PROJECT REVIEW

"Development of tests for detecting hypoxia-inducible gene doping to enhance athletic performance"

S. Lahiri, T.S. Khurana (University of Pennsylvania, Pennsylvania, USA)

Numerous pharmacological and/or genetic strategies exist that simulate the effects of hypoxia at the cellular level and increase expression of hypoxia-induced genes such as hypoxia-inducible factor (HIF), its downstream targets such as erythropoietin (EPO) and consequently increase red blood cell production. Many of these strategies / molecules were developed to treat diseases such as the anemia associated with chronic renal failure and indeed are mainstays in the medical management of these conditions. However, these strategies have great potential to be abused by elite athletes seeking to gain a (unfair) competitive advantage, since they are, 1) currently available and 2) not efficiently detectable by current anti-doping protocols (particularly the newer molecular / gene-based strategies that are subject of this proposal).

Prime amongst the pharmacological and / or genetic strategies that have great doping potential are those aimed at modulating hypoxia-induced genes by inhibiting HIF-prolyl hydroxylase (HIF-PHD), a key regulatory enzyme that regulates HIF. Both small molecule inhibitors against HIF-PHD (*such as iron chelators or small molecules are able to efficiently inhibit HIF-PHD*) and direct gene transfer (i.e. RNAi-based) strategies knocking down HIF have been shown be effective. Of particular concern regarding 'doping' by athletes is the availability drugs/small molecules such as clinically used iron chelators (desferrioxamine-DFO, ciclopirox olamine-CPX) and small molecules (e.g. FG-2216, FG-4519) developed by FibroGen, that are currently in human Phase II/III trials.

Currently, tests do not exist to detect hypoxia induced gene 'doping' with pharmacological and/or genetic strategies such as those listed above. The fact that a number of web-based resources and pharmaceutical firms currently offer 'HIF-PHD inhibitory reagents, underscores the need for rapidly developing a test to detect hypoxia induced-gene doping not just the currently described reagents but also those that would be used in the near future, such as gene transfer using RNA-based inhibitors (such as RNAi molecules against HIF).

Thus, the challenge is to develop standardized detection tests that would be specific, sensitive and standardized enough to hold up to legal challenges that anti-doping agencies would almost certainly face by athletes caught using these tests. These challenges are not insurmountable; we propose to develop a robust and standardized, blood-based test using '*molecular signatures*' of hypoxia that will detect all the different forms (of small molecule and gene-based) hypoxia-induced gene doping that are currently in use (or likely to be developed in the near future) with great sensitivity and specificity to serve this need.

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Results and Conclusions

Numerous physical, pharmacological and/or genetic strategies exist that simulate the effects of hypoxia at the molecular and cellular level and increase expression of hypoxia-induced genes such as hypoxia-inducible factor (HIF), its downstream targets such as erythropoietin (EPO) and consequently increase red blood cell production. While hypoxia was classically achieved by exposure to high altitude (hypobaric hypoxic exposure), there are currently numerous methodologies for achieving hypoxia-induced gene doping including chambers (normobaric hypoxia), chemicals and genetic manipulation. Our basic hypothesis is that exposure to different types of hypoxia lead to both a unique *'molecular signature'* specific to the type of hypoxia as well as core *'molecular signature'* irrespective of the type of hypoxia. Testing the '*molecular signatures of hypoxia*' using blood samples from athletes will detect all the different forms (of physical, small molecule and gene-based) hypoxia-induced gene doping that are currently in use (or likely to be developed in the near future) with great sensitivity and specificity. Identification and definition of these *molecular signatures* would allow detection of hypoxia-inducible gene doping and preclude abuse by elite athletes seeking to gain a (unfair) competitive advantage using this strategy. The project was funded to provide proof of concept that the '*molecular signatures*' (in peripheral blood) are distinct for different types of hypoxia-induced doping.

We have been able to demonstrate that different hypoxic conditions tested experimentally (hypobaric/high-altitude, normobaric/ chambers or tents and chemical) have both a unique *'molecular signature'* specific to the type of hypoxia as well as core *'molecular signature'* irrespective of the type of hypoxia. To be comprehensive and rigorous experiments were conducted both in laboratory conditions and in field studies on Mt. Everest at altitudes up to c. 8400 mts. The *'molecular signature'* generated during the pilot studies have been deposited into the WADA Informatics website and GEO databases (GEO Accession Numbers GSE 15894, 15901 and 15902). Further we have been able to validate the data by Taqman based qPCR assays. These findings are of great relevance for development of anti-doping efforts as it provide the first evidence that sensitive and specific anti-doping tests to detect hypoxia-inducible gene doping can indeed be developed using the bioinformatically generated *'molecular signatures'* we have identified. Based on the progress and tangible resources that we have generated, we are confident of achieving our overall goals to develop sensitive and specific tests to comprehensively and rigorously detect hypoxia induced gene doping and preclude abuse by elite athletes seeking to gain a (unfair) competitive advantage using these strategies.