Hypoxia inducible factor 2α/insulin-like growth factor receptor signal loop supports the proliferation and Oct-4 maintenance of mouse germline stem cells

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Submitted on January 3, 2014; resubmitted on February 14, 2014; accepted on February 21, 2014

ABSTRACT: Hypoxia inducible factor 2α (HIF-2α) is critical for primordial germ cell (PGC) survival as knockout of HIF-2α (HIF-2α−/−) decreases both expression of Oct-4 and PGC number in genital ridge. Hypoxia is known to stabilize HIF-2α protein from proteasomal degradation. However, little is known about the hypoxia-associated endocrine signaling in HIF-2α expression. The current work demonstrates a role for an endocrine insulin-like growth factor-I receptor (IGF-IR)-PI3K/Akt-mTOR-HIF-2α regulatory loop in the proliferation and Oct-4 maintenance of PGC-like alkaline phosphatase positive mouse germline stem cells (AP+ GSCs). We found that hypoxia greatly increased the cell proliferation and the levels of nuclear Oct-4/HIF-2α protein of AP+ GSCs. The hypoxic-AP+ GSCs presented stronger stemness ability for germ cell differentiation than normoxic, with expressions of c-KIT (differentiation germ cell marker), VASA (differentiation germ cell marker) and SCP3 (meiotic marker) using a renal capsule transplantation assay. Meanwhile, hypoxia significantly increased the expression levels of secreted-IGF-I and IGF-IR. The IGF-I dose dependently increased the HIF-2α expression levels in AP+ GSCs; and, the inhibition of IGF-IR by RNA interference (shIGF-IR) or LY294002 (PI3K inhibitor)/Rapamycin (mTOR inhibitor) effectively suppressed the IGF-I- and/or hypoxia-induced HIF-2α and Oct-4 expression, suggesting that the IGF-IR and its downstream Akt/mTOR signaling are involved in the IGF-I/hypoxia effects. Additionally, knockdown of HIF-2α dramatically suppressed Oct-4 and IGF-IR protein levels in AP+ GSC cells. In conclusion, the present study demonstrates a regulatory loop of IGF-IR-PI3K/Akt-mTOR-HIF-2α in proliferation and Oct-4 maintenance of PGC-like AP+ GSCs under hypoxia. This finding provides insights into the niche endocrinology underlying early germ cell development.

Key words: germline / tissue-specific stem cells / niche growth factor / oxygen tension / self-renewal

Introduction

Germline stem cells (GSCs) are cells that are involved in gamete production. During embryogenesis, GSCs include primordial germ cells (PGCs), gonocytes (post-migratory PGCs) and spermatogonial stem cells. Germ cell development begins with early PGC specificity and competence from epiblasts, which is followed by late proliferation and migration to genital ridges. Defects in either proliferation or in the maintenance of Oct-4 expression in early germ cell development lead to an insufficient number of germ cells in the embryonic gonads, which can result in infertility and/or extragonadal germ cell tumors (Hoei-Hansen et al., 2006). Both internal gene regulation and external niche signals are involved in germ cell development. In mice, early PGCs are first detected at the base of the allantois in gastrulating E7.25 embryos (Ginsburg et al.,...
1990). The internal transcription factors Froggils/Stella (Saitou et al., 2002) and Blimp I (Ohinata et al., 2005) were shown to confer PGC specificity from epiblasts; and the external niche endocrine growth factor BMP4/8b is responsible for PGC competence (Ying et al., 2000, 2001; Lawson et al., 2003). After specification by proximal epiblasts, PGCs undergo proliferation and migration to the embryonic genital ridge. A physiological hypoxic environment (1–5% O2) has been hypothesized to play an important role in embryogenesis and early germ cell survival (Scortegagna et al., 2003; Covello et al., 2006).

Niche hypoxia is known to stabilize the hypoxia-inducible protein (HIF) by preventing its proteosomal degradation (Ginouves et al., 2008). It is well documented that HIF-2α is expressed in tissues where Oct-4 is also highly expressed, e.g. blastocyst-stage, E7.5 embryos and PGCs. Increasing HIF-2α expression by generating HIF-2α knock-in mice enhanced Oct-4 protein expression, while loss of HIF-2α severely reduced the number of embryonic PGCs in the genital ridge (Covello et al., 2006) and led to azoospermia (Scortegagna et al., 2003). These observations suggest that HIF-2α expression is required for Oct-4 expression and for PGC survival. However, the niche endocrine signaling pathways linking HIF-2α expression, Oct-4 expression and survival of early pluripotent GSCs has not yet been identified.

