Final: 4-29-16

Quenching the Cellular Hypoxia Alarm Quells FOP Flare-Ups

Frederick S. Kaplan, M.D.

Eileen M. Shore, Ph.D.

Robert J. Pignolo, M.D., Ph.D.

Corresponding author:

Frederick S. Kaplan, M.D.

Isaac & Rose Nassau Professor of Orthopaedic Molecular Medicine

Chief, Division of Orthopaedic Molecular Medicine

Perelman School of Medicine

The University of Pennsylvania

C/o Department of Orthopaedic Surgery

Penn Musculoskeletal Center - Suite 600

3737 Market Street

Philadelphia, PA 19104

Tel: 215-294-9145

Fax: 215-222-8854

Email: frederick.kaplan@uphs.upenn.edu

When English cleric Joseph Priestly, French chemist Antoine Lavoisier and Swedish pharmacist Carl Scheele discovered oxygen c.1774, they showed that air was not inert and tackled the timeless question of why and how things burn. But, in the rare and disabling genetic disorder fibrodysplasia ossificans progressiva (FOP), it is ironically the lack of oxygen – or more precisely the cellular response to the lack of oxygen that fans the flames of FOP flare-ups. What fuels those lesional fires and keeps them burning?

After extensive investigation beginning in 2009, this week, scientists from **The Center for Research in FOP and Related Disorders** at the University of Pennsylvania provided the first answers to these elusive questions in the nearly 280 years since John Freke first described FOP. Writing in **The Journal of Bone and Mineral Research**, University of Pennsylvania researcher Haitao Wang and colleagues announced a major breakthrough in understanding FOP, and identified new targets for possible treatment. In their paper, **Cellular Hypoxia Promotes Heterotopic Ossification by Amplifying BMP Signaling,** the authors examined the critical role of tissue hypoxia (oxygen starvation) and the cellular response to tissue hypoxia in the induction and amplification of FOP lesions, also known as “flare-ups.”

The research team, led by Drs. Robert Pignolo and Frederick Kaplan, showed that cells from FOP lesions in humans and in a genetic mouse model of FOP are markedly hypoxic (oxygen starved), that hypoxia triggers a molecular alarm called HIF-1α (pronounced “hif one alpha”), that HIF-1α amplifies bone morphogenetic

protein (BMP) signaling in the oxygen starved cells and stimulates heterotopic ossification. Most importantly, by disabling HIF-1α through genetic or pharmacologic means, BMP signaling is restored to normoxic levels in human FOP bone progenitor cells and profoundly reduces heterotopic ossification and resultant disability in a mouse model of FOP.

In 2006, researchers at **The Center** announced the discovery of the FOP gene and the recurrent mutation in the BMP type I receptor called Activin Receptor A type I (ACVR1) that occurs in all individuals who have classic FOP. The classic mutation in ACVR1 (mACVR1) causes the ACVR1 protein, a cell surface receptor, to be mildly overactive, thus stimulating the BMP pathway continuously, like a faucet that drips water when it should be turned-off. However, despite the presence of mACVR1 in all FOP patients, individuals with FOP do not form bone continuously but rather episodically during flare-ups, an important clue that suggested that "something else" could add to fuel the process of lesion formation. A tantalizing lead came from studying FOP lesions themselves. Importantly, all FOP flare-ups, whether spontaneous or triggered by trauma, are associated with inflammation, a well-known cause of oxygen starvation (hypoxia) to cells and tissues. Might oxygen – or the lack of it – be playing a role?

Every cell needs oxygen in order to generate energy and maintain viability. In health, blood carries oxygen to the body's cells and tissues. Every cell generates energy from

the nutrients and oxygen it receives, and uses that energy to maintain the cell membrane, make new proteins, and sustain viability.

Over eons, cells evolved an intricate alarm system to respond to hypoxic conditions. The cellular response to hypoxia is controlled by the hypoxia-inducing factor (HIF) family of proteins, mainly HIF1-α – a molecular alarm that allows a cell to respond instantaneously to the imminent danger of oxygen starvation. Every cell constantly produces HIF1-α but rapidly destroys it when the cell has an adequate supply of oxygen. When a cell is oxygen starved, the enzymes that inactivate HIF1-α instantly cease to function, allow HIF1-α to escape destruction, enter the nucleus of the cell, and trigger an alarm that instructs genes to adapt to a low oxygen microenvironment and thus survive.

Hypoxia can occur for many reasons, but in early FOP flare-ups, we speculated that hypoxia might result from the edema-laden and inflammatory microenvironment where oxygen supply to the damaged tissue is impaired and oxygen-demand by the damaged cells greatly exceeds its supply.

To begin, we examined lesional biopsies of FOP flare-ups that had been obtained mistakenly from patients before the diagnosis of FOP had been correctly made. Additionally, we examined FOP lesions from mice genetically engineered to form FOP-like heterotopic bone. In humans and in mice, we found that the cells of early inflammatory FOP lesions were profoundly hypoxic (oxygen starved).

In order to understand the implications of oxygen starvation in FOP lesions, we examined the effects of hypoxia in human osteoprogenitor cells (SHED cells; see **The Tooth Ferry Program at the FOP Lab: SHEDding Light on FOP** – in The Twenty-Fourth Annual Report of the Fibrodysplasia Ossificans Progressiva Collaborative Research Project) directly from FOP patients and controls. These stem-like progenitor cells can be coaxed to differentiate *in vitro* into fibrous cells, cartilage cells, or bone cells – the types of cells that progressively populate FOP lesions on their way to becoming bone.

When SHED cells from FOP patients were oxygen starved, they formed cartilage, an obligate precursor to bone formation in FOP. Surprisingly, FOP SHED cells also exhibited a dramatic increase in both the intensity and the duration of BMP signaling under hypoxic conditions. Even if BMPs were blocked from reaching the FOP cells, mACVR1 remained extremely active in hypoxic conditions, far above its basal level in normoxic conditions (a normal oxygen microenvironment). This suggested that the increased BMP signaling observed under hypoxic conditions is the result of a change occurring inside the cell that affects the traffic and transit of the receptor itself, rather than from a change occurring outside the cell from BMPs or other ligands acting on the receptor. But why should the oxygen supply to an FOP cell be linked to the traffic and transit of the receptor or to the level of BMP signaling? And, if there was a link, what was it? We began to investigate.

An unexpected clue came from the field of cancer research from scientists who had recently discovered that hypoxia prolonged the activity of cell surface receptors by delaying their delivery to the cell's garbage disposal where they are normally destroyed after use. If a cell did not have ample oxygen to generate energy to make new proteins, it adapted by conserving its old proteins – like keeping an old car in tough economic times rather than discarding it and buying a new one. An old car may not run as well as a new car, but it would get you where you want to go – and you would not go broke if you broke down! Cancer researchers found that this adaptive response helped normal cells adapt to hypoxic conditions but ironically stimulated cancer cells by conserving mutant receptors that were driving the cancer process. Could something similar be occurring in FOP?

ACVR1 is, in fact, a cell-surface receptor. We reasoned therefore that hypoxia, a microenvironmental condition that we had documented in early FOP lesions, might cause a disastrous retention of mACVR1 in FOP lesional cells - similar to the way that hypoxia caused cancer cells to retain their mutant receptors and thus fuel the cancer process.

To test this hypothesis, we examined the effect of hypoxia on ACVR1 activity in SHED cells from FOP patients and controls. We were astonished at what we found. Under normoxia (a normal oxygen microenvironment) in control and FOP SHED cells, ACVR1 was cycled, after use, to the cell's garbage disposal. However, under hypoxia, mACVR1 (in FOP SHED) cells was retained in endosomes (cellular vesicles), where it continued

to signal for a longer duration and at elevated levels. But why should this be? We were determined to delve deeper and decipher the mechanism of this sabotage.

We returned to cancer research for more clues. We reasoned that both cancer (almost all types) and cartilage (the obligate pre-bone tissue that occurs in FOP lesions), thrive in hypoxic conditions (whereas most tissues suffocate under hypoxia). In cancer, researchers found that HIF1-α, the molecular alarm that signals danger to the cell's genes in an hypoxic microenvironment, abruptly shuts-off Rabaptin-5 production, a critical protein that helps escort receptors to the cell's garbage disposal. As a result, the mutant receptors hung around longer, and continued to fuel the cancer. When we subjected FOP SHED cells to the same hypoxic conditions that existed in cancer cells (and that we had demonstrated in FOP lesions), we found exactly the same thing that the cancer biologists had found: HIF1-α disabled Rabaptin-5. As a result, mACVR1 was retained inside the cell's endosomes allowing hypoxic cells to transmit dangerous BMP signals from retained mACVR1 at higher levels and for a longer duration, in effect dramatically amplifying an already overactive signal from the mutation alone. Amazingly, when we restored Rabaptin-5 levels in the cells to normal, the cells breathed a sigh of relief as elevated BMP levels returned to normoxic levels, indicating that Rabaptin-5 was mediating the process.

Most importantly, when the HIF1-α alarm was silenced, either genetically or with drugs, Rabaptin-5 levels were restored to normal, mACVR1 was appropriately escorted to the cell's garbage disposal, and BMP signals fell to the baseline levels that existed in

normoxia - indicating that HIF1-α was driving the entire process. So, while FOP is not cancer, the two conditions have something fundamentally in common. Both FOP and cancer have exploited the cellular hypoxia-HIF1-α alarm system to their advantage, by prolonging the activity of mutant receptors that dramatically amplify already overactive signaling pathways and drive their unique processes to their inexorable conclusions. Essentially, the hypoxic cells in FOP lesions trigger an alarm (HIF1-α) only to discover that the fire engine summoned to douse the fire, instead sprays gasoline on it! In essence, not only does mACVR1 alter the basal threshold for heterotopic bone formation, but the tissue microenvironment that it helps create triggers a cellular response that amplifies the damage.

Although many chemical compounds inhibit HIF-1α in cells, only a few have been shown to effectively inhibit HIF-1α *in vivo*. Could these same pharmacologic inhibitors that quenched HIF1-α in FOP cells *in vitro* do the same in FOP lesions *in vivo*, restoring amplified BMP signals to normoxic levels, and inhibiting heterotopic ossification and subsequent disability in FOP mice? To investigate, we pre-treated FOP mice with either a placebo or one of three pharmacologic inhibitors that quenched HIF1-α activity, and then induced FOP flare-ups with an intramuscular injection. We found that heterotopic ossification was greatly reduced and movement was preserved in the FOP mice receiving any of the three HIF1-α inhibitors compared to control mice who received a placebo.

The HIF-1α inhibitors examined in both the SHED cells and the animal models were apigenin, imatinib and PX-478. Apigenin is a naturally occurring HIF-1α inhibitor found in parsley and other food sources. Imatinib, an inhibitor used to treat several cancers, is a potent HIF-1α inhibitor. Although imatinib has off-target effects, it was selected because of its availability and low toxicity. PX-478 is a potent, experimental HIF-1α inhibitor.

Our study showed that imatinib, apigenin and PX-478 potently inhibited dysregulated BMP signaling induced by HIF-1α *in vitro*, as well as heterotopic ossification following tissue injury in a mouse model of FOP. In all cases, it is possible that these compounds also affect heterotopic ossification by mechanisms other than HIF-1α-BMP pathway crosstalk**.** The implications for targeted clinical trials and for compassionate clinical use of HIF-1α inhibitors are promising, but little data exist on critical issues of dosing, duration, timing, rebound, resistance or long-term safety of any of the HIF-1α inhibitors. At the present time, caution is advised.

In a related study published in **The Proceedings of the National Academy of Medicine**, Agarwal and her colleagues from The University of Michigan showed that inhibition of HIF1-α prevents both trauma-induced and genetic heterotopic ossification. Using different approach, the study confirms the veracity of HIF1-α as a target in HEO.

The groundbreaking studies described here support that FOP lesions thrive in a hypoxic microenvironment – not simply due to oxygen deprivation, but due to a maladaptive

response to hypoxia by the HIF1-α molecular alarm, similar to events that occur in cancer. Importantly, by silencing HIF1-α, the amount of heterotopic ossification and the resultant functional disability are greatly reduced in a genetic mouse model of FOP.

Generation of a hypoxic microenvironment in injured skeletal muscle appears to be a critical step in the formation of heterotopic bone. Our studies demonstrate that BMP signaling in FOP lesions is BMP ligand-independent under hypoxic conditions in both an injury-induced animal model of FOP and in spontaneous lesions from FOP patients. However, injury-induced inflammation is a complex physiologic response to tissue damage, involving immune cells, progenitor cells, and secreted hormone-like factors. Thus, while hypoxia mediates ligand-independent BMP signaling, other responses in the local microenvironment could be ligand-dependent and result in a more complex regulation of heterotopic ossification in FOP.

Although this study focused on the cellular response to hypoxia in the development of FOP flare-ups, it also verified the existence of cellular hypoxia in non-genetic forms of FOP – thus opening the door to modulating the cellular response to hypoxia in more common forms of heterotopic ossification.

Our study provides critical insight into the role of cellular hypoxia in the episodic induction and amplification of FOP flare-ups and establishes that cellular oxygen sensing through HIF1-α is a critical regulator of the BMP pathway and heterotopic ossification in FOP. Most importantly for individuals with FOP, our study identifies HIF1-

α as a robust therapeutic target for FOP – vital new knowledge that will likely contribute to the development of more effective treatments for FOP and related common disorders of heterotopic ossification. Usually, when the oxygen is low, the fire goes out. For FOP lesions, as with cancer, when the oxygen is extinguished, the fire grows. Quenching the cellular hypoxia alarm quells FOP flare-ups.

SUMMARY:

- FOP lesions in humans and mice are profoundly hypoxic (oxygen starved).
- HIF1-α is a molecular alarm that regulates the cellular response to hypoxia.
- HIF1-α promotes cartilage formation, the obligate scaffold for heterotopic ossification in FOP.
- HIF1-α promotes cartilage formation by regulating BMP signaling.
- HIF1-α amplifies mutant BMP signaling by retaining mACVR1 in the internal signaling pathway (endosomes) and delaying its transport to the protein garbage disposal.
- HIF1-α turns-off Rabaptin-5, a key escort protein that delivers ACVR1 to the protein garbage disposal.
- Pharmacologic or genetic inhibition of HIF1-α restores normal cellular Rabaptin-5 levels, diminishes retention and signaling of mACVR1, reduces BMP signaling and inhibits heterotopic ossification in a mouse model of FOP.

Acknowledgements:

We thank Celeste Simon for critical reading of the manuscript and helpful suggestions. This work was supported in part by the International Fibrodysplasia Ossificans Progressiva Association (IFOPA), the Center for Research in FOP and

Related Disorders, the Ian Cali Endowment for FOP Research, the Whitney Weldon Endowment for FOP Research, the Isaac and Rose Nassau Professorship of Orthopaedic Molecular Medicine (to FSK), the Cali-Weldon Research Professorship in FOP (to EMS), the Rita Allen Foundation, the Roemex Fellowship for FOP Research (to HW), the Brinkman Family, the McGuire FOP Research Fund, the Ashley Martucci FOP Research Fund, the Penn Center for Musculoskeletal Disorders, and the National Institutes of Health (NIH R01-AR41916).