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The genetics of exceptional longevity identifies new druggable targets for vascular protection and repair

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Abstract

Therapeutic angiogenesis is a relatively new medical strategy in the field of cardiovascular diseases. The underpinning concept is that angiogenic growth factors or proangiogenic cells could be exploited therapeutically in cardiovascular patients to enhance native revascularization responses to an ischemic insult, thereby accelerating tissue healing. The initial enthusiasm generated by preclinical studies has been tempered by the modest success of clinical trials assessing therapeutic angiogenesis. Similarly, proangiogenic cell therapy has so far not maintained the original promises. Intriguingly, the current trend is to consider regeneration as a prerogative of the youngest organism. Consequentially, the embryonic and foetal models are attracting much attention for clinical translation into corrective modalities in the adulthood. Scientists seem to undervalue the lesson from Mother Nature, e.g. all humans are born young but very few achieve the goal of an exceptional healthy longevity. Either natural experimentation is driven by a supreme intelligence or stochastic phenomena, one has to accept the evidence that healthy longevity is the fruit of an evolutionary process lasting million years. It is therefore extremely likely that results of this natural experimentation are more reliable and translatable than the intensive, but very short human investigation on mechanisms governing repair and regeneration. With this preamble in mind, here we propose to shift the focus from the very beginning to the very end of human life and thus capture the secret of prolonged health span to improve well-being in the adulthood.

Keywords: Angiogenesis; Ischemia; Aging, Genome-Wide-Association-Studies; Nitric oxide

Abbreviations

BPIFB4: bactericidal/permeability-increasing fold-containing family B member

CVD: cardio vascular disease

CHD: coronary heart disease

MI: myocardial infarction

HF: heart failure

LI: limb ischemia

AD: Alzheimer disease

AAV: adeno associated virus

APOE: apolipoprotein E

eNOS: endothelial nitric oxide synthase

nNOS neuronal nitric oxide synthase

iNOS: inducible nitric oxide synthase

VEGF-A: vascular endothelial growth factor-A

LLIs: long-living individuals

GC: genetic component

SNPs: single nucleotide polymorphisms

FOXO3A: forkhead box O3A

GWAS: genome-wide association studies

LAV: longevity associated variant

RV: rare variant

WT: wild type

SOD: Superoxide dismutase

ER: endoplasmic reticulum

UPR: unfolding protein response

MNC: mononuclear cell

SDF-1α: stromal cell-derived factor 1α

MCP-1: monocyte chemoattractant protein-1

BH4: tetrahydrobiopterin

BH2: dihydrobiopterin

RNS: nitric radical species

ROS: reactive oxygen species

HSPs: heat shock proteins

NO: nitric oxide

cGMP: cyclic guanosine monophosphate

PKB: protein kinase B

PKA: protein kinase A

HSP90: heat shock protein 90

L-NAME: N-nitro-L-arginine methyl ester

PAD: peripheral artery disease

CLI: critical limb ischemia

L-arg: L-arginine

1. Introduction

There are an estimated 7 million people living with cardiovascular disease (CVD) in the UK and 160,000 people die each year because of CVD. Coronary heart disease (CHD) caused by the narrowing of arteries that feed the heart is the UK's single biggest killer, being responsible for ~73,000 deaths each year, an average of 200 people each day. Acute myocardial infarction (MI), which is caused by the occlusion of a coronary artery, represents the most harmful form of CHD. Most of the current treatments are palliative, i.e. they reduce symptoms associated with heart dysfunction, without providing a definitive repair. Consequently, CHD patients undergo a progressive decline in the pumping function of the heart that ultimately leads to heart failure (HF). Today, post-infarct HF is the leading cause of invalidity, hospitalization, and mortality in patients over 65. Limb ischemia (LI), is also caused by arterial occlusion and manifests as claudication, foot ulcers, and gangrene. Revascularization by angioplasty is often unfeasible or ineffective, hence many LI patients are left with no therapeutic option rather than foot amputation, which is associated with a yearly mortality rate >25% (1).

Regenerative medicine aims to provide a definitive treatment of ischemic complications by promoting endogenous mechanisms of repair and delivering supply-side boosts of cells, genes, or proteins. Unfortunately, both gene therapy and cell therapy failed to achieve the initial promises and researchers are now trying to understand the reasons for the unsuccessful translation of preclinical studies. In addition, there is a growing interest in untangling the mechanisms that allow an efficient regeneration during early stages of the life, but are attenuated with aging.

A novel way to pursue an effective regenerative product would be to start from a solid genetic understanding of the causes of disease and its predisposing factors. The most important risk factor for CVD is aging, and, recently, huge efforts have been done to halt the aging process in order to prevent cardiovascular complications. On the other hand, CVDs represent the most important cause of death for both middle-age and elderly people. For instance, a follow-up study on a population of 14345 individuals of 44 years old men showed that 300 out of the 914 death for all causes died for CVD in the follow-up of 11.4 years. Men who maintained or improved fitness had 30% and 40% lower risk of corresponding mortality, respectively (2). This data could be interpreted in two different ways: 1) exercise training is the only possible way to reduce mortality; 2) among people analyzed, the ones genetically predisposed to live longer are biologically younger and thus have a better attitude to fitness that further contributes to the reduced mortality. The second key of interpretation is also in keeping with a fundamental shift in the regenerative medicine strategy. While most attention has been focused on correcting mechanistic targets responsible for failed regeneration, a more effective approach would be to determine and exploit the factors that allow

some individuals to avoid CVD or cope better with CVD when it occurs. This is the case of the long-living individuals (LLIs), i.e. the small number of (1/5000 born) people that survive to be 100 years old, and their closest relatives and offspring, which have higher probability to live long and healthy (3, 4). Centenarians are either healthy or survivors of diseases of aging, such as diabetes, cancer, and CVD. Thus, studying the centenarians' genetic code could disentangle how these individuals delay aging and escape or experience attenuated forms of CVDs.

In this review, we will discuss mechanisms of regenerative angiogenesis instrumental to therapeutic revascularization of ischemic tissues. In particular, we will focus on the novel approach of interrogating the genetics of exceptional longevity to unravel regulators of vascular function that could be exploited in effective proangiogenic therapies.

2. Reparative angiogenesis for CVD

The knowledge of the different phases of the angiogenic process, the cells involved, and the factors released have been exploited to treat aging-related diseases in which angiogenesis is dysfunctional. The process of vessel remodeling in the adult occurs through sprouting of new vessels from existing ones. This process known as angiogenesis is guided by endothelial cells (ECs) that switch from a quiescent to an active state in the presence of proangiogenic factors as reviewed in (5). During the process, specialized ECs known as tip ECs, following proangiogenic cues, move towards the zone that needs to be vascularized. The cells behind the tip are known as stalk ECs and form the body of the nascent vessel. In addition, $CD34^+$ cells derived from bone marrow (BM) and released in the circulatory system upon activation by physical or chemical stimuli, formerly known as endothelial progenitor cells (EPCs), also contribute to the process mostly *via* the paracrine activation of resident ECs (6).

The possibility to restore angiogenesis therapeutically was applied for the treatment of coronary artery disease (CAD) and peripheral vascular disease (PVD) especially through the administration of proangiogenic factors like VEGF-A (7). Indeed vascular endothelial growth factor-A (VEGF-A) triggers angiogenesis and also improves vessel function through the activation of eNOS (with the consequent production of NO) and activation of prostacyclin (PGI_2) (8). However, despite the success of preclinical studies in animals, clinical trials in humans showed poor or no efficacy. The same results were obtained using other angiogenic factors like FGF-2 (9) or other technologies like gene therapy methods (10). Most of the negative outcomes are related to bias in the study design, but also to the fact that angiogenic factors activity is more complex than expected. For instance VEGF *per se* is not sufficient to maintain vessels stabilized for a long time. In addition, the VEGF dosage should be finely tuned to avoid aberrant or poor angiogenesis (11).

The modulation in angiogenesis through cell therapy was also tested using BM-derived cells. These studies demonstrated that transplantation or chemokine-induced mobilization of BM cells in patients with CAD (reviewed in (12)) or PAD (13) improve ischemic tissue functions although only modestly. However, BM cells are not homogeneous as they include hematopoietic ($CD45^+$) and so-called endothelial progenitors ($CD34^+$) as well as non-hematopoietic ($CD45^-$) cells. Therefore, better isolation protocols and more specific markers are needed. Furthermore, more efforts should be done to better understand their mechanisms of action, including physical or paracrine interactions with resident cells. In addition, it is necessary to consider that there are inter-individual differences in the level of these cell populations which are affected by aging especially when associated with diseases like diabetes, hypertension and others (14). In this complex context, new tools to promote a controlled and physiologic angiogenesis are needed.