Gaps in the understanding of hypoxia-associated niche endocrine signals in germ cell development may be due to the lack of an appropriate in vitro stem cell model and/or the generous use of serum in culture medium. Although serum-containing medium should enhance the proliferation of stem cells, undefined components and batch variations of serum (Barnes and Sato, 1980) can greatly reduce cell stemness and prevent the identification of potential factors that control stem cell fate. For this reason, we previously established a serum-free culture system to generate pluripotent GSCs from wild-type neonatal mouse testes (Huang et al., 2009). These pluripotent GSCs display early germ cell characteristics including strong alkaline phosphatase activity (AP), expression of the cell surface proteins stage-specific embryonic antigen-1 and CD49f, and expression of PGC-related genes (Oct-4, Nanog, Blimp 1). They also exhibit migration, multiple differentiation abilities in vitro and chimera/teratoma formation in vivo (Huang et al., 2009). As these early germ cells express strong AP activity, we designated them ‘AP+GSCs’. The serum-free AP+GSC cell platform provides an in vitro cell model to examine the niche hypoxia effect on endocrine signals in cell proliferation and Oct-4 maintenance during early germ cell development.

Here, using this serum-free culture system, we demonstrate a hypoxia-mediated IGF-I receptor (IGF-IR)-HIF-2α signaling loop involved in mediating proliferation and Oct-4 maintenance in mouse AP+GSCs. These findings advance our understanding of the regulatory capacity of niche hypoxia. We also demonstrate the existence of an IGF-IR-HIF-2α signaling loop that regulates proliferation and Oct-4 maintenance in early AP+GSCs.

**Materials and Methods**

**Cultivation of mouse AP+GSCs in serum-free culture medium**

Newborn ICR mice were obtained from the National Laboratory Animal Center and National Applied Research Laboratories (Taipei, Taiwan). Mouse AP+GSCs were generated as previously described (Huang et al., 2009). More than 40 mice were used for a single experiment. In brief, testes from 0 to 2 days post-partum newborn ICR mice were collected and briefly washed in Hank’s buffer (Gibco BRL, Grand Island, NY, USA) containing penicillin (100 units/ml) and streptomycin (100 µg/ml) before treatment with 0.1% protease type-XIV (Sigma, St. Louis, MO, USA) in MCDB-201 medium (Sigma) at 4°C for 16–20 h. Digested tissues were transferred to SMEM medium (Sigma) containing 10% fetal calf serum, and filtered through a 70-µm nylon cell strainer to remove cell debris. In general, one testis yielded ~1.5 x 10⁶ cells. For AP+GSC colony formation, total testicular cells were re-suspended in basic culture medium (BM) composed of MCDB-201 medium supplemented with 1 x insulin, transferrin and selenium and 10 ng/ml of epidermal growth factor (Gibco BRL). For AP+GSC colony formation, the testicular cells were seeded on a laminin-coated culture plate at a density of 8 x 10⁶ cells/cm² and cultivated at 37°C in a 5% CO₂ incubator under 21, 10 or 5% oxygen concentrations for 7 days. The AP+GSC colonies were collected for gene and protein expression analysis.

**Purification of CD49f+ mouse GSCs**

CD49f+ GSCs were purified using a magnetic activated cell sorting system (MACS). Briefly, the total testicular cells were re-suspended in 0.5-mL blocking buffer and then incubated with specific PE-conjugated primary antibodies against CD49f (10 mg/ml, eBioscience, CA, USA) for 1 h on ice. CD49f-labeled cells were washed twice with phosphate-buffered saline (PBS) containing 2% BSA, then incubated with anti-PE Microbeads (Miltenyi Biotec, CA, USA) for 40 min at 4°C. Stained cells were separated through MACS column (Miltenyi Biotec) according to the manufacturer’s recommended protocol. The CD49f-positive cells were then used in experiments.

**AP activity assay**

Clumped GSC colonies or CD49f+ GSCs in serum-free medium were fixed with 3.7% paraformaldehyde for 2 min at room temperature. The AP activity of these GSCs was examined using an AP detection kit according to the manufacturer’s instructions (Chemicon, Hampshire, UK).

**Construction of short hairpin RNA and generation of transgenic mouse GSCs**

shControl (TRCN0000072246), shGF-IR#1 (TRCN0000023491) and shGF-IR#2 (TRCN0000023489) plasmids were purchased from National RNAi Core, Taiwan. Double-stranded hairpin oligonucleotides designed to target the mouse HIF-1α cDNA (NM_010431) 376–398 (5′-GAACTAAC TGGACACAGTGTT-3′), mouse HIF-2α cDNA (NM_010137) 2052–2070 (5′-GATGAGGTCTGCAAAGGAC-3′), shRNA#1) and 87–105 (5′-GGAGAGGAGGTCTTTTCTAT-3′, shRNA#2) of the HIF-2α gene were cloned into the BamHI/NotI site of pGSHI-GFP vector to generate shHIF-1α and shHIF-2α. The mouse transgenic GSCs were generated by electroporation with plasmid (15 µg). The electroporation was performed using an electroporator (BTX) at 250 V for 3 pulses, each pulse lasting 0.1 ms, 250 ms apart.

**Bromodeoxyuridine incorporation assay**

Cell proliferation was determined using immunocytochemical staining based on bromodeoxyuridine (BrdU, Sigma) incorporation by DNA synthesis. Cells were seeded on 12-well plates and incubated under normoxia or hypoxia. After 24 h of incubation, BrdU was added to a final concentration of 0.1 µM, and cells were incubated at 37°C for an additional 24 h. Excess BrdU in the medium was removed by three washes with PBS, and then the BrdU-incorporated cells were detected using immunocytochemical staining.
RNA isolation and reverse-transcription polymerase chain reaction

The AP⁺ GSC colonies and CD49f⁺ GSCs were collected and the total RNA was extracted with an RNeasy Micro Kit (QIAGEN, Valencia, CA, USA), according to the manufacturer’s instructions. Three micrograms of total RNA was used to synthesize complementary (c)DNA with a random primer (Invitrogen, Carlsbad, CA, USA). cDNA synthesis was performed at 42°C for 50 min in a final volume of 20 μl, according to the manufacturer’s instructions for Superscript III reverse transcriptase (Invitrogen). PCR was performed with Platinum Taq™ Polymerase (Invitrogen) and the reverse-transcription polymerase chain reaction (RT–PCR) amplifications were titrated to within a linear range of amplification. The primer pairs, accession numbers, and primers for beta-globin and AP activity were designed for the GSCs in this study. A 120-bp fragment of AP activity was amplified using the following primers: forward primer, 5'-GATGTTGACCTTCTCAAGGC-3'; reverse primer, 5'-TCCATGCTGCTGCTTGGATT-3'. The PCR products were separated by agarose gel electrophoresis, and the DNA bands were visualized with ethidium bromide under UV light. RT–PCR analysis of AP activity of the colonies was assessed. The size and number of GSC colonies expressing AP activity were assessed for statistical analysis.

For examination of HIF-2a expression, particularly in AP⁺ GSC cells, CD49f⁺ AP⁺ GSCs were collected by MACS and cultivated under hypoxia (5% O₂) or under normoxia (21% O₂) with IGF-I treatment for 24 h. Cell lysates were collected for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and western blotting analysis.

Immunostaining

For the BrdU incorporation assay, primary cultured cells were fixed with 3.7% paraformaldehyde at room temperature for 30 min. After fixation, cells were washed twice with PBS, and then treated with 2 N HCl at room temperature for 20 min, followed by neutralization with 0.1 M sodium borate at room temperature for 2 min. After three washes with PBS, immunocytochemical staining was performed on the cells. To detect Oct-4 expression in primary cultured cells, cells were fixed in methanol:acetone (1:1) at room temperature for 10 min. For other antigens, cells or tissues were fixed with 3.7% paraformaldehyde at room temperature for 30 min. After fixation, cells were rinsed twice with PBS and then treated with PBS containing 0.1% Triton-X 100 (PBST) at room temperature for 10 min, and blocked with bovine serum albumin (BSA; 5 mg/ml) in PBST for 1 h at room temperature. For confocal spectroscopic fluorescence, the AP⁺ GSC colonies were immuno-probed at 4°C overnight with the following antibodies: anti-Oct-4 (sc-5279 and sc-9081), anti-IGF-I (sc-9013) and anti-IGF-IR (sc-7952) (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA). Anti-ctk (CD117, Clone 553352, BD Biosciences, San Jose, CA, USA); anti-HIF-1α (NB100-105) and anti-HIF-2α (NB100-122) (Novus Biologicals, Littleton, CO, USA); anti-BrdU (M0744) (DakoCytomation, Carpintera, CA, USA); anti-Scp3 (ab15093) (Abcam, Cambridge, MA, USA); anti-Vasa (R&D, Minneapolis, MN, USA) and anti-β-Actin (A544, Sigma). The experimental conditions are listed in Supplementary data, Table S1. Specific labeling of primary antibodies was detected with Cy3-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA). The nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole) (Sigma). All cells were covered with an anti-fading reagent (Vector Laboratories, Burlingame, CA, USA) and analyzed with a fluorescence microscope (Olympus, Melville, NY, USA).

Renal capsule grafting

Eight-week-old NOD-SCID mice were obtained from the BioLasco Taiwan Co., Ltd (Taipeh, Taiwan). Normoxic AP⁺ GSCs or hypoxic AP⁺ GSCs, both together with testicular stromal cells (1 × 10⁶ cells), were engrafted beneath the renal capsule of NOD-SCID mice (n = 5) using the method described at: http://mammary.nih.gov/tools/mousework/Cunha001. Eight weeks after transplantation, host mice were sacrificed and the transplanted kidneys were retrieved, fixed and sectioned for further histological analysis. All animal experiments were approved by the Animal Care and Use Committee of Taipei Medical University and performed in accordance with the Institutional Animal Care and Use Committee guidelines of Taipei Medical University.

Statistical analysis

All experiments were repeated at least three times with individual samples. Data are expressed as the mean ± standard deviation (SD). Differences in means were assessed by t-test or one-way analysis of variance and post hoc test (GraphPad InStat 3.0, GraphPad Software, Inc., La Jolla, CA, USA).

Results

Hypoxia increases mouse AP⁺ GSC proliferation

Niche oxygen level influences stem cell self-renewal and differentiation. To test the effect of oxygen tension on GSCs, serum-free medium was used to generate AP⁺ GSCs (Huang et al., 2009) under normoxia (21% O₂) and hypoxia (5% O₂). As shown in Fig. 1A, compared with the normoxic condition, hypoxia increased AP⁺ GSC proliferation, evidenced by the larger size of those colonies (Fig. 1A, b versus a); the greater percentage of colonies > 100 μm (⁎⁎⁎P < 0.001, Fig. 1Ac) and by the higher percentage of BrdU⁺ GSC cells in the total hypoxic GSC population (Fig. 1B). In addition, RT–PCR analysis of AP⁺ GSC colonies showed that hypoxia increased Cyclin D1 and c-Myc gene expression levels in AP⁺ GSCs (Fig. 1C). Together these results demonstrate that hypoxia stimulates the proliferation of mouse AP⁺ GSCs.
Hypoxia increases expression of stemness-related genes in mouse AP+ GSCs

To examine the effect of hypoxia on gene expression, AP+ GSCs were collected for real-time quantitative RT–PCR analysis. As shown in Fig. 2, normoxic AP+ GSCs expressed ~70% of the level of Oct-4 mRNA expressed by mouse embryonic stem cells (ESCs). Hypoxia increased the Oct-4 mRNA level of AP+ GSCs from 70 to ~115% with that of ESCs (Fig. 2A). We found that the hypoxic effect on the expression of stemness-related genes (e.g., Oct-4, Nanog, Sox2, and Klf-4) was inverse to the oxygen concentration: the lower the culture oxygen concentration, the higher the level of stemness-related gene expression in AP+ GSCs (Fig. 2B). Western blot analysis confirmed that hypoxia increased Oct-4 protein expression in AP+ GSCs (Fig. 2C) and immunocytochemical staining showed that nuclear Oct-4 levels in AP+ GSCs increased under hypoxic culture conditions (Fig. 2D and E, ***P < 0.001).

Hypoxia maintains AP+ GSC stemness ability for germ cell formation

Given the finding that hypoxia increases the expression levels of stemness-related genes, we compared normoxic- and hypoxic AP+ GSCs for their ability to form germ cells in vivo. Renal capsule transplantation in NOD-SCID mice was used in this experiment, as the renal capsule lacks endogenous GSCs and is not the physiological niche for GSCs. As shown in Fig. 3, when compared with the hypoxic AP+ GSCs, the normoxic AP+ GSCs did not form significant seminiferous-like bulb in renal capsule transplantation (Fig. 3A). The hypoxic AP+ GSC group (H) displayed a larger and more-notable bulb than the normoxic AP+ GSC group (N) (Fig. 3A and B). Histological staining revealed a marked seminiferous tubule-like morphology, suggesting that the potential of hypoxic AP+ GSCs in germ cell formation (5/5, 100%) (Fig. 3C and 3–I, HE staining, indicated by dash line). Germ cell differentiation of AP+ GSCs was also examined by immunostaining. Figure 3D shows positive c-Kit
immunostaining of cells located in tubules (Fig. 3D-I). The c-Kit+ cells were predominantly located at the basement of the tubules, which is similar to the location of differentiated spermatogonia type B cells in the post-natal testis (i.e. testicular basal membrane) (inset of Fig. 3D-II, indicated by black arrowhead). Further examination revealed positive immuno-recognition of Vasa+ cells located one-cell distance from the tubular basement, which is consistent with what is seen during the normal spermatogenesis process (Fig. 3E and E–I). Cells located in tubular lumens stained positive for the meiosis-specific marker Scp3, demonstrating the potential of meiosis in the AP+GSC differentiation process (Fig. 3F and F–I). These observations demonstrate a better germ cell formation ability of hypoxic versus normoxic AP+GSCs in vivo, and further support that hypoxia has a positive effect on proliferation and Oct-4 maintenance in AP+GSCs.

IGF-IR signaling regulates hypoxia-induced cell proliferation and Oct-4 expression in AP+GSCs

We found that under hypoxic conditions, secreted IGF-I in medium (Fig. 4A and B) as well as IGF-IR protein expression in AP+GSCs (Fig. 4C) were increased. To determine whether hypoxia-induced proliferation and Oct-4 expression in AP+GSCs are mediated via IGF-IR signaling, the molecular inhibitor PPP (a IGF-IR phosphorylation inhibitor), LY294002 (LY, a PI3-kinase inhibitor) or PD98059 (PD, a MAPK inhibitor) was used under hypoxic conditions. As shown in Fig. 4D, compared with the normoxic condition, hypoxia significantly enhanced the proliferation and AP activity of AP+GSCs (panel e versus a), and the IGF-IR signaling inhibitors PPP and LY markedly suppressed the hypoxia-induced AP+GSC proliferation and AP activity (panels f and g versus e). Consistent with these results, hypoxia markedly enhanced Oct-4 expression in AP+GSCs (Fig. 4E and F), and PPP and LY significantly suppressed the hypoxia-induced Oct-4 protein expression in AP+GSCs (Fig. 4F). Quantitative analysis is shown in Fig. 4G. In our experiments, the MAPK inhibitor PD did not affect AP+GSC proliferation, AP activity (Fig. 4D-h) or Oct-4 protein expression (Fig. 4F) in either normoxic- or hypoxic conditions. These results strongly suggest a crucial role for IGF-IR/PI3K-Akt signaling in the downstream regulation of hypoxia-induced germ cell proliferation and Oct-4 maintenance.

IGF-I/IGF-IR signaling regulates HIF-2α expression in AP+GSCs

CD49f is an AP+GSC cell surface marker (Huang et al., 2009). To further determine the signaling specific for AP+GSCs, CD49f+GSCs were isolated by an MACS. As shown in Supplementary data, Fig. S1, the MACS-purified CD49f+GSCs exhibited strong AP activity (both of the Fast Red Violet/Napthol AS-BI phosphate, red; and 5-Bromo-4-chloro-3-indolyl phosphate/Nitroblue tetrazolium, BCIP/NBT, blue) and expressed the pluripotency-related genes Oct-4, Nanog, Sox2 and Blimp1.

Hypoxia is known to stabilize the HIF-2α protein, which has been shown to be associated with Oct-4 expression in PGC cells (Covello et al., 2006). Given that IGF-IR signaling regulates AP+GSC Oct-4 levels under hypoxia, we examined whether IGF-I/IGF-IR signaling also regulates HIF protein expression in AP+GSCs. As shown in Fig. 5A, IGF-I increased
both HIF-2α and Oct-4 expression levels in a dose-dependent manner under normoxia. The Oct-4 protein expression levels induced by IGF-I were coincident with the HIF-2α levels in AP+GSCs. In contrast, IGF-I did not affect HIF-1α protein expression in AP+GSCs (indicated with an open triangle in Fig. 5A). The IGF-I effect on HIF-2α translational expression was further confirmed by treating AP+GSCs with the proteasome inhibitor MG132 (Fig. 5B and C).

The role of IGF-IR in HIF-2α expression was examined using RNA interference targeting of endogenous IGF-IR (shIGF-IR). We tested six different shIGF-IR constructs, and the knockdown efficiency of shIGF-IR was examined using HEK293T cells. Two constructs with different knockdown efficiencies were selected for IGF-IR knockdown experiments in AP+GSC cells (Supplementary data, Fig. S2, #1 and #2). The shIGF-IR #2 effectively suppressed the IGF-I-induced protein expression of HIF-2α as well as Oct-4 expression in AP+GSCs (Fig. 5D).

The role of IGF-IR downstream signaling in HIF-2α expression was further examined using the signaling inhibitors PI3K, LY294002, and Rapamycin (mTOR inhibitor). Figure 5E shows the effective suppressive effects of LY and Rapamycin on the IGF-I-induced protein expression of HIF-2α and Oct-4 in AP+GSCs. These results suggest that downstream PI3K/Akt-mTOR signaling is involved in IGF-I/IGF-IR-mediated expression of HIF-2α and Oct-4 in AP+GSCs under normoxia.

Figure 3 Hypoxia increased the germ cell formation ability of AP+GSCs in vivo. Renal capsule transplantation of (A) normoxic AP+GSCs or (B) hypoxic AP+GSCs. (C) C-I, Hematoxylin and eosin staining of the hypoxic bulb tissue section in (B). Dash line indicates the seminiferous-like structure. C-II, higher magnification of dash-lined area in C-I. (D) Immunostaining of tissue section in (B) with antibody against c-Kit. D-I: magnification of c-kit immunostaining shown in D-I boxed area. D-II: c-kit immunostaining shown in D-II boxed area. Black arrowheads indicate the basal membrane location of c-Kit+ cells. (E) Vasa immunostaining of tissue section in (B). E-I, higher magnification of boxed area in (E). White arrowheads indicate the non-basal membrane location of Vasa+ cells. (F) Scp3+ cells in (B). F-I, higher magnification of boxed area in (F). Bar = 25 μm.
Hypoxia maintains Oct-4 and HIF-2α expression levels through IGF-IR/PI3K-Akt/mTOR signaling

The crucial role of IGF-IR and its downstream PI3K-Akt/mTOR signaling in hypoxia-induced Oct-4 and HIF-2α expression in AP^+ GSCs was further demonstrated using shIGF-IR (#2) and small molecule inhibitors by western blotting (Figs 6A and B) and immunocytochemical staining (Fig. 6C). Pre-suppression of IGF-IR signaling effectively decreased the expression levels of Oct-4 and HIF-2α protein in AP^+ GSCs under hypoxia. Together, these results support an important role for IGF-IR/Act/mTOR signaling in hypoxia-induced HIF-2α expression in mouse AP^+ GSCs.

HIF-2α regulates hypoxia-induced IGF-IR expression in AP^+ GSCs

To clarify whether IGF-IR regulates Oct-4 expression through HIF-2α, RNA interference targeting HIF-2α (shHIF-2α) was used with IGF-I treatment. Two shHIF-2α plasmids with different knockdown efficiencies (#1 and #2) were used in this study. As shown in Fig. 6D, the shHIF-2α#2 effectively suppressed IGF-I-induced HIF-2α as well as...
Oct-4 expression. This result demonstrates a critical role for HIF-2α in IGF-I/IGF-IR-mediated Oct-4 expression. Interestingly, we found that shHIF-2α not only effectively suppressed the translational level of Oct-4, but also suppressed IGF-IR expression levels in AP+GSCs (Fig. 6D). This result suggests that there is a regulatory loop of IGF-IR and HIF-2α in AP+GSCs under hypoxia. This hypothesis is further supported by the experiment of HIF-2α knockdown in hypoxic AP+GSCs. As shown in Fig. 6E, the shHIF-2α effectively suppressed IGF-IR protein expression in AP+GSCs under hypoxia condition. Together these results demonstrate a critical role for hypoxia-induced HIF-2α in IGF-IR expression and Oct-4 maintenance in mouse AP+GSCs.

**Discussion**

Hypoxia (low oxygen) is a physiological condition during early embryogenesis. It occurs predominately in tissues undergoing rapid growth, and has been shown to promote the survival of stem cells, including neural crest stem cells (Morrison et al., 2000; Studer et al., 2000), hematopoietic stem cells (Danet et al., 2003), human ES cells (Ezashi et al., 2005), induced pluripotent stem cells (Yoshida et al., 2009) and PGCs (Scortegagna et al., 2003; Covello et al., 2006). Hypoxia inducible protein HIF-2α has been associated with early PGC development (Covello et al., 2006). When PGC cells migrate from the hindgut to the genital ridge, they maintain their Oct-4 levels and AP activity, and increase in cell number from 50 (E8.5 embryos) to 20,000 (E12.5 genital ridges) (Covello et al., 2006). Experiments with transgenic mice demonstrated that loss of HIF-2α HIF2α−/− severely reduced the number of PGCs from 20,000 to ~20 in E12.5 genital ridges (Covello et al., 2006) and led to azoospermia (Scortegagna et al., 2003). As HIF-2α directly regulates Oct-4 (Covello et al., 2006) and c-Myc (Keith and Simon, 2007) expression, the regulation of HIF-2α expression undoubtedly will affect Oct-4 expression and stem cell proliferation. Hypoxia is known to stabilize HIF-2α protein stability; however, to date the hypoxia-induced niche endocrine signal regulating HIF-2α expression during early germ cell development has not been identified.
The fate of stem cells is dependent on internal gene regulation and external niche signaling. In germ cell development, internal genes, such as the transcription factors *Fragilis*, *Stella* and *Blimp1*, regulate the emergence of PGCs (Saitou et al., 2002; Ohinata et al., 2005); and the external BMP4 signal enables PGC competence (Ying et al., 2000, 2001; Lawson et al., 2003). After PGCs are competent, they continue to proliferate and migrate to the genital ridge; however, the mechanisms regulating these processes are unclear. Our findings reveal that a hypoxia-induced regulatory loop of IGF-IR-HIF-2α signaling regulates cell proliferation and Oct-4 maintenance during the time AP⁺GSCs are migrating to the genital ridge (Fig. 7). These findings contribute important insight into our understanding of niche hypoxia crosstalk and endocrine signaling during early germ cell development.

The level of Oct-4 expression is tightly associated with cell fate determination during embryogenesis (Niwa et al., 2000). In male GSCs, Oct-4 expression levels are different in PGCs, gonocytes and spermatogonial...
stem cells (Pesce et al., 1998). The important role of Oct-4 levels in PGC cell fate has been documented previously with a conditional Cre/loxP gene-targeting strategy (Kehler et al., 2004). Reported regulators of Oct-4 level include EpCAM (Huang et al., 2011), estrogen (Zhang et al., 2008; Jung et al., 2011), SUMO1/sentrin-specific peptidase proteins (Wu et al., 2012), IGF-I/IGF-IR (Bendall et al., 2007; Huang et al., 2009) and HIF-2α (Covello et al., 2006). In our experiments, hypoxia elevated the expression of stemness-related genes (Oct-4, Sox2, Nanog and Klf-4) in mouse AP⁺ GSCs. Notably, hypoxia induced Oct-4 levels to a level similar to that of mouse ES cells (Fig. 2). Our results suggest that hypoxia increases the expression of IGF-I/IGF-IR to enhance a niche endocrine IGF-IR signaling loop (Fig. 4A–C) that also stimulates the expression of HIF-2α (Fig. 5). This model is supported by the fact that PPP significantly decreased hypoxia-induced colony formation (Fig. 4) and the shIGF-IR inhibited IGF-I- and/or hypoxia-induced HIF-2α as well as Oct-4 expression level in AP⁺ GSCs (Figs 5 and 6).

The role of IGF-IR signaling in the regulation of proliferation and pluripotency of stem cells has been documented (Huang et al., 2009; Li and

Figure 7 In vitro model for the IGF-IR-HIF-2α regulatory loop in hypoxia-induced Oct-4 expression in AP⁺ GSCs. (A) Proliferation and Oct-4 maintenance of AP⁺ GSCs during early germ cell development. (B) In vitro model for IGF-IR-HIF-2α signaling loop of mouse AP⁺ GSCs under hypoxic conditions. AP, alkaline phosphatase; HIF, hypoxia inducible protein; IGF-I, insulin-like growth factor-I; IGF-IR, insulin-like growth factor-I receptor; PHD, prolyl hydroxylases.
In line with our current work, the activation of the PI3K-Akt pathway was demonstrated to promote PGC proliferation, as well as the conversion into teratoma or pluripotent embryonic germ cells (Kimura et al., 2003; Moe-Behrens et al., 2003). Additionally, the PI3K/Akt signal axis has been shown to crosstalk with self-renewal mechanisms in ES cells (Watanabe et al., 2006; McLean et al., 2007). Consistent with this result, IGF-I was also reported to co-operate with basic fibroblast growth factor in the self-renewal of human ES cells (Bendall et al., 2007).

Similar to our work, the IGF-I/IGF-IR was associated with the expression of HIF-1α and/or HIF-2α in cells of somatic lineage or cancers (Akeno et al., 2002; Carroll and Ashcroft, 2006; Catrina et al., 2006). Interestingly, in our study, we found that knockdown of HIF-2α decreased IGF-IR expression in AP+ GSCs (Fig. 6D and E). This result is consistent with results demonstrating that human cancers converging at the HIF-2α oncogenic axis require IGF-IR activation (Franovic et al., 2009). HIF-2α is known to directly regulate Oct-4 and c-Myc (Covello et al., 2006; Keith and Simon, 2007); therefore, the signaling regulators of HIF-2α expression will also mediate both Oct-4 expression and proliferation. Our data demonstrate that niche hypoxia not only stabilizes HIF-2α by preventing proteosomal degradation, but also stimulates the expression of IGF-1 and IGF-IR (Fig. 4). Autocrine IGF-IR activation increased the HIF-2α expression (Fig. 5), while the up-regulation of HIF-2α resulted in up-regulation of IGF-IR expression, both of which can be up-regulated by hypoxia-induced IGF-1. Together these results strongly suggest that niche hypoxia strengthens an endocrine loop of IGF-1/IGF-IR-HIF-2α signaling involved in the stimulation of proliferation and in the maintenance of Oct-4 in early mouse AP+ GSCs (Fig. 7).

Conclusion
This study provides the first evidence, using primary AP+CD49f+ GSCs and serum-free medium, to demonstrate how niche hypoxia co-operates with its associated IGF-IR-PI3K/Akt-mTOR-HIF-2α signaling loop to stimulate cell proliferation and maintain Oct4 levels in early AP+ GSCs. Our finding provides important insight into the niche hypoxia and its associated endocrinial signal in early germ cell development.

Supplementary data
Supplementary data are available at http://molehr.oxfordjournals.org/.

Authors’ roles
T.Y.L. designed and performed the experiments, analyzed and interpreted the data, manuscript preparation and gave final approval. M.H.L. designed and performed the experiments, analyzed and interpreted the data, P.C.W., H.L.C., Y.L.W., J.H.C., Y.K.H., S.Y.G. contributed to perform experiments and data analysis. H.N.H. contributed to data interpretation and manuscript revision. Y.H.H. conceived the study, analyzed and interpreted the data, wrote the paper and gave final approval.

Funding
This work was supported by grants (NSC96-99-3111-B-038-001, NSC100-2321-B-002-048, NSC(100-102)-2321-B-038-001 and NSC99-2628-B-038-009-MY3) from the National Science Council, Taiwan, and by MOHW103-TD-B-111-01 from Comprehensive Cancer Center of Taipei Medical University.

Conflict of interest
The authors of the study have no conflict of interests to report.

References