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Cardiac Adaptation to Hypoxia: Novel Mechanisms and Therapeutics for Acute Myocardial Infarction

CONFIRMATION REPORT

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Cardiac Adaptation to Hypoxia: Novel Mechanisms and Therapeutics for Acute Myocardial Infarction

Introduction

Hypoxia is a physiological status in which the body or a part of the body fall short in adequate nutrient and oxygen supply at the tissue level. The heart is one of the most sensitive organs to hypoxia because it requires sustained and abundant energy supply from aerobic respiration. Hypoxia is one of dominant donors to the great morbidity and mortality rates related to a broad range of cardiovascular diseases (CVDs), such as acute myocardial infarction (AMI), heart failure (HF), atherosclerosis and pulmonary arterial hypertension[1,2]. Ischemia/reperfusion (I/R) injury as a predominant physiological injury resulted from hypoxia is an ongoing central challenge for addressing the public health concerns related to cardiovascular disease. Studies have shown that living at high altitude leads to hypoxia-resistant adaptations, in both fetal and adult hearts [3,4]. Understanding the mechanisms that underlie natural adaptations to hypoxia in cardiomyocytes could provide a unique perspective for the exploration of novel therapies for myocardial I/R injury. Moreover, current therapies for AMI lack the ability to establish cardioprotection to prevent cell death and cardiac remodelling associated with ischemia. Particularly, prognosis of AMI patients largely depends on the severity of ischemia/reperfusion injury[5]. As such, therapies protecting cardiomyocytes from I/R injury remain a priority of AMI patients.

1. Cardioprotection therapy is needed for AMI

Ischaemic heart disease (IHD) is a significant public health burden in many decades. There are 643,000 adults IHD patients in Australia, which accounted for ~ 4% of the national population from 2014 to 2015[6]. IHDs cost a huge direct economic burden to the society as well as indirect cost caused by patient productive life years shrinking[7,8]. AMI is a kind of life-threatening IHDs that attacks hearts when coronary blood flow is stopped by vessel

obstruction suddenly, causing a sudden oxygen transport decrease for cardiomyocytes and leading to tissue damage. AMI is diagnosed by the trace of myocardial necrosis in the series of a clinical manifestation. AMI is a main cause of mortality and hospital admissions around the world [9]. In 2015, there were an estimated 7.29 million patients that were diagnosed with AMI in the whole world[10]. In the US, about 1 million myocardial infarctions occur annually [11]. The mortality from AMI remains unignorable, and the morbidity of post-myocardial infarction HF is increasing.

1.1 The treatment window for successful intervention after AMI is very narrow

Currently, AMI treatment options can be divided into two categories: surgies (percutaneous coronary intervention (PCI) and coronary artery bypass grafting (CABG)) or intervention with fibrinolytics. Generally, AMI includes both non-ST segment elevation myocardial infarction (NSTEMI) and ST segment elevation myocardial infarction (STEMI)[12]. For STEMI patients, PCI must be done within 90 minutes of the onset of chest pain. If the surgery cannot be done within 90 minutes [13], the patients should be started on fibrinolytics within 60 minutes. For complicated NSTEMI patients, immediate PCI or CABG also are required within a narrow treatment window [14]. Ensuring AMI patients receive treatment within the required time is challenging, especially for STEMI patients who require surgery within 90 minutes. Two studies of US national registries of myocardial infarction suggested that the in-time rate of patients who need PCI re-vascularization is only 4.2%[15]. Even though the treatment window was enlarged to 2 hours, only 15% of patients could receive PCI in time. After normalization, the weighted mean FMC-to-door time (the time between first medical contact and arrival at hospital door) was 41 min (n=101 646; 95% CI 39 to 43, range 21-88) according to two independent reviews including 100 studies (125,343 patients) conducted in 20 countries [16]. Based on this, less than 40 min is provided in hospital for surgical intervention for STEMI patients who need PCI.

The prognosis of AMI patients largely depends on treatment timing. In ischemic heart diseases, irreversible cardiomyocyte damage occurs after ~20 min of ischemia[17]. The infarction size grows rapidly as time goes on (**Fig. 1** The relationship between time after onset of ischemia and tissue injury.)

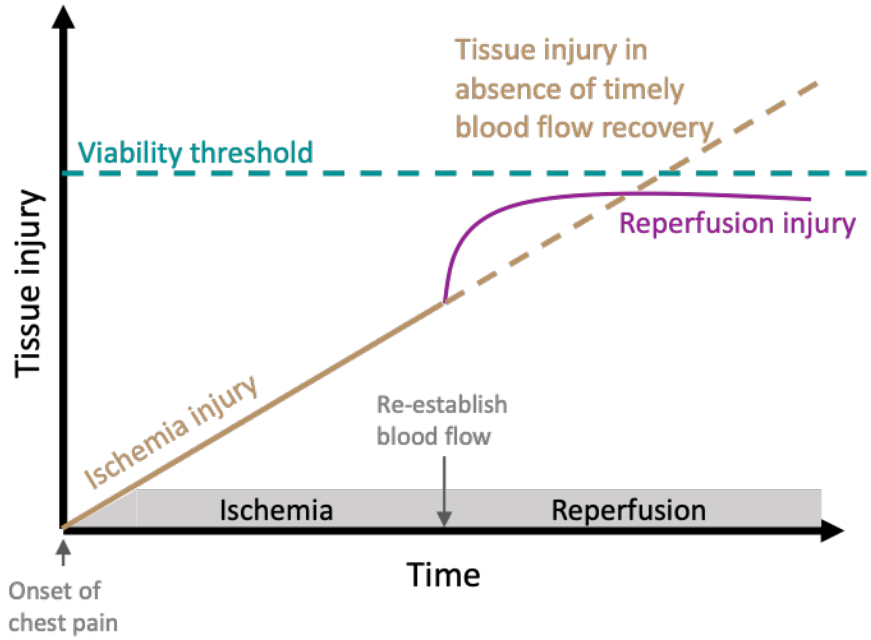


Fig. 1 The relationship between time after onset of ischemia and tissue injury. Tissue injury caused by ischemia is linearly correlated with the time it takes to re-establish blood flow. Reperfusion therapy exacerbates the tissue injury, but is necessary and the sooner it occurs, the less severe the injury will be. [17,18]

1.1.1 In-hospital mortality and long-term mortality are both related to the delay of reperfusion treatment.

A very strict treatment window for AMI is set by almost all clinical guidelines including the ACC/AHA/SCAI jointly guidelines on PCI, the ESC Guidelines for the management of AMI in patients suffering ST-segment elevation and Australian clinical guidelines for the management of acute coronary syndromes[19-21]. Reducing time delays in primary angioplasty benefits patients and relieve emergency physicians' pressure by decreasing both in-hospital and long-term mortality. A large scale research which includes 62,470 patients at 973 hospitals from 1999 to 2002 showed that the shorter door-to-needle times were, the lower in-hospital mortality was in STEMI patients who received fibrinolytic therapy [22]. For patients with less than 30 minutes door-to-needle times, in hospital mortality was only 2.9%. Among patients with 31-45 minutes door-to-needle times, in hospital mortality increased to 4.1%. A meta-analysis concluded that emergency medical services (EMS) accounts for half of the total system delay in STEMI rescue. Shorter EMS delay was also significantly correlated to lower short-term mortality in patients receiving prehospital thrombolysis ($p=0.018$) [16].

Although almost all guidelines and studies claimed the treatment window for AMI is very narrow, the longest treatment window remains inconclusive. A SCAAR registry study, a Swedish registry, illustrated that for STEMI patients, more than 1-hour delays in FMC-to-PCI were highly relevant to mortality increasing, possibly mortality in severe heart failure accounted for a large percentage. A goal of keeping FMC-to-PCI less than 1 hour might save patient lives is recommended in this research[23]. But in the latest AHA/ACC clinical guideline, the FMC-to-PCI time for acute STEMI patients was still set as < 120 min [21]. Beyond in-hospital mortality, long-term risk is also affected by the delayed initiation of reperfusion treatment. For the patients who received primary PCI to recover blood supply after AMI, there is a significant relationship between time to treatment and long-term mortality - the relative 1-year mortality rate increases by 7.5% for every 30 minutes delay in coronary blood supply re-establishment. This relationship exists in AMI patients with some other risk factors but not for low risk patients [22,24,25]. The time between symptom onset and the restoration of blood flow is critical to decrease the tissue injury in hearts and lower AMI patients' risk of death.

1.1.2 Delay of primary reperfusion therapy leads to worse cardiomyocyte injury

The delay of reperfusion treatment has other effects on clinical consequences besides mortality. The risk of recurrent infarction and infarction area size is a predominant consequence of delayed reperfusion observed by clinical researchers. In a study of 357 consecutive STEMI patients, the re-infarction rate has a positive relevance with the delay of reperfusion treatment[26]. In a one-year follow-up study, patients who originally suffered from an AMI whom were taken to the hospital within the first 3 hours for treatment has a 1.6% myocardial infarction reoccurrence, whereas patients whom were taken to the hospital longer than 3 hours after suffering from AMI has a 9% reoccurrence. In the same study, 0.6% of patients who had longer than 1 hour surgery processing time times re-attacked by MI at 1 year follow-up; few of patients with shorter than 1 hour surgery processing time had re-infarction at 1 year. The results of measuring the infarct size of patients with acute anterior STEMI without cardiogenic shock by cardiac magnetic resonance showed total ischemic time and preprocedural thrombolysis in MI were correspond to infarct size increasing on 3 to 5 days after PCI [27]. Biochemical indicators also

perform worse with treatment delay. Highly sensitive troponin T test is a relatively reliable tool to estimate the risk of death or recurrent ischemia from AMI for patients and this biomarker level is also affected by reperfusion time after onset of chest pain. Patients who arrived in hospital later from symptoms onset gain a significantly higher peak CK-MB (creatine kinase - myocardial isoenzyme) and higher troponin T levels than early arriving patients [26]. Generally, delays of reperfusion therapy profoundly exacerbate the myocardium damage, which is consistent with the relationship between delays of primary treatment and mortality increasing of AMI patients. Thus, cardioprotection seems necessary to AMI patients gain more favourable clinical outcomes.

1.2 Cardioprotection is needed by current clinical therapy for AMI

Although timely myocardial reperfusion with thrombolytic therapy or primary PCI rescued many patients with AMI, patients who underwent reperfusion therapy still had a quite mortality rate[28]. For STEMI patients undergoing PCI, the in-hospital mortality rate is between 6% and 14%[29]. The size of the myocardial infarct size determines the prognostic risk of patients with AMI[30]. There is an urgent need for treatment strategies that limit infarct size. Conversely, no cardiomyocyte protection therapy in current clinical guideline, even no one therapy for protecting myocardium in AMI rescuing is approved by government. Moreover, MI after PCI is an important safety issue. Biochemical indicators of MI still elevate in post-PCI patients, which means the reperfusion surgeries cannot solve all problems and cardiac protecting treatment is still required by patients after surgeries [31].

1.2.1 Current Treatment for AMI do not provide cardioprotection

The present treatments for AMI are predominantly reperfusion surgeries and thrombolysis. The mechanical reperfusion surgeries include primary PCI and CABG; the thrombolysis means to dissolve clots in coronary artery which are obstructing blood flow by drugs. Physicians' strategies for STEMI patients, emergency reperfusion is via fibrinolytic drugs, PCI, or, occasionally, CABG. For NSTEMI patients, reperfusion is via PCI or CABG surgery. The workflow of diagnosis and treatment can be summarized as follows:

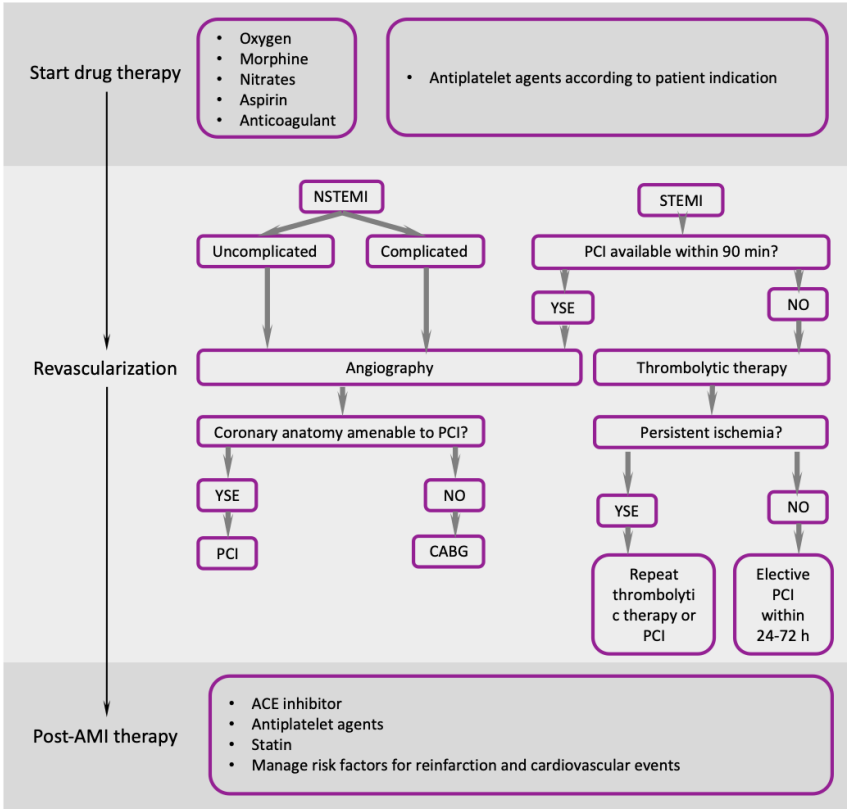


Fig. 2 AMI diagnosis and treatment workflow according to international clinical guidelines [19,21]. Drug therapy is administrated to patients on ambulance to relieve symptoms. In hospital, surgeries including PCI and CABG are standard treatment according to current clinical guidelines. After revascularization, risk management of re-infarction and cardiovascular events becomes the first priority. ACE inhibitor, antiplatelet agents, statin are used to reduce risk for patients.

In addition to reperfusion therapy, antiplatelet drugs, anticoagulants, nitrates, beta-blockers, statins are 4 mainstream kinds of drug interventions topping up the reperfusion therapies according to clinical guidelines and physician practice:

- Antiplatelet drugs, like aspirin, aim to stop further platelet activation and aggregation, and parenteral anticoagulation [32].
- Anticoagulants are used to stop disrupted atherosclerotic plaque to active the coagulation pathway, which followed by thrombin. Rivaroxaban, dabigatran and apixaban are all anticoagulants [32].

- Nitrate is a classic type of clinical drugs for ischemic heart disease. It can slacken vascular smooth muscle and the vasodilator effects are evident in systemic arteries including coronary vessels to recovery blood flew partially [31].
- Beta blockers reduce local ischemic chest pain. It reduces myocardial oxygen demand by lowering heart rate and blood, as well as relaxing blood vessels [33], thereby limiting the infarction size by reducing cardiomyocyte workload [34]. But it remains in debate that whether beta-blocker can decrease the long-term mortality or not [35].

1.2.2 Reperfusion therapies cannot solve every problem of myocardial infarction

Clearly, the most helpful way to limit infarct size is timely and complete revascularization. Due to the pathological mechanism of AMI, it is inevitable that reperfusion also increases reperfusion injury in addition to ischemic injury, and thus, it leads to a further increase in infarct size[36,37]. Cardioprotection interventions show positive outcome after primary reperfusion surgery in some clinical practices. It is up to 50% of infarction size can be reduced by interventions during myocardial reperfusion, proving reperfusion phase-specific detrimental events induced further infarction in heart[17,38]. While optimizing selective PCI and other large cardiovascular procedures (except CABG), surgeons are constantly seeking additional heart protection to reduce perioperative myocardial infarction.[39] Infarction size still increases after PCI and CABG noticed by troponin elevation in post-PCI patients [31,40].

1.2.3 Challenges to myocardium protection therapy development for AMI

In recent years, many therapeutic strategies for AMI via myocardium protection arise. However, none of them have launched in the clinic so far. Intravenous glucose potassium insulin (GIK) is a therapy based on metabolic regulation that attempts to limit myocardial infarct size. In ambulances, GIK was performed in patients with AMI suspected of acute coronary syndrome, and cardiac arrest and in-hospital mortality were lower in GIK treated group compared with placebo-treated group, but in In the comparison of the primary endpoints of AMI, no significant differences in primary clinical endpoints. [37] Levosimendan resists ischemic by opening ATP-sensitive potassium channels in vascular smooth muscle cells. It is a drug used to treat acute and compensatory heart failure. Levosimendan has

been proved the effect of prevents myocardial apoptosis during ischemia in large animal experiment models[41]. Although the original indication of Levosimendan is heart failure, it reduced ischemia side effect of revascularization surgeries in several phase I clinical trials of postconditioning ischemia-reperfusion study[42-44]. The cardioprotection function of levosimendan has been shown by many preclinical and primary clinical evidence. It might be a promising candidate for myocardium protection in AMI therapy. Colchicine is another promising candidate for cardioprotection. It has been shown to have excellent anti-inflammation and cardioprotection characteristics through immune-modulating in both pre-clinical studies and early stages of clinical trials. There are some large scale clinical trials of colchicine cardioprotective characteristics are in process, including COLCOT (the full form is Colchicine Cardiovascular Outcomes Trial; ClinicalTrials.gov identifier: NCT02551094) – a phase III study involved ~4800 patients in[45]. Still, some candidates failed in late stages despite success early on. Ciclosporin A is one of these tragedies. Although it showed clinical benefits in Phase I and some Phase II studies, no significant difference of surrogate endpoints or biomarkers were found in Phase III study[46]. Here summarize drug candidates for cardioprotection in AMI and their development stages in Supplemental material, **Table S1.** In the past decades, few candidates achieved success after Phase II trials. Moreover, none of candidates are novel compounds or new biologics. Not only novel compounds and biologics are needed but also novel targets on cardiomyocyte protect pathway.

2. Cytobiology and molecular biology of myocardial I/R injury

Myocardial I/R is a pathological condition identified by initially limiting the blood supply to the heart and then restoring perfusion and reoxygenation[5,47]. Insufficient supply of oxygen and nutrients leads to a set of sudden biochemical and metabolic changes in the myocardium. Finally, ischemia/ reperfusion activates of cell death programs. Meanwhile, the injury will be followed by transcriptional reprogramming, autoimmunity, and innate and adaptive immune activation[2,37].

2.1 Cytobiology of myocardial ischemic/reperfusion injury

Insufficient supply of oxygen and nutrients during ischemia results in a series of sudden biochemical and metabolic changes in the myocardium. Deletion of oxygen prevents

oxidative phosphorylation, leading to mitochondrial membrane depolarization and ATP depletion, thereby inhibiting myocardial contractile function [17]. As a response to mitochondrial membrane depolarization, the function of F1F0 ATPase is reversed to maintain mitochondrial membrane potential, resulting in ATP hydrolysis and increased mitochondrial inorganic phosphate[48]. Any available ATP decomposition will exacerbate this process, making the situation worse quickly. Due to ATP depletion, energy-consuming Ca^{2+} efflux activation is reduced and the re-uptake of calcium by the sarcoplasmic reticulