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Recommended Citation

Bruinsma, Tyler J.; Curwick, Kevin T.; Ellman, Leah; Grootwassink, Jared M.; and O'Toole, Thomas M., "HLHS is caused by the up regulation of HIF1 α due to hypoxia caused by a polymorphism in eNOS" (2015). *Celebrating Scholarship & Creativity Day*. 61.
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HLHS is caused by the up regulation of HIF1 α due to hypoxia caused by a polymorphism in eNOS.

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Background Information

Hypoplastic left heart syndrome (HLHS) is a rare congenital heart defect occurring in fewer than 0.5% of live births in the United States (Fruitman, 2000). It is characterized by a critically underdeveloped left ventricle with accompanying imperfections including septal defects, under-sized aorta, and underdeveloped bicuspid and aortic semilunar valves (Mayo Clinic Staff, 2012; CDC, 2013). HLHS is detectable on ultrasound at the end of the first trimester of pregnancy allowing for early diagnosis and potential treatment *in utero*. While a protocol involving three surgeries exists to treat the condition, there is no cure for HLHS. Even with treatment, many patients eventually require a heart transplant due to congestive heart failure.

Hypothesis and Rationale

We hypothesize that HLHS is caused by an up-regulation of hypoxia-inducible factor 1 α (HIF1 α) caused by prolonged hypoxia in the developing heart. Furthermore, we propose that a polymorphism in the endothelial nitric oxide synthase gene (*eNOS*) causes a decrease in basal levels of nitric oxide (NO), a potent vasodilator, in the blood stream and contributes to hypoxia. HIF1 α expression, brought about by hypoxia and other environmental factors, regulates a number of transcription factors and genes vital to normal cardiogenesis. These factors combine to form a perfect storm in the developing heart, leading to the development of HLHS.

When working properly, the eNOS enzyme promotes production and regulation of NO which dilates blood vessels and allows for proper blood flow throughout the body. eNOS is expressed in endothelial cells during early stages of cardiogenesis and naturally depletes starting as early as embryonic stage 14.5 (between days 33-36 of pregnancy) with only small traces remaining at birth (Bloch et al., 1999). Many researchers have identified that a polymorphism on exon 7 of the *eNOS* gene, G894T, leads to a greater risk for congenital heart defects during early cardiogenesis (van Beynum et al., 2008; Bloch et al., 1999; Feng et al., 2002; Shaw, et al., 2005; Zhou et al., 2014). The polymorphism corresponds to a down regulation of NO, leading to an inability to control blood pressure, cardiomyocyte apoptosis, and cell growth in cardiogenesis (Feng et al., 2002). Specifically, van Beynum et al. (2008) demonstrates a positive relationship between the G894T polymorphism and structural deformations of the heart. Feng et al. (2002) attributes the identified structural deformations to the increased and uncontrolled cardiomyocyte apoptosis found as a result of eNOS deficiency. Another result of the polymorphism is a decrease in available cyclic guanosine monophosphate (cGMP) (Bloch et al., 1999), which relates to the mechanism of vasodilation by NO (McQuillan, Leung, Marsden, Kostyk, & Kourembanas, 1994). Due to downregulation of NO, blood flow becomes restricted during cardiac development and prolonged hypoxia arises as a detriment to cardiogenesis.

Hypoxia-inducible factor 1 α (HIF1 α) is a dimer of the heterodimeric transcription factor, HIF1 (Ziello, Jovin, and Huang, 2007). According to Balligand, Feron, and Dessy, (2009) HIF1 α is activated through various pathways, including hypoxia. HIF1 α is hydroxylated in the combined presence of oxygen and prolyl hydroxylase (PHD), and is then degraded after binding to the von Hippel Lindau protein (pVHL). This process acts to regulate levels of HIF1 α during periods of normal oxygenation. When confronted with conditions, such as hypoxia, that prevent HIF1 α 's

hydroxylation, HIF1 α is able to stabilize and complete the HIF1 complex after migrating to the cell nucleus. This contributes to increased levels of HIF1 α during periods of low oxygenation (Balligand, Feron, and Dessy, 2009). While low oxygenation has been shown to increase levels of HIF1 α , many other factors such as increased stress, NO levels, and inflammation have also been shown to increase levels of HIF1 α (Gorlach, 2009). Chronic diseases, such as cardiovascular disease, diabetes, and autoimmune diseases as well as chronic environmental stress all contribute to higher levels of inflammatory proteins. If an epidemiological connection can be found among mothers of HLHS patients who have any of these diseases or environmental factors, increased HIF1 α levels may be preliminarily implicated as the cause of HLHS.

Gaber and colleagues (2013) studied left ventricle tissue samples from aborted, second trimester fetuses who were found to have HLHS and compared them to control samples obtained in a similar manner. HLHS samples were found to have higher nuclear expression of HIF1 α . Furthermore, the phenomenon was mimicked *in vitro* using human pluripotent stem cells (hPSC's). hPSC's subjected to a hypoxic state during cardiac differentiation showed elevated HIF1 α levels. The authors of the study indicate that this data points strongly to the assertion that hypoxia can reprogram the developing heart (Gaber, et al., 2013). This new data aligns with existing evidence that HIF1 α plays an essential role in cardiac development by regulating key genes in ventricular formation (Comperolle et al., 2003).

According to Krishnan et al, (2008) it has also been determined that HIF1 α contributes to the regulation of *NKX2.5*, *TBX5*, and *MEF2C*. *NKX2.5* and *MEF2C* are expressed very early on in the heart field of the developing embryo and continue to be expressed into the late stages of development. They are considered some of the earliest cardiac indicators (George, Colombo, Tergoff, 2014; Srivastava, Olson, 2000). *NKX2.5* is also vital to the development of ventricular identity in the transition from the anterior-posterior patterning to the left-right patterning in the developed heart. Region specific knockouts of *NKX2.5* exhibit elevated levels of Bmp10 which leads to hypertrophy of the developing ventricles (Gilbert, 2010). *TBX5* is expressed exclusively in the left ventricle asymmetric to *TBX20* in the right ventricle during heart patterning and ventricular septation. Together these genes are vital for normal ventricular septum formation and normal ventricular development (Gilbert, 2010).

HIF1 α is believed to regulate *TBX5* and *NKX2.5* by binding to Hypoxia Response Elements (HREs) contained within the promoters of the respective gene on the DNA. By binding to the promoter, the transcription of the gene is down regulated. (Krishnan et al., 2008). Experiments have shown that irregularly low doses of HIF1 α result in the up-regulation of *NKX2.5*, *TBX5*, and *MEF2C* (Bohuslavova, et. al. 2013). Therefore, high doses of HIF1 α likely result in the down regulation of these specific factors. The evidence-based roles of these downstream factors further implicate our proposed pathway connecting hypoxia and HIF1 α to the development of HLHS.

The full phenotypic scope of irregular expression of HIF1 α and its regulation of downstream proteins and transcription factors is currently not well understood. There is, however, phenotypic evidence that, in tandem with our proposed pathway, points to the potential role of hypoxia and HIF1 α in HLHS. Research has found that hearts afflicted with HLHS exhibit reduced levels of connexin 43 in intercalated discs (Mahtab, et. al, 2012), and that connexin 43 may be reduced under the same hypoxic conditions that promote HIF1 α expression (Xianghong, et. al, 2013). Additionally, z-discs interact with titin and α -actinin to function properly, and both titin and α -actinin have been exhibited in decreased levels when HIF1 α levels are reduced (Krishnan). Therefore, it is possible that the inverse may be true, where increased levels of HIF1 α lead to an overexpression of titin and α -actinin. Alpha-actinin is highly important in heart sarcomere function, and unusual expression may be linked to HLHS (Chiu, et. al, 2010; Wang, 2013). The importance

of these findings is not necessarily about how they function to create HLHS (as much information in that area has yet to be discovered). Rather, their importance lies in their ability to link HIF1 α to the disease.

Significance and Innovation

A preliminary epidemiological study could provide a level of validity for our hypothesis. If mothers of HLHS patients tend to have inflammatory protein-causing diseases, our hypothesis connecting HIF1 α to HLHS would be preliminarily supported. Additionally, we propose a series of simple laboratory tests. First, we propose the sequencing and analysis of HLHS patient samples for the eNOS polymorphism G894T. Next, we propose a simple ELISA assay to test for abnormal levels of HIF1 α in cardiomyocytes derived from induced pluripotent stem cells (iPSCs) from HLHS patients. If a relevant correlation between the presence of a G894T and elevated levels of HIF1 α exists, our pathway would be supported. In the future, it would be possible to perform both of these tests *in utero*, allowing for early diagnosis and potential prophylaxis. Tests may also be performed on the parents prior to pregnancy to screen for a predisposition to the G894T polymorphism or elevated HIF1 α .

In the event that early screenings using our proposed tests indicate a predisposition to HLHS, we hypothesize that it is possible to prevent the development of HLHS by attempting to treat the abnormal phenotype created by this polymorphism. This may be possible through the use of ACE inhibitors, statins or vasodilators, which have been proven to activate eNOS (Balligand, Feron, & Dessy, 2009). This would not only remedy the vasoconstriction caused by a lack of NO due to abnormal levels of eNOS, but it would also provide one mechanism to avoid the accumulation of HIF1 α , preventing the abnormal downstream regulation of *NKX2.5*, *TBX5*, and *MEF2C*.

Our hypothesis is significant because if supported it relies on a simple genetic test to determine if potential parents are at risk for passing on the polymorphisms to their offspring. If parents choose to have a child despite the risk there is the potential for treatment *in utero* that would remove the environmental conditions (hypoxia) that lead to the development of HLHS. The combination of genetic screening and *in utero* treatment could lead to the cure for HLHS ensuring no more children are born with this heart defect. Ultimately our proposed pathway is novel because it connects a polymorphism in *eNOS* to hypoxia, elevated HIF1 α , various cardiac progenitors, and finally the development of HLHS.

Summary

The G894T polymorphism in *eNOS* during early stages of embryonic cardiogenesis results in a reduction of basal NO production, which results in vasoconstriction and prevention of HIF1 α degradation. As a result, prolonged hypoxia in the developing heart brings about an accumulation of HIF1 α . When combined with environmental factors such as inflammation and depleted levels of NO, the uncontrolled levels of HIF1 α down regulate *TBX5*, *NKX2.5*, and *MEF2C*; genes responsible for development of the left ventricle. Compounded with data demonstrating that degradation of eNOS results in uncontrolled cardiomyocyte apoptosis, the greater expression of HIF1 α results in an underdeveloped left ventricle corresponding to HLHS. Simple parental PCR testing for the G894T polymorphism in *eNOS* and ELISA assay for observing abnormal levels of HIF1 α in iPSCs can result in an accurate diagnosis through our proposed mechanism of HLHS. A proposed treatment regimen calls for ACE inhibitors or statins to promote eNOS activation, leading to greater prevalence of NO, which eliminates one factor contributing to HIF1 α abundance.

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