Determining the role of hypoxia-induced autophagy in the survival of tumor-promoting macrophages

Candidacy Exam Proposal

November 30, 2016
Ovarian Cancer Background

Epithelial Ovarian Cancer Progression

- The current standard of care starts with surgical tumor de-bulking followed by platinum-based chemotherapy.
- Most patients develop platinum-resistant (cisplatin) ovarian cancer, which is currently considered incurable.
- The tumor microenvironment has been linked to the ineffectiveness of chemotherapy treatments and the promotion of tumor progression.
The tumor microenvironment is a complex network of multiple cell types both localized and recruited.
Tumor cells recruit and differentiate circulating monocytes into macrophages

- **M1-polarized** tumor associated macrophages (TAMs) are the most abundant immune cell found within the ovarian cancer TME.

- **M2-polarized** TAMs are more abundant in ovarian cancer TME than other cancer types, and high levels are associated with a poorer prognosis for ovarian cancer patients.

- **Mainly anti-cancer**
  - M1-polarized
  - Inflammatory
  - Immune stimulating

- **Mainly Pro-cancer**
  - M2-polarized
  - Wound healing
  - Immune suppressive
  - Pro-angiogenic
Cross-talk between TAMs and tumor cells promote tumorigenesis

Promote Angiogenesis

Endothelial cells

VEGF
TGFβ
IL-8
CXCL8

M2-polarized TAMs

Recruit and polarizes monocytes

CCL2
CSF-1
IL-4
IL-10
IL-13

Tumor cell

Promote Invasion

IL-10
IL-12
CD163
MMP-9
CCL22
IL-6

Immune suppression

PD-L1
CCL22
TGFβ

T cell
Hypoxia is a key feature of the tumor microenvironment.

- In normoxia the transcription factor hypoxia-inducible factor alpha-1 (HIF-1α) is degraded by proteasomes.
- Hypoxia-induced autophagy in tumor cells is mediated by HIF-1α, and helps to maintain their energy balance.
Autophagy (Self-eating) is an intracellular recycling mechanism.

Autophagy is an evolutionarily conserved catabolic process that is used to deliver cytoplasmic materials, including damaged organelles, misfolded or unused proteins, to the lysosome for degradation.

Mizushima N, Komatsu M. Cell 2011;147(4) 728-74
Activation of autophagy in cells

Nutrients availability and certain growth factors block the activation of autophagy.

Nutrient depletion and cellular stress can activate autophagy.
The complex interplay between autophagy and apoptosis in hypoxia

The Bcl2 family comprises anti-apoptotic (pro-survival) and pro-apoptotic members.

- In response to hypoxia, HIF-1α blocks p53 transcriptional activation of pro-apoptotic genes.
- HIF-1α transcribes pro-autophagy genes that release the breaks on autophagy.

Proposed model for p53/HIF-1α interaction
Evidence of autophagy as a pro-survival mechanism

- *Atg5*-deficient mice die in neonatal stages due to depletion of amino acids and metabolic insufficiency.

- While overexpression of *Atg5* in mice extends their lifespan.

- In cancer cells, metabolic stress induces autophagy, which is sustained when apoptosis is blocked.

- Stress-induced autophagy in mesenchymal cells and provides support to breast tumor cells both *in vitro and in vivo* through production of anti-apoptotic factors.

- Hypoxia induced autophagy in cardiomyocytes significantly increases cell viability and helps to overcome hypoxic cell injury.
Induction of autophagy is essential for monocyte-macrophage differentiation

- Monocytes cultured in GM-CSF and CSF-1 (not shown) differentiated into macrophages.
- Autophagy inhibitors blocked the differentiation and lead to death of monocytes.

Autophagy as a mechanism of survival of mature macrophages has yet to be elucidated.
Hypothesis: Hypoxia-induced autophagy has a dual function in EOC, where it mediates the survival of macrophages and aids in tumor progression.

Rationale: Revealing mechanisms of tumor support in ovarian cancer, may aid in the development of novel therapeutic targets for ovarian cancer patients.
Specific Aims

Aim 1. Examine the role of hypoxia in the promotion of tumorigenesis through macrophage signaling.

-I hypothesize that M2-polarized macrophages will increase the secretion of tumor invasion and angiogenesis promoting factors in response to hypoxia.

Aim 2. Investigate the effect of hypoxia on the induction of autophagy in M2-polarized macrophages.

-I hypothesize that elevated levels of HIF-1α in macrophages in response to hypoxia, will lead to the activation of pro-autophagic pathways and the down-regulation of apoptotic activity leading to cell survival.
Aim 1. Examine the role of hypoxia in the promotion of tumorigenesis through macrophage signaling.

Aim 1a. Determine if hypoxia leads to increased production of ovarian cancer promoting factors, VEGF and MMP-9 by M2-polarized macrophages.

Aim 1b. Elucidate the effect of hypoxia on M2-polarized macrophage promotion of ovarian cancer metastasis.

Aim 1c. Determine if inhibition of HIF-1α diminishes the effect of hypoxia on M2-polarized macrophage signaling.
3D spheroid cell culture model

- *In vitro* 3D cell culture models are widely recognized as more physiologically relevant systems compared to 2D formats.

- The 3D models reflect more accurately the complex *in vivo* microenvironment.

U-shaped well bottoms, with low attachment coating.
Approach: Aim 1a Investigate the effect of hypoxia in the transcriptional activation of genes involved in cell survival and tumor-promotion by macrophages
AIM 1a. HIF-1α expression increases in both macrophages and ovarian cancer cells in response to hypoxia.
AIM 1a. Secretion of MMP-9 by macrophages co-cultured with ovarian cancer cells will increase in response to hypoxia.

*Similar results expected for VEGF.*
Approach: Aim 1b. Elucidate the effect of hypoxia on M2-polarized macrophage promotion of ovarian cancer metastasis.

Culture 3D cell spheroids

- Ovarian cancer cells alone
- Macrophages + Ovarian cancer cells

Add matrigel basement membrane
Control (no matrix)

Matrigel Tumour spheroid

3D Tumor cell Invasion Assay

Incubate 3D spheroid co-cultures in hypoxia or normoxia with matrigel for 48hrs

Confirm secretion of MMP-9 by ELISA

Confirm HIF-1α expression by western blot using control cells

Quantify by microscopy
Aim 1b. Increased production of MMP-9 by macrophages in response to hypoxia leads to increased invasion of ovarian cancer tumor cells.
Aim 1b. Increased production of VEGF by macrophages in response to hypoxia leads to increased migration of endothelial cells.

- Macrophages alone
- Ovarian cancer cells alone
- Macrophages + Ovarian cancer cells

Migrated endothelial cells in response to secreted growth factors:

- Normoxia
  - 0hrs
  - 48 hrs

- Hypoxia
  - 0hrs
  - 48 hrs

Number of migrated cells:

- 0hrs
- 48 hrs

** and * indicate statistical significance.
Approach: Aim 1c. Determine if inhibition of HIF-1α diminishes the effect of hypoxia on M2-polarized macrophage signaling.

1. Confirm HIF-1α expression by western blot using control cells or WT-Macrophages alone.
2. Perform an ELISA to measure MMP-9 and VEGF production.
3. Create HIF-1α knockout M2-macrophages with CRISPR/Cas 9 system.
4. Validate knockouts by western blot.
5. Create 3D cell spheroids.
Aim 1c. HIF-1α expression increases after incubation in hypoxia in WT-macrophages. Which is not seen in HIF-1α −/− macrophages. Hypoxic HIF-1α KO macrophages co-cultured with ovarian cancer cells produce less MMP-9 than WT-macrophages.

**Western blot of HIF-1α expression**

<table>
<thead>
<tr>
<th></th>
<th>Normoxia (21% O₂)</th>
<th>Hypoxia (1% O₂)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WT macrophages</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIF-1α</td>
<td></td>
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<tr>
<td>β-Actin</td>
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<tr>
<td><strong>HIF-1α −/− macrophages</strong></td>
<td></td>
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<tr>
<td>Ovarian cancer cells</td>
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<tr>
<td><strong>WT-M2</strong></td>
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<td>HIF-1α</td>
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<td>β-Actin</td>
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<tr>
<td><strong>HIF-1α −/− M2</strong></td>
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</tbody>
</table>

(Time in hours)
AIM 1c. Ovarian cancer cells cultured with HIF-1α−/− macrophages travel less distance than those cultured with WT-macrophages in hypoxia.
Aim 1. Conclusions

• HIF-1α expression is increased in macrophages and ovarian cancer cells following incubation in hypoxia.

• Macrophages co-cultured with ovarian cancer cells produce higher levels of tumor promoting factors which lead to increased tumor invasion and endothelial cell migration.

• Silencing HIF-1α expression in macrophages leads to lower production of tumor promoting factors by hypoxic macrophages, which in turn decreases the invasion of ovarian cancer cells.
Aim 1: Pitfalls and alternative approaches

2. Although the majority of macrophages found in the ovarian cancer tumor microenvironment are M2-polarized, it would be of interest to use M1-polarized macrophages as an alternative or even in comparison to observe how hypoxia affects these pro-inflammatory cells.

1. If HIF-1α knockout doesn’t yield expected results: Alternatively, NF-κB may be a good alternative target. Although HIF-1α is considered the master regulator of the cells stress response, NF-κB is also involved in the production of cytokines and cell survival in response to stress.
Aim 2. Investigate the effect of hypoxia on the induction of autophagy in M2-polarized macrophages.

Aim 2a. Identify if autophagosome formation is increased in M2-polarized macrophages in a HIF-dependent matter.

Aim 2b. Determine if HIF-dependent autophagy reduces apoptotic activity.

Aim 2c. Determine if HIF-dependent autophagy affects cell viability.

Aim 2d. Determine if inhibition of autophagy leads to revived apoptotic activity in M2-polarized macrophages.
Aim 2a. Identify if autophagosome formation is increased in M2-polarized macrophages in a HIF-dependent matter.

M2-polarized macrophages

Incubate WT-HIF-1α and knockout HIF-1α macrophages for 24hrs in hypoxia.

Measure autophagosome formation by Western blot analysis of LC3-I and LC3-II

And

Visualize autophagosome formation by fluorescently-tagging LC3 with antibodies
Aim 2a. Autophagosome formation will be higher in response to hypoxia in WT macrophages versus HIF-1α knockout macrophages.

Western blot of HIF-1α and LC3 expression

<table>
<thead>
<tr>
<th></th>
<th>WT macrophages</th>
<th>HIF-1α Knockout macrophages</th>
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<tbody>
<tr>
<td>HIF-1α</td>
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<td>β-Actin</td>
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<tr>
<td>LC3-I</td>
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<td>LC3-II</td>
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Approach: Aim 2b. Determine if HIF-dependent autophagy reduces apoptotic activity.

Determine the apoptotic threshold of macrophages in hypoxia.

HIF-1\(\alpha\) WT and knockout macrophages

Time Course Experiment

0min, 30 mins, 1hr, 3hrs, 6hrs, 12hrs, 18hrs, 24hr, 36hrs, 48hrs, 72hrs.

Harvest cells and perform co-immunoprecipitation of BCL-2 with anti-beclin1 and anti-BNIP3

Measure and analyze autophagsome formation by western blot and fluorescent microscopy

Perform annexin V staining with flow cytometry to measure apoptotic cell death
Aim 2b. If hypoxia induces autophagy in macrophages, Co-IP of BCL with anti-BNIP3 will show WT macrophages have increasing levels of BNIP3 attached to BCL2 in hypoxia. While BCL-2/Beclin-1 interaction will decrease with time in hypoxia.

<table>
<thead>
<tr>
<th>Normoxia</th>
<th>BCL2</th>
<th>WT macrophages</th>
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<tbody>
<tr>
<td>Beclin 1</td>
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<tr>
<td>BNIP3</td>
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<td>β-Actin</td>
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<th>Hypoxia</th>
<th>BCL2</th>
<th>WT macrophages</th>
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<tr>
<td>Beclin 1</td>
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Western blot showing Co-IP of BLC2/BNIP3 and BCL2/Beclin1

HIF-1α knockout would look similar to WT macrophages in cultured in Normoxia.
Aim 2b. If autophagy is increased due to HIF-1α expression in response to hypoxia, WT-macrophages will show decreased apoptotic activity.

**Annexin V Staining Results**

**Percent of apoptotic cells in normoxia**

<table>
<thead>
<tr>
<th>Time</th>
<th>WT Macrophages</th>
<th>HIF-1a KO Macrophages</th>
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<tbody>
<tr>
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<td>72 hrs</td>
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**Percent of apoptotic cells in hypoxia**

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<th>Time</th>
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<td>72 hrs</td>
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</table>

Potentially apoptotic threshold?

* indicates statistical significance.
Approach: Aim 1c. Determine if HIF-dependent autophagy affects cell viability.

Determine cell viability

HIF-1α WT and knockout

0 min, starting at apoptotic threshold continue to culture cells up to 48 hrs after threshold

Measure and analyze autophagosome formation by western blot and fluorescent microscopy

Perform MTT assay to measure cell viability
Aim 1c. If hypoxia induces autophagy in macrophages, HIF-1α WT macrophages will have higher cell viability than HIF-1α⁻/⁻ macrophages in hypoxia.
Aim 2d. Determine if inhibition of autophagy leads to revived apoptotic activity in M2-polarized macrophages.

HIF-1α WT and knockout macrophages

Incubate for 24hrs in normoxia and hypoxia with DMSO, 1, 5, and 10 μM of 3-MA

Perform western blot of apoptosis related proteins caspase-3 and caspase-9. Measure autophagic activity by western blot analysis of LC3-I and LC3-II expression. Measure HIF-1α expression by western blot.
Aim 2d. Inhibition of autophagy with increasing doses of 3-MA, leads to decreased expression of autophagic proteins and increased apoptotic proteins in WT-macrophages cultured in hypoxia.
Aim 2. Conclusions

- Apoptotic activity is decreased in M2-polarized macrophages cultured in hypoxia.

- Autophagy is induced as a mechanism of survival by M2-polarized macrophages in response to hypoxia.

- Inhibition of autophagy with 3-MA, reverses the effects of HIF-induced autophagy.
Aim 2 Pitfalls and Alternatives

1. To confirm that HIF-1α knockout is responsible for the increase in autophagy and decrease in apoptosis in hypoxic macrophages, a HIF-1α rescue experiment can be conducted.

   - If HIF-1α \(-/-\) macrophages transiently transfected with a plasmid containing the sgRNA resistant cDNA of HIF-1α.

2. If increased levels of autophagy are not observed in macrophages in response to hypoxia:
   - An alternative experiment could determine if macrophages use NK T cell surveillance escape as a mechanism of survival in ovarian cancer in response to hypoxia.

   Natural killer T cells have been shown to specifically target M2-polarized macrophages in a CD1d, dependent matter in the neuroblastoma tumor microenvironment. M2 macrophages escape death by down-regulating these surface markers. It would be interesting to address whether a similar behavior is seen ovarian cancer in response to hypoxia.
Acknowledgements

Special Thanks to:

Candidacy Examining Committee
Background support
Slides Start Here
TAM numbers in ovarian cancer

- The role of TAMs in cancer progression has been investigated in many types of human malignancies, including lung cancer, oral squamous cell carcinoma, esophageal cancer, gastric cancer, pancreatic cancer, liver cancer, intrahepatic cholangiocarcinoma, colorectal cancer, thyroid cancer, breast cancer, endometrial cancer, cervical cancer, bladder cancer, and prostate cancer.

1. serous,
2. mucinous,
3. undifferentiated ovarian cancer histotypes most frequently display TAM infiltration.
Evidence that TAMs proliferate in breast cancer tissue.

Brown cells with blue nucleus staining indicate proliferating cells.
Autophagy in the innate immune response
Macrophage background

• Granulocyte-macrophage colony-stimulating factor (GM-CSF), also known as colony stimulating factor 2 (CSF2), is a monomeric glycoprotein secreted by macrophages, T cells, mast cells, NK cells, endothelial cells and fibroblasts that functions as a cytokine.
Mechanisms of cisplatin resistance

• Alterations in DNA repair mechanisms and slowing of cell cycle kinetics.

• Reduction in the intracellular accumulation of the platinum compounds by decreased uptake or increased efflux.

• Increased DNA methylation leading to genes involved in metabolism and stress-response pathways.

• Inactivation of apoptosis and activation of autophagy.

• Increased self-renewal capabilities (cancer stem-cell like characteristics).

• Induction of angiogenesis through modifications of neighboring stromal cells.

The tumor microenvironment is critical to the majority of these mechanisms of resistance.
Stress-induced autophagy in mesenchymal cells provide tumor support

- Transwell assay showing that serum starved mesenchymal cells support the survival of the breast cancer cell line, MCF-7 in serum deprivation.

- Western blot showing upregulation of indicated autophagy proteins in serum deprived mesenchymal cells.

Pochampally et al. Carcinogenesis, 2011
Hypoxia-induced autophagy mediates cisplatin resistance in lung cancer

- Hypoxia significantly increased cell viability upon treatment of cisplatin, as compared with that in cells under normoxic condition.

- The expression of Hif-1α was increased along with the time that cells were exposed to hypoxic conditions within 24 h.

Liu et al, Scientific Reports, 2014
Extra and Alternative Experiments
Slides Start Here
Since p62 accumulates when autophagy is inhibited, and decreased levels can be observed when autophagy is induced, p62 may be used as a marker to study autophagic flux.
RT-PCR Assay to detect RNA expression

Provides information at the transcriptional level about genes that being expressed at the protein level.
Alternative induction of HIF-1α

- Use the drug Cobalt (II) Chloride
In vivo analysis of hypoxia

Transduce ovarian cancer cells with a HIF-1α Luciferase-reporter construct.

1. Control mice are sacrificed after day 1.
2. Mice are sacrificed at Day 14.

Collect tumors:
1. Perform IHC staining for M1/M2 macrophages.
2. Stain tissue samples for autophagy markers.
Ovarian cancer mouse models

For studying the mouse immune system:
• C57BL/6 mice with ID8 (mOVCAR cell line) – Currently only established ovarian cancer mouse cell line.

For studying human immune response in mouse model:
• MSTRG mouse model (support enhanced engraftment of human hematoipoietic cells) They also have human SIRPα-encoding BAC transgene. This transgene enables mouse phagocytes to tolerate and not engulf engrafted human cells expressing CD47.
Mouse models

• Carcinogen induced tumor models

• Syngeneic tumor models (self-tumor/self immune system)- Pros: Can study the microenvironment and immune response to drugs, gene alterations, and much more. Cons: Mouse immune response isn’t always exactly what happens in humans.

• Genetically induced tumor models (Transgenic mice)- Alter genes in specific tissues using CRE-LOXP system.
• Pros: Can be used to study the response of tumors to gene alterations and observe changes in drug response.
• Cons:

• Humanized mouse models- Expresses human cytokines/chemokines, low to no mouse T-cells and B-cells, and NK cells.
• Pros: Allows for engraftment of human BM derived cells.
• Cons: Still artificial system, not fully human model.

• Xenograft/ PDX models- Pros: Used to the tumor response to drugs mainly in vivo.
• Cons: No immune system, can’t fully study the immune response in these models.
Endothelial cell migration assay

<table>
<thead>
<tr>
<th></th>
<th>WT Macrophages</th>
<th>HIF-1α⁻/⁻ Macrophages</th>
<th>Ovarian cells alone</th>
<th>WT Macrophages + Ovarian cells</th>
<th>HIF-1α⁻/⁻ Macrophages + Ovarian cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Normoxia:</strong></td>
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<tr>
<td>0hrs</td>
<td><img src="image1" alt="Image" /></td>
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<td><strong>Hypoxia:</strong></td>
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<td><img src="image18" alt="Image" /></td>
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Migrated endothelial cells in response to secreted growth factors

**Graph:**
- **X-axis:** Time (0hrs, 48hrs)
- **Y-axis:** Number of migrated cells
- **Legend:**
  - WT-macrophages alone
  - HIF-1α⁻/⁻ macrophages alone
  - Ovarian cancer cells alone
  - WT-macrophages + Ovarian Cancer cells
  - HIF-1α⁻/⁻ macrophages + Ovarian Cancer cells
Assay Protocols
Start Here
Mimicking Hypoxia in cell culture
The CRISPR/Cas9 system can completely knockout target genes with a high rate of efficiency.

Single guide RNA (sgRNA) directs the cas9 nuclease to the specific site of cleavage.

Cas9 creates DNA double strand breaks.

Homologous DNA templets with desired modifications can be joined by homologus recombination.
Transwell migration assays

1. Cell suspension placed in upper chamber
2. 24-48 hr
3. Invasive cells pass through basement membrane layer and cling to the bottom of the Boyden chamber membrane. Non-invasive cells stay in the upper chamber
4. After removal of non-invasive cells, invaded cells are stained and quantified

Legend:
- Media/FBS
- Cells
- Staining Solution
- Serum Free Media
- Basement Membrane Layer
Co-IP

**Co-Immunoprecipitation**

1. Lyse cells
2. Incubate with antibody bound to bead which binds to protein-protein complex
3. Wash and elute protein
4. Analyze by western blot
Annexin V staining

Annexin V staining

= Phosphatidylserine
MTT cell viability assay

1. Add MTT reagent
2. Incubate
3. Add Detergent to solubilize formazan
4. Incubate
5. Record absorption
Small molecule inhibitors and other Targets of Macrophages

<table>
<thead>
<tr>
<th>Pathway Recruitment</th>
<th>Target</th>
<th>Efficacy in Murine Models</th>
<th>Clinical Compounds</th>
<th>Clinical Trials in Solid Tumors $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11b</td>
<td>CSF-1R</td>
<td>single agent (GBM, PDAC), chemotherapy, radiation, angiogenesis inhibitors</td>
<td>PLX3397, AMG820, IMC-CS4LY3022855, RG7155/RG85609554</td>
<td>NCT01596751 (O); NCT01444404 (C); NCT0346345 (O); NCT01004861 (O); NCT01346358 (O); NCT02858813 (O); NCT01494068 (O); NCT02031012 (O)</td>
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<td>CCL2</td>
<td>single agent (metastasis, PDAC)</td>
<td>carlumab</td>
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<tr>
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<td>FcγR</td>
<td>chemotherapy</td>
<td>clazakizumab</td>
<td>NCT00433446 (C);</td>
</tr>
</tbody>
</table>

$^a$ Clinical Compounds
$^b$ Clinical Trials in Solid Tumors

<table>
<thead>
<tr>
<th>TNF-α</th>
<th>mitogen-activated protein kinase inhibitors</th>
<th>adalimumab, certolizumab, golimumab, infliximab</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activation CD40</td>
<td>single agent (PDAC), chemotherapy</td>
<td>CP-870,893</td>
</tr>
</tbody>
</table>

O, ongoing; C, completed.

- Only targets with clinical compounds are listed.
- Data obtained from https://clinicaltrials.gov.
Alternative transcription factors mediated by hypoxia

FIG. 1. Hypoxia upregulates various transcription factors (such as HIF, AP-1, and NF-kB) and chaperone proteins (such as Hsp and UPR), as well as OPN. These hypoxia-responsive factors especially interact with HIF by activating and protecting it. HIF also regulates gene transcription that is involved in tumor progression such as metabolism, angiogenesis, tissue remodeling, apoptosis, and erythropoiesis. AP-1, activator protein-1; HIF, hypoxia-inducible factor; Hsp, heat shock protein; NF-kB, nuclear factor-kB; OPN, osteopontin; UPR, unfolded protein response.