Reg Ost
H2 (-) H2 (+)
F
PPARγ transcript
14
12
10
8
6
4
2
0
Reg Adipo Reg Adipo
H2(-) H2(+)


A
bFGF (pg/10⁶ cells)
P<0.005
H2(-)
H2(+)
H2 preTx
B
HGF (pg/10⁶ cells)
P<0.01
C
VEGF (ng/10⁶ cells)
D
SDF-1 (pg/10⁶ cells)
E
IL-6 (ng/10⁶ cells)
F
IFN-γ (-)
PGE2 (pg/10⁶ cells)
G
IFN-γ (+)
H
IDO transcript (fold)
P<0.05
P<0.01
I