Targeting Aurora Kinase A Inhibits Hypoxia-mediated Neuroblastoma Cell Tumorigenesis

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Abstract. *Background/Aim: It is unknown whether hypoxia regulates aurora kinase A (AURKA), a serine/threonine kinase, in neuroblastoma to stimulate cell growth or migration. We sought to determine whether AURKA mediates hypoxia-induced regulation of neuroblastoma tumorigenicity. Materials and Methods: Human neuroblastoma BE(2)-C cells were treated with $CoCl_2$, a chemical hypoxia mimetic, and MLN8237, a pharmalogical inhibitor of AURKA, to assess cell viability, colony formation and transwell migration. Focal adhesion kinase (FAK) expression was analyzed after silencing of AURKA under normoxic vs. hypoxic conditions. Results: Hypoxia up-regulated expression of AURKA mRNA and protein. $CoCl_2$ stimulated cell proliferation and migration, while inhibiting colony formation. MLN8237 reduced colony formation and cell migration. Silencing of AURKA reduced expression of FAK and pFAK under normoxia and hypoxia. Conclusion: Hypoxia positively regulates AURKA expression. Hypoxia-induced stimulation of colony formation and migration is, in part, mediated by AURKA. These findings establish that AURKA is a critical regulator of hypoxia-mediated tumor progression in neuroblastoma.*

Neuroblastoma is an aggressive extracranial solid tumor that affects infants and children. It is characterized by several features, such as rapid tumor growth and frequent metastasis. The development of multi-modality therapy has had some impact on the treatment of neuroblastoma; however, patients with advanced-stage tumors are difficult to cure, with a dismal long-term survival of 40%. One factor known to contribute to aggressive tumor behavior is a hypoxic tumor environment (1).

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The disorganized vasculature often found in solid tumors prevents the supply of consistently oxygenated blood. The hypoxic microenvironment selects-out tumor cells by up-regulating certain factors, such as hypoxia-inducible factor (HIF)-1α, which then allow tumor cells to adapt and survive under these conditions. HIF-1α does not undergo degradation, and becomes stabilized under hypoxic conditions, leading to the activation of downstream targets, such as vascular endothelial growth factor (VEGF) and carbonic anhydrase IX (2, 3). Hypoxia is known to cause neuronal changes in neuroblastoma that promotes an aggressive tumor phenotype (3). Additionally, up-regulation of HIF-1α is associated with clinically more aggressive neuroblastoma and poor overall survival (4).

Overexpression of aurora kinase A (AURKA), a member of a family of mitotic serine/threonine kinases, has been associated with disease progression and, subsequently, poor prognosis in neuroblastoma (5). Our laboratory has shown that it is involved in the regulation of angiogenesis and tumorigenicity in neuroblastoma (6). The function of AURKA under hypoxic conditions in neuroblastoma has not been extensively studied but others have demonstrated that the transcriptional regulation of AURKA is increased by both hypoxia and HIF-1α (7). Furthermore, in renal cell carcinoma, HIF-1α stabilization induces AURKA expression and promotes an aggressive tumor phenotype (8). These studies clearly highlight that hypoxia and HIF-1α stabilization can induce the activity and expression of AURKA, which subsequently results in sustaining a malignant phenotype *via* features such as cell proliferation and tumor metastasis.

Given the lack of research describing the behavior of AURKA under hypoxia in neuroblastoma, we sought to determine whether hypoxic conditions altered its regulation and if it mediated hypoxia-induced tumorigenicity.

Materials and Methods

Materials. Primary antibodies against AURKA and cell lysis buffer were obtained from Cell Signaling Technology (Beverly, MA,

USA); focal adhesion kinase (FAK) and phosphorylated (p)-FAK from BD Biosciences (San Jose, CA, USA). Antibody against β-actin, fetal bovine serum (FBS) and cobalt (II) chloride hexahydrate were from Sigma (St. Louis, MO, USA). NuPAGE Novex 4-12% Bis–Tris Gel and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA, USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies against mouse and rabbit IgG were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Chemiluminescence (ECL) HRP substrate were purchased from Millipore (Billerica, MA, USA) and Perkin Elmer (Alameda, CA, USA). MLN8237 was from Selleckchem (Houston, TX, USA).

Cell culture, plasmids and transfection. Human neuroblastoma cell line, BE(2)-C, was purchased from the American Type Culture Collection (Manassas, VA, USA). Primary neuroblastoma cell line, JF, was a gift from Dr. Jason Shohet from the Texas Children's Hospital. Cells were maintained in RPMI-1640 medium with L-glutamine (CellGro Mediatech, Inc. Herndon, VA, USA) supplemented with 10% FBS. Cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Hypoxic conditions were maintained in an incubation chamber that contained 1% O_2, 6% CO_2 and 93% N. Short hairpin (sh)RNA against AURKA (shAURKA) and non-targeting control (shCON) were purchased from Sigma-Aldrich. For transfection, cells were plated in 6-well plates and transfected with shRNA using Lipofectamine 2000 as per the manufacturer's protocol.

Cell viability and soft agar colony-formation assays. Cells were seeded onto 96-well plates at a density of 1×10^4 cells per well in RPMI culture media with 10% FBS. After allowing 24h for attachment, the cells were treated with control media (RPMI culture media with 10% FBS) or medium with cobalt (II) chloride hexahydrate (100 μM or 200 μM) in triplicates. The cell number was assessed using Cell Counting Kit-8 (Dojindo Molecular Technologies, Rockville, MD) for cell viability after 24 and 48 hours. For soft agar assay, cells were trypsinized and resuspended in RPMI 1640 media containing 0.4% agarose and 10% FBS. Cells were overlaid onto a bottom layer of solidified 0.8% agarose in RPMI-1640 media containing 5% FBS, at a cell density of 3×10^3 cells per well in a 12-well plate. Cells were treated with control media or a combination of medium with MLN8237 (50, 100 or 500 nM) and cobalt (II) chloride hexahydrate (100 μM) and incubated for three weeks. Colonies were photographed under an inverted microscope Olympus CKX41 (Olympus, Center Valley, PA, USA), and quantified.

Migration assay. Cells were initially seeded in 6-well plates. After 24 h of incubation, the media were changed and individual wells were treated with either cobalt (II) hexahydrate (0 μM), cobalt (II) chloride hexahdryate (100 μM), cobalt (II) chloride hexahdryate (100 μM) plus MLN8237 (500 nM) or MLN8237 (500 nM) alone. Subsequently, transwell filters (8 μm) were coated on the lower side with 5 μg/ml collagen type I (BD Biosciences) overnight and then blocked with 2.5% BSA/phosphate buffered saline (PBS) for 1 h. The previously treated cells (1×10^5) were trypsinized, collected in serum-free media and added to the upper side and incubated for 4 h. Cells were fixed with 4% paraformaldehyde, stained with 4',6-diamidino-2-phenylindole (DAPI) and counted. The assay was performed in duplicates, and counting was from five randomly selected microscopic fields (×200 magnification).

Immunoblotting and reverse transcription and real-time polymerase chain reaction (PCR). BE(2)-C cells were seeded in 6-well plates and incubated for 24 h. Cells were then treated with cobalt (II) chloride hexahydrate (100 μM) for 0 and 30 min and 1, 2, 4, and 8 h. Whole-cell lysates were prepared using cell lysis buffer with 1 mM PMSF and incubated on ice for 30 to 60 min. Total protein (50 μg/lane) was resolved on NuPAGE Novex 4-12% Bis–Tris gels and electrophoretically transferred to polyvinylidene difluoride membranes. Non-specific binding sites were blocked with 5% milk in TBST (120 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween 20) for 1 h at room temperature (RT) or overnight at 4˚C. Target proteins were detected by using rabbit or mouse anti-human antibodies (1:500 to 1000 dilution) for 3 h at RT or overnight at 4˚C. The membranes were washed three times and incubated with secondary antibodies (1:5000 dilution) conjugated with HRP. Immune complexes were visualized using the enhanced ECL system. Equal loading and transfer were confirmed by blotting the same membrane with β-actin antibody. Data are representative of three independent experiments.

Prior to real-time PCR analysis, BE(2)-C cells were seeded in 6-well plates and incubated for 24 hours. Cells were then treated with cobalt (II) chloride hexahydrate (100 μM) for 0 and 30 minutes. Total RNA was isolated using RNAqueous™ kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Isolated RNA was used to synthesize cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Austin, TX, USA). *AURKA* mRNA levels were measured by quantitative real-time PCR using SsoFast™ EvaGreen Supermix with CFX96 Real-Time PCR System (Bio-Rad, Hercules, CA, USA).

Statistical analysis. Data were expressed as means±SEM; statistical analyses were performed using one-way analysis of variance for comparisons between the treatment groups. A *p*-value less than 0.05 was considered significant.

Results

Hypoxia increased mRNA and protein expression of AURKA and cell growth in neuroblastoma. We first began our study by identifying what effect hypoxia had on the mRNA levels of AURKA. Human neuroblastoma BE(2)-C cells were treated with $CoCl_2$ for 30 and 60 min. As compared to controls (10% FBS RPMI medium), *AURKA* mRNA levels increased by approximately 1.5-fold after $CoCl_2$ treatment (Figure 1A). This activity level rapidly diminished by 60 min to less than the baseline activity observed in the control cells. We subsequently examined the protein expression of AURKA after treatment with $CoCl_2$ (100 μM), a hypoxia mimetic, over a time course (0, 30 min and 1, 2, 4, 8 h). By 30 min, there was an increase in protein expression of AURKA compared to 0 min (Figure 1B); this peaked at 1 h, and then returned to baseline by 2 h. In addition to having a positive effect on the protein expression of certain genes, hypoxia has been shown to promote cell growth and a survival advantage to cells that have lost their apoptotic potential (9). The viability of neuroblastoma cells was assessed after treatment with $CoCl_2$ (100 μM). Cell

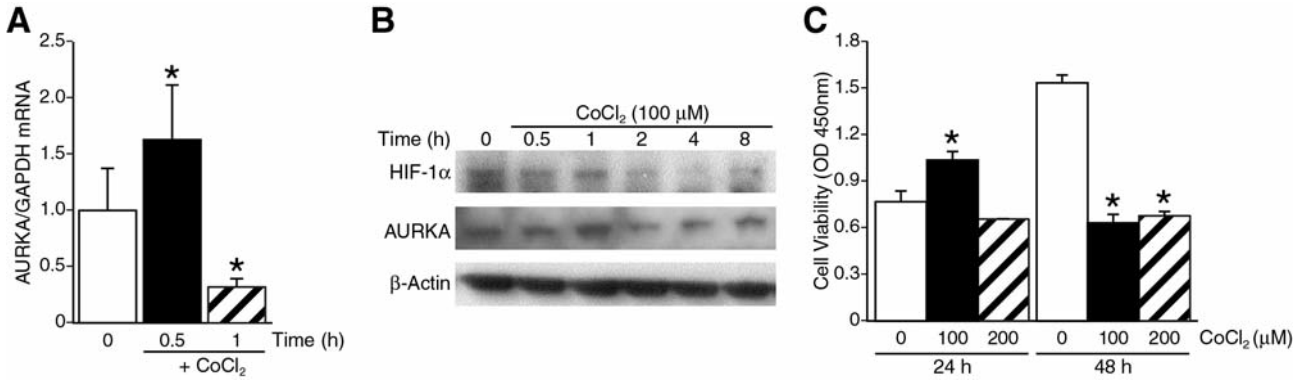

Figure 1. *Effect of hypoxia on aurora kinase A (AURKA) expression and cell growth in BE(2)-C cells. A: AURKA mRNA levels after 30 and 60 min exposure to $CoCl_2$ treatment as compared to controls. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. B: AURKA protein expression was examined by immunoblotting after $CoCl_2$ treatment over a time course. HIF-1α expression demonstrates specificity of treatment to $CoCl_2$, a hypoxia mimetic. β-Actin was used as a loading control. C: $CoCl_2$ treatment (100 μM) increased cell viability as measured by CCK-8 kit at 24 h in comparison to controls (10% FBS RPMI medium without $CoCl_2$). This effect was attenuated at 48 h of CoCl2 treatment with both 100 and 200 μM dosages (Data represent mean±SEM; *p<0.05 vs. control, n=3 for each treatment group).*

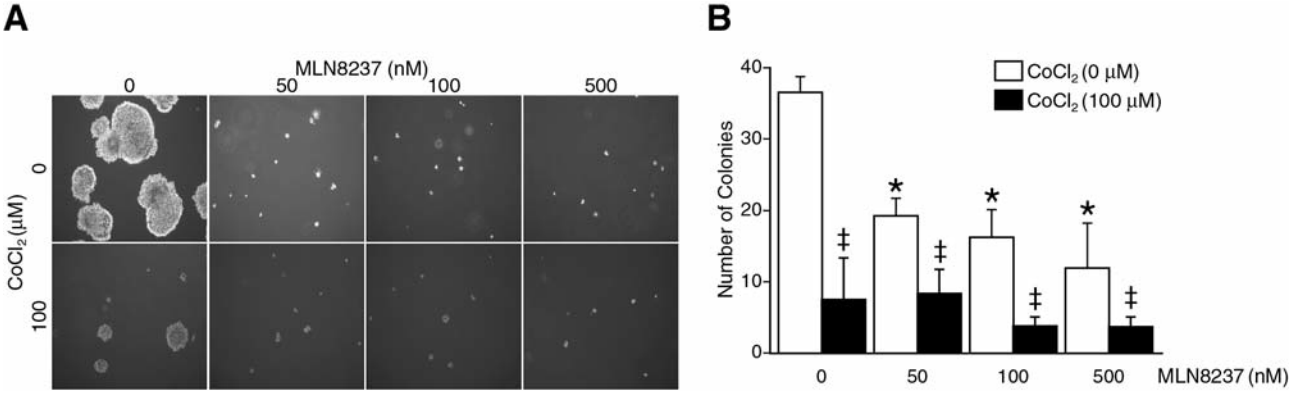

Figure 2. *Inhibition of aurora kinase A (AURKA) reduced soft agar colony formation by BE(2)-C cells irrespective of $CoCl_2$. A: Increasing concentrations of MLN8237, a specific chemical inhibitor of AURKA, (50, 100, 500 nM) reduced soft agar colony formation (top row) as compared to controls (10% FBS RPMI media). Treatment with $CoCl_2$ (100 μM) reduced the size and number of individual colonies formed (bottom row), as compared to controls. B: Quantitative representation of soft agar colonies in response to treatment with MLN8237 with or without $CoCl_2$ (mean ± SEM; *p<0.05 vs. control, ‡p<0.05 vs. without $CoCl_2$, n=3 for each treatment group).*

proliferation was significantly increased at 24 h compared to control cells (Figure 1C). By 48 h, we observed a reversal of these changes with a large increase in control cells compared to cells treated with $CoCl_2$. These findings indicate that acute hypoxia activates AURKA in neuroblastoma cells. In the case of mRNA transcription, this effect is quickly lost and subsequently decreased. Although protein expression is also slightly increased, it subsequently plateaus. Hypoxia may initiate a stress response resulting in the acute and transient activation of *AURKA* mRNA transcription and protein expression to promote cell proliferation and survival, as this gene is known to do in neuroblastoma (10).

Inhibition of AURKA reduced soft agar colony formation irrespective of $CoCl_2$. Colony formation is an *in vitro* measure of anchorage independence, known to be an indicator of a tumor's metastatic potential *in vivo*. As compared to untreated controls, cells treated with increasing concentrations of MLN8237 (50, 100 and 500 nM), a specific inhibitor of AURKA, demonstrated a decrease in size and number of colonies formed (Figure 2A; top row). Under treatment with $CoCl_2$, colony size and number remained diminished in comparison to that of the control group (Figure 2A; bottom row). The observed reduction in colony formation was statistically significant (Figure 2B). Based on these findings,

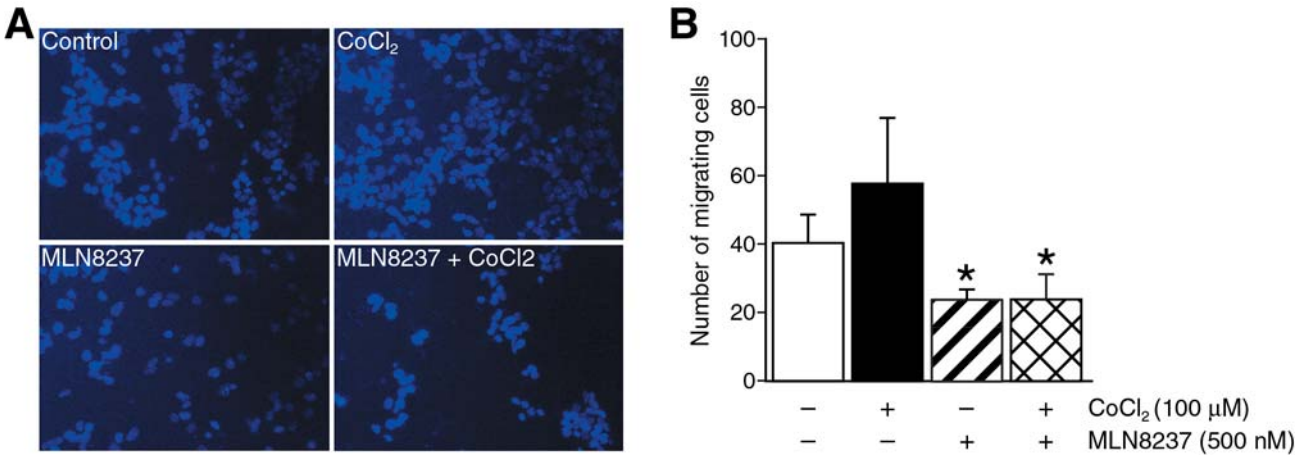

Figure 3. *Inhibition of aurora kinase A (AURKA) reversed hypoxia-mediated increases in JF cell migration. A: Cell migration of primary human neuroblastoma JF cells increased with $CoCl_2$ treatment (top right) as compared to controls (10% FBS RPMI medium). MLN8237, a specific chemical inhibitor of AURKA, reduced cell migration with and without $CoCl_2$. Representative images of staining for counting are shown (×200 magnification). B: Quantitative representation of JF cell migration (*p<0.05 vs. control).*

we speculate that AURKA is involved in mediating anchorage independence, and therefore may be critical in promoting tumor metastasis in neuroblastoma.

Inhibition of AURKA reversed hypoxia-mediated increase in cell migration. Hypoxia is known to promote changes in the tumor microenvironment that can lead to an increase in cancer cell migration and invasion (11). We plated primary neuroblastoma cell line, JF, in transwell plates coated with collagen I. We found increased JF neuroblastoma cell migration after $CoCl_2$ (100 μM) treatment (Figure 3A; top right) and significant attenuation of this migration after treatment with MLN8237 (500 μM) (Figure 3; bottom row), compared to controls. These results show that hypoxia induces a stimulatory effect on neuroblastoma cell migration and that AURKA is necessary for this process to occur, given the decrease observed after AURKA inhibition with and without the presence of $CoCl_2$. Our findings highlight another way in which AURKA could be involved in hypoxia-mediated tumor progression.

Silencing AURKA inhibited hypoxia-induced increase in FAK and p-FAK. FAK is a non-receptor cytoplasmic tyrosine kinase known to regulate cellular adhesion, growth, survival and migration (12). In cancer cells, up-regulation of FAK is associated with increased invasiveness and metastasis of tumor cells (13). AURKA has been shown to regulate key factors of cell migration and adhesion (14). Given this, and our data demonstrating reduced *in vitro* neuroblastoma cell migration with inhibition of AURKA, we next attempted to identify the effect of silencing AURKA on FAK expression. We used stably-transfected AURKA-silenced BE(2)-C cells (shAURKA) established in our laboratory (2, 6). Under normoxic (O_2, 21%) and hypoxic (O_2, 1.1%) conditions, silencing of AURKA resulted in decreased expression of FAK and p-FAK, compared to controls (shCON) (Figure 4). Cells were cultured under hypoxia for 48 h. We also noted that the expression of p-FAK was significantly increased compared to its expression under normoxic conditions. These results indicate that hypoxia induces neuroblastoma cell migration and positively effects the expression of the phosphorylated form of FAK, which we have previously shown is associated with metastases in neuroblastoma (15). Additionally, these findings suggest that AURKA may be an upstream regulator of hypoxia-induced migration *via* the regulation of FAK.

Discussion

The oncogenic role of AURKA in neuroblastoma and in other types of cancer is well established, in terms of its effect on mitosis, cell proliferation and tumorigenesis. Yet despite this extensive research, very little is known about the role or behavior of AURKA in mediating tumor progression under hypoxic conditions for neuroblastoma. We created hypoxic conditions *via* chemical treatment with $CoCl_2$ (a hypoxia mimetic) and a hypoxic chamber to examine its effect on *AURKA* transcription and protein expression and several characteristics associated with tumor progression including cell growth, soft agar colony formation and cell migration. $CoCl_2$ treatment resulted in a rapid increase in *AURKA* mRNA and protein levels, and cell proliferation. Both MLN8237, a chemical inhibitor of AURKA, and $CoCl_2$ treatment resulted in decreased colony formation. Hypoxia

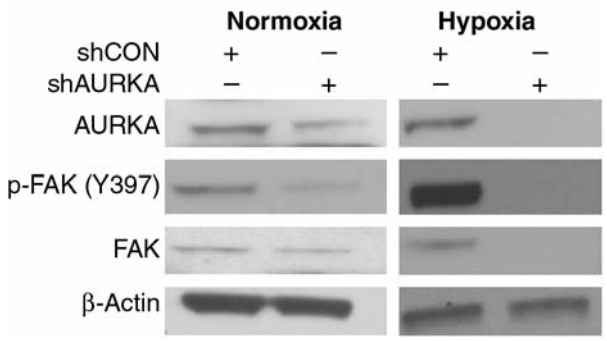

Figure 4. *Silencing of aurora kinase A (AURKA) using shAURKA inhibited hypoxia-induced increases in focal adhesion kinase (FAK) and p-FAK. Under normoxic conditions (21% O_2; left panel), AURKA, FAK and p-FAK expression was decreased in BE(2)-C/shAURKA in comparison to control cells, BE(2)-C/shCON, as assessed by immunoblotting. After 48 h of hypoxia (1.2% O_2; right panel), the expression of total FAK and p-FAK in BE(2)/shCON cells increased as compared to those same cells collected under normoxic conditions. AURKA, FAK and p-FAK expression decreased in BE(2)-C/shAURKA in comparison to control cells, BE(2)-C/shCON. β-Actin was used as a loading control.*

induced cell migration but these effects were attenuated by MLN8237 treatment. Finally, after silencing AURKA, we were able to demonstrate a decreased expression of FAK and p-FAK, proteins known to be critical in cell migration and tumor metastasis, under normoxic and hypoxic conditions. In the present study, we found that hypoxia activates AURKA expression in neuroblastoma cells. Overall, we introduce the concept that AURKA is a critical regulator of hypoxia-mediated tumor progression in neuroblastoma.

Hypoxia is a known stressor in cancer cells. As the hypoxic tumor microenvironment evolves, genetic changes occur to sustain tumor cell viability under the stresses of cycling between normoxic and hypoxic conditions, most notably the stabilization of HIF-1α. Our study does not establish a direct link between HIF-1α and AURKA with respect to transcriptional regulation or post-translational protein stabilization, nor the changes seen in cell proliferation, migration or colony formation. Rather, we found that hypoxia clearly has a direct positive effect on the up-regulation of *AURKA* and the phenotypic changes seen in cell growth and migration in neuroblastoma.

The changes seen in the mRNA levels of *AURKA* and protein expression under hypoxic conditions were transient, further suggesting this gene's pattern of behavior may vary under acute *vs*. chronic hypoxia. Another tumor of neuroendocrine origin, prostate cancer, has been shown to exhibit similar behavior under hypoxic conditions, with chronic hypoxia (≥24 h) inducing increased cell death and reduced cell proliferation (16). Based on the results we have shown, it cannot be said with any certainty that HIF-1α mediates the changes seen in AURKA. However, we speculate given the wide range of downstream targets that HIF-1α has been shown to effect, under hypoxic conditions, *AURKA* may be one of those genes in neuroblastoma.

Hypoxia promotes the migration and invasiveness of tumor cells *via* the regulation of genes that control the breakdown of the extracellular matrix and those genes that are responsible for cellular adhesion and migration (17). Soft agar colony formation, a measure of anchorage independence, and the migration assay are two *in vitro* studies that can give us insight into what early stages of the migration/invasion cascade may look like. Furthermore, both colony formation, and migration share regulation *via* a common signaling cascade involving SRC, another non-receptor tyrosine kinase, and FAK (12, 18). We noted a decrease in colony formation and migration that were mediated through AURKA inhibition *via* treatment with MLN8237. In light of this, we speculated that AURKA might regulate genes involved in this pathway.

Activation of FAK can occur *via* auto-phosphorylation at tyrosine site 397 (Y397), a critical event linking this kinase to signaling pathways that regulate cell proliferation, survival, migration and invasion. SRC can also phosphorylate and activate FAK at Y397 (19). A recent study in our laboratory has shown that FAK acts as a downstream target of gastrin-releasing peptide receptor signaling, to regulate liver metastasis in neuroblastoma, and that its overexpression leads to increased cell growth and anchorage independence (15). In addition to gastrin-releasing peptide receptor signaling, hypoxia is known to increase FAK expression and result in increased migration of cells (20, 21). In recent years, others have also described the regulation of FAK *via* AURKA, citing that after knockdown of *AURKA*, there was a decrease in FAK expression and invasiveness of cancer cells both *in vivo* and *in vitro* (14). In our study, hypoxia increased protein expressions of FAK and p-FAK (Y397) in BE(2)-C/CON cells, compared to BE(2)-C/CON cells cultured under normoxic conditions. Moreover, with silencing of *AURKA*, there was a significant decrease in the expression of both total FAK and p-FAK (Y397) under both normoxia and hypoxia. AURKA is known to phosphorylate proteins in its role as an oncogene (22). It could be that it functions in this same capacity to phosphorylate FAK, leading to its activation and regulation.

We have identified a new potential role for AURKA in mediating tumor progression of neuroblastoma cells under conditions of hypoxia. The sum of this work proposes that hypoxia has a positive effect on cell proliferation and migration. In addition to this, inhibiting AURKA attenuated phenotypic changes seen in colony formation and migration. Finally, we propose a new interaction between AURKA and FAK with the former activating FAK through its kinase activity. If true, this would explain the decrease in migration and colony

formation after treatment with MLN8237. Furthermore, since these preliminary data seem to indicate AURKA may be central to pathways involved in anchorage independence and tumor migration, it establishes a rationale for targeting AURKA in the treatment of metastatic neuroblastoma. This study also leaves open a new avenue of investigation into how hypoxia regulates *AURKA*, since we have yet to define the mechanism of how this oncogene is up-regulated under these conditions. Altogether, we provide further evidence as to why AURKA should continue to be an important therapeutic target in treating aggressive neuroblastoma.

Acknowledgements

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