Hypoxia Increases Metastatic Potential of Osteosarcoma Cells in vitro
Li D, DiResta G, Matsubara T, Kakunaga S, +Healey JH
Memorial Sloan-Kettering Cancer Center, New York, New York healeyj@mskcc.org

Introduction
Hypoxia within the cancers is an independent marker of a poor prognosis in many solid tumors. HIF-1α expression, a marker for tissue hypoxia, correlates with metastatic disease in osteosarcoma [1]. The mechanism is unknown. We simulated hypoxia in an in vitro cell culture system for two human cell lines. U2OS and MG-63 were cultured in two growth incubators whose gas mixtures were independently adjusted to 2% O₂, simulating hypoxia; and 21% O₂ simulating normoxic conditions. We hypothesized that 2% O₂ increases the metastatic potential of human osteosarcoma cells.

Materials and Methods. Cell lines and cell culture. U2OS, and MG-63 were obtained from the ATCC (Manassas, VA). U2OS cells were cultured in McCoy’s 5A medium supplemented with 10% fetal bovine serum, pen/strep. MG-63 cells were cultured in ATTC-formulated Eagle’s Minimum Essential Medium supplemented with 10% fetal bovine. For all experiments both cell lines were used at passages 4-10. Cells were cultured under normoxic conditions (21% O₂, 5% CO₂, balance N₂) within a NuAire IR Sensor Stacked AutoFlow NU-8700 incubator and under hypoxic conditions (2% O₂, 5% CO₂, balance N₂) within a NuAire US AutoFlow NU-4950 incubator. Cell Proliferation, Cycle & Apoptosis Assays. Both cell lines were seeded in T75 flasks under normoxic and hypoxic conditions. For cell proliferation, cells are harvested at time points: 0, 24, 48 and 72 hours. For cell cycle, cells are harvested at 5 time points: 6, 12, 24, 48, and 72 hours. The media was decanted and cells were harvested from culture flasks by trypsinization 5×10³ cells were incubated for 15 minutes with 0.5 ml PI/RNASE staining buffer (BD Pharmingen) at room temperature and Flow Cytometry analyzed with a FACS Calibur instrument (Becton Dickinson).

Apoptosis analysis was per the Roche Annexin-V-FLUOS Staining Kit protocol. Test validity was checked by inducing apoptosis in OS cells with ultraviolet (UV) irradiation at 254nm at a dose of 240 mJoules/cm², using a Stratagene Stratalinker UV Crosslinker (Stratagene, La Jolla, CA). MG-63 cells were cultured for another 7 and 24 hours and U2OS cells were cultured for 4.5 and 5.5 hours.

Boydenn Chamber Migration & Invasion assays. Migration and invasion assays were performed with Boyden chambers (BD Biosciences) with 8-Am filters, and BD BioCoat™ Matrigel™ Invasion Chambers. MG-63 Cells (1.5×10⁵) and U2OS cells (1×10⁵) were seeded in the upper chamber compartment containing media with 1% FBS; for 24 h at 37°C and the cells present in the lower chamber were counted. All experiments were done twice.

cDNA array screen. U2OS cells grown under different conditions were harvested with Trizol. cDNA array analysis was performed by the MSKCC Genomics core facility. Data was analyzed using “supervised gene selection”. For each comparison between the two incubator O₂ gas conditions the present/absent filter, replica noise filter, and T-test were applied. RT-PCR was used to confirm the change of RNA expression level of selected genes. The RNA from U2OS cells grown under normoxic and hypoxic conditions was harvested and 1 ug of total RNA was reverse-transcribed using the Thermoscript RT-PCR system (Invitrogen) at 52°C for 1 hour. cDNA was used in a Q-PCR reaction using an iCycler (Bio-Rad), and pre-designed TaqMan ABI gene expression assay. Amplification was carried for 40 cycles (95 °C for 15 secs., and 60°C for 1 min.). All were done x3

Results
Neither cell line showed significantly different proliferation rates under normoxic and hypoxic conditions. However U2OS cells showed higher total viable cell counts under hypoxic conditions at 72 hrs than cells grown under normoxic conditions (p=0.075). Under normoxic conditions in the G1 phase, the MG-63 cell line had 10% more cells at all the time points: 6, 12, 24, 48 and 72 hours than the hypoxic group. The U2OS cell line showed the same trend. More cells in the G1 phase is considered an arrest of the cell cycle. In both cell lines, hypoxic conditions slightly inhibited cell cycle progression. Both MG-63 and U2OS cells grown under hypoxic and normal conditions displayed similar apoptotic and necrotic cell number, and under hypoxic conditions showed greater resistance to UV radiation. The Annexin V and PI assay show that hypoxic U2OS had fewer apoptotic cells and MG-63 had fewer necrotic cells compared to normoxic conditions. This finding suggests that hypoxia enhances the survival of both cell lines.

Migration and invasive assay. Both cell lines had significantly more migration ability under hypoxia (Figure) MG-63, p=0.0002; U2OS, p=0.0006). But the invasive index was statistically equal.

cDNA array and RT-PCR From the cDNA array screen, we observed PFKFB4, BNIP3, UPK1A and ANKPD37 to be up-regulated under hypoxic conditions, while HMOX1 and HES1 were observed to be down-regulated. The RT-PCR results confirmed these findings for all genes except ANKPD37.

Discussion
Hypoxia is a powerful selective factor for solid tumors [2]. Hypoxia is associated with tumor progression, increased aggressiveness, enhanced metastatic potential and poor prognosis [1]. Our study was done at a pO₂ of 2%, to simulate a mild in vivo hypoxic condition and to give the cultured cells greater opportunity to adapt and evolve rather than die.

Both cell lines displayed a more invasive phenotype in a 2% O₂ atmosphere. Unlike extreme hypoxia (<0.2% O₂), proliferation is not suppressed at mild hypoxia. On the contrary, the proliferation rate of U2OS cell line under 2% O₂ level was moderately increased over the 21% O₂ level. Flow cytometry showed more cells in phase G1 when cultured in a 2% O₂ atmosphere, suggesting that this gas level could induce a slight cell cycle arrest. Though the mechanism is not clear, both cell lines showed more ability to resist UV radiation under 2% O₂ level. Cells exposed to severe and prolonged hypoxia may undergo apoptosis, whereas cells exposed to acute and mild hypoxia may adapt to this environmental stress and survive. Reduced apoptosis and increased migration suggest that mild hypoxia promotes metastatic potential of these OS cells.

PFKFB4, BNIP3 and UPK1A expression was up-regulated under hypoxia while HMOX1 expression was down-regulated. HES1 was down-regulated up to 48 hours. These hypoxia induced changes are enticing therapeutic targets.

Conclusions
Under the 2% O₂ conditions, 1.both cell lines had a slowed cell cycle and increased migration, 2.the cells were more tolerant to UV-induced apoptosis 3.these phenotypes increase metastatic potential.

References