3. ENOS is a central player in cardiovascular homeostasis

Since the discovery of angiogenic growth factors, it was clear that signaling pathways converge on NO production by eNOS in the endothelium and in circulating angiogenic cells (22). In this section, we briefly summarize current evidence on how eNOS activity impacts on the health of the cardiovascular system, and how researchers have attempted until now with a various degree of success to intervene on eNOS to improve vascular function.

3.1 The eNOS signaling pathway and the control of angiogenesis

ENOS is the endothelial-specific form of a family of Ca^{2+}-dependent enzymes that regulate NO production from L-arginine. In addition to endothelial cells, eNOS is present on circulating blood cells, including circulating angiogenic MNCs, and on circulating microparticles, therefore its regulation impacts on the overall homeostasis of the vascular tree controlling blood flow restoration via angiogenesis, vasculogenesis, and arteriogenesis (23-26). Together with neuronal NOS (nNOS), eNOS is constitutively expressed. On the other hand, a third form of NOS, inducible NOS (iNOS), is produced in response to insults in monocytes/macrophages, but also in MNCs with angiogenic activity, with recent evidences pointing to a potential role of iNOS in the control of angiogenesis (27-29).

The enzymatic function of NOS is modulated by co-factor-mediated dimerization and by selective phosphorylation (30, 31). Lack of functional co-factors, tetrahydrobiopterin (BH4) being the crucial one, impairs dimerization, with the consequent switch from NO generation to superoxide anions production, a process known as NOS uncoupling (32). BH4 oxidation generates

dihydrobiopterin (BH2) that in turn contributes to uncoupling (33). Since BH2 competes with BH4 for eNOS binding, the ratio BH4:BH2 becomes central in coupling (34).

The vascular endothelium is the primary target of NO that 1) regulates vascular tone and cell migration, 2) has an antithrombotic effect, and 3) modulates inflammation (35). The abrogation of NO production is linked to alterations in endothelial function, consisting in vessel rigidity, increased the adhesiveness of endothelial cells, increased inflammation, and risk of thrombi formation (36). Such a condition, known as endothelial dysfunction or endothelial activation, is a hallmark of many CVDs. ENOS plays a central role in endothelial homeostasis since it can either produce NO contributing to endothelium quiescence or generate reactive oxygen species (ROS) thus triggering endothelium activation. Under certain circumstances, the eNOS switch from an NO producing to an ROS-producing enzyme is advantageous to implement host defense responses to infections, but a persistent ROS production becomes detrimental when exceeding the ROS detoxifying machinery of the cell (36). ROS exacerbate endothelial activation by reacting with NO to produce nitric radical species (RNS) (37), thus leading to eNOS uncoupling (38). The functional alterations of the vascular endothelium reflect on the functionality of entire organs and tissues being the cause of disease conditions. ENOS activation is strictly related to angiogenesis. Indeed, signaling pathways activated by proangiogenic factors leads to NO formation *via* eNOS activation (8, 39). Generated NO affects also vascular smooth muscle cells biological function contributing to vessel relaxation needed for efficient angiogenesis and arteriogenesis. Of note, the NOS inhibitor N-nitro-L-arginine methyl ester (L-NAME) is able to block VEGF-mediated angiogenic response (40). Moreover, in eNOS knockout mice, ischemic neovascularization is impaired with VEGF administration being unable to restore it (35).

Further supporting the central role of eNOS in vascular regeneration, eNOS knockout mice, and L-NAME treated mice show impaired proangiogenic cell mobilization from the bone marrow in response to exercise training (41). These proangiogenic cells are also dependent on eNOS for their efficient functionality in the vascularization process (42, 43).

3.2 ENOS/NO-based therapies for vascular diseases

ENOS is crucial for blood flow restoration upon ischemia due to its ability to control angiogenesis by inducing EC proliferation and migration, vasculogenesis *via* activation of circulating proangiogenic cell migration, and arteriogenesis by NO-mediated vasodilation (44). Moreover improving eNOS/NO system may be the basis of treatment for most if not all the diseases of the cardiovascular system as briefly described below. NO availability is fundamental to prevent atherosclerosis development and progression. However, the positive effect of dietary

supplementation with L-arginine in animal models appears not to be long term effective. This may be due to eNOS uncoupling and consequent generation of counterproductive anions. NO unbalance also affects the coronary arteries, which loose a proper vasodilatory activity and become prone to atherosclerotic remodeling. Reduced expression of eNOS was observed in the endothelium of coronary vessels of patients with HF (45). In addition, NO availability is instrumental to the outcome of patients with acute MI (46). However, rescuing cardiac function by NO after MI needs a fine tuning of eNOS activity, as pointed out by the evidence of a detrimental effect of bearing high levels of eNOS activity in transgenic mouse models (47).

Of note, polymorphisms in eNOS gene have been reported. The 786T/C polymorphism in the promoter of the eNOS is associated with decreased enzyme abundance and cardiovascular mortality (48). In addition, studies on the three NOS isoforms documented the existence of an association between polymorphic variants of eNOS, nNOS and iNOS and susceptibility to CHD (49).

From all of the above, it is clear why several lines of research were developed in the attempt of improving NO generation by eNOS or providing NO to the vasculature. Therapeutic strategies aimed at recovering functional eNOS coupling are based on antioxidant treatments combined to BH4 administration (50, 51). Infusion of BH4 in patients with hypercholesterolemia or type II diabetes proved to restore-NO mediated vasodilation, but this approach may hide potential negative side effect of altering BH4:BH2 ratio (52-54). Statins represent a powerful tool to improve NO generation since their use results in BH4 promotion and eNOS phosphorylation through AKT and PKA activation (55). In addition to ameliorating endothelial dysfunction in diabetics, statins and thiazolidinediones are effective in improving circulating proangiogenic cell function and number (56-58). NO donor drugs have been developed, as for example S-nitrosothiols that have been effectively studied in trials using coated stent and other devices to prevent neointima formation (59). Combining the beneficial effect of a statin with NO donor approach, the insertion of an NO-donating moiety to a different class of drugs has been investigated with successful results for ischemic vascular regeneration as documented by our group and others (60, 61).

4. Genetic approaches to unravel the cause of exceptional longevity

Genetic studies on exceptional longevity have so far disclosed the presence of protective variants that buffer the effect of deleterious variants (15). This could explain why, with the exception of apolipoprotein E (APOE) epsilon 4, an allele that is associated with increased risk of CVD and Alzheimer disease (AD), no risk factors have been associated with reduced risk of reaching human exceptional longevity (16). A possible explanation of why, despite the buffering role of protective

variants, APOE isoform epsilon 4 negatively impact on exceptional longevity, is its interference with stem cell survival (17), that could also influence the susceptibility to, and severity of CVD and AD.

Dealing with genetics of complex traits is not easy, since association of a given allele and a phenotype is often not replicated in independent studies and this could be related to lack of power of the study, which in turn depends on the number of hypotheses tested, the population size adopted, and the frequency of the polymorphism interrogated (18). In addition, genetic admixture between cases and controls inflated the results generating false positives, a problem now avoided by the genetic component (GC) analysis based on the investigation of the entire genome trough chips (see below). While candidate genetic studies, being hypothesis-driven, loose most of their novelty (see for example APOE and forkhead box O3-FOXO3A), Genome-Wide-Association-Studies (GWAS) and now full-genome re-sequencing could unravel completely unexpected new players. Unfortunately, especially with re-sequencing, millions of genetic variants are interrogated making the results less significant due to the need of correcting for multiple testing. Thus, study design in genetic association studies needs to accomplish the goal of being hypothesis-free with a limited number of hypotheses tested.

With the aforementioned difficulties in mind, we have focused in the last 5 years on the characterization of a secreted protein, namely bactericidal/permeability-increasing fold-containing family B member 4 (BPIFB4) that emerged from a GWAS on an Italian set of LLIs and was validated in a subsequent follow up done on two independent populations from Germany and USA. Data showed the consistent enrichment for homozygous for the minor allele rs2070325 (I229V) of BPIFB4 in LLIs. The BPIFB4 locus is under balancing selection. (i.e. it is enriched of highly frequent polymorphisms as consequence of an evolutionary force) and it is characterized by high levels of Linkage disequilibrium that has been described between rs2070325 polymophism and additional three polymorphisms (19). Thus, the rs2070325 variation (Ile229Val) of BPIFB4 (identifier: P59827.2) is strongly correlated with rs2889732 (Asn281Thr), rs11699009 (Leu488Phe) and rs11696307 (Ile494Thr) generating two alternative haplotypes: WT (Ile229/Asn281/Leu488/Ile494-BPIFB4) and LAV (Val229/Thr281/Phe488/Thr494) isoforms.

BPIFB4 is expressed in undifferentiated and highly proliferative tissues, and in pathologic conditions like cardiac hypertrophy. The mechanism of action of BPIFB4 still remains unclear but its overexpression induces activation of the stress response (HSPs) and protein homeostasis (translation, ribosome biogenesis, spliceosome). Since these two processes promoted by BPIFB4 are lost during aging leading to apoptosis and contributing to age-related dysfunction of endothelial cells (20), we postulate a role of BPIFB4 in healthy cardiovascular aging. In line with this

hypothesis, BPIFB4 serum levels are higher in healthy-LLIs compared to frail-LLIs and young controls (21). Similar differences are seen in CD34+ progenitor cells of LLIs, which expressed higher levels of BPIFB4 RNA than cells from young controls (19). Hence, the expression of BPIFB4 is somehow related to both increased life expectancy and reparative cells activation. Importantly, old mice showed a decline of BPIFB4 expression at the vessels level as compared to young animals (19). In addition, we showed that mononuclear cells (MNCs) isolated from a/a homozygous carriers for rs2070325 (LAV) had higher levels of activated eNOS than non-carriers pointing to beneficial effects of LAV genotype and an involvement of eNOS in the LAV-associated benefit. Indeed, *in vivo* administered LAV-BPIFB4 improved eNOS activity and endothelial function measured in explanted mesenteric vessels, and reduced blood pressure in both young and old mice. To be noted, the above mentioned therapeutic effects were not observed with the administration of the WT-BPIFB4, reinforcing the genetic finding and the protective role of LAV genotype. BPIFB4 knock down in isolated vessels showed decreased eNOS and impaired endothelial function further confirming a central role of BPIFB4 in eNOS modulation. Adeno Associated Virus- (AAV)-LAV-BPIFB4 therapy in old mice showed a full recovery of both endothelial function and eNOS activity, to levels comparable to the young mice.

The mechanism underpinning the eNOS activation by LAV-BPIFB4 needs further investigation. Initial observations from our group indicate that LAV-BPIFB4 phosphorylation at serine 75 by the stress kinase PERK regulates the recruitment of 14-3-3 and heat Shock Protein (HSP90), which together interact with eNOS and other proteins forming an activated complex that cooperates to NO generation (**Figure 1**). Of note, LAV-BPIFB4 is involved in angiogenesis promotion. Intravenous injection of LAV-BPIFB4 supports reparative vasculogenesis and enhances the recruitment and homing of circulating proangiogenic cells in a mouse model of limb ischemia. Furthermore, LAV-BPIFB4 therapy induced VEGF-A together with Superoxide dismutase 2 (SOD2)/Superoxide dismutase 3 (SOD3) expression in ischemic muscles (19). These data link the expression of LAV-BPIFB4 in LLIs with a greater availability of proangiogenic cells, a higher antioxidant efficiency, and activation of molecular pathways crucial to an efficient angiogenesis.

5. Conclusions

In ancient times, explorers have incessantly searched for the fountain of youth, a source of magical water capable of reversing the aging process. This was the motivational tactic for the King Ferdinand's crew leading to the eventual discovery of Florida. By 2020, for the first time in history, people ≥65 years of age will outnumber children (<5 years of age) in the world. Therefore, scientists are urgently asked to find solutions to maintain the old population healthy and active.

Identifying genetic variations in centenarians might represent the winning strategy to treat or prevent the onset of diseases at earlier stages of life and aid in the discovery of new pathways to promote longevity with less disease. We have provide prototypical examples of such an approach. Further studies are necessary to validate the therapeutic and safety profile of BPIFB4 therapy in large animals before starting a definitive experimentation in a first-in-man clinical trial for CVD.

Figure Legends

Figure 1. Mechanism of action of the longevity-associated variant (LAV) of BPIFB4. A) Cellular stress induces protein kinase RNA-like endoplasmic reticulum kinase (PERK) activation with subsequent phosphorylation of LAV-BPIFB4, which then binds to the 14-3-3 protein. **B**) The 14.3.3/LAV BPIFB4 complex selectively binds phosphorylated endothelial nitric oxide synthase (eNOS) the activation of which is mediated by a yet to be identified kinase (question mark). Other players, such as heat shock protein (HSP) 90, are recruited to increase the production of nitric oxide (NO). Increased NO production enhances endothelial function and promotes angiogenesis.

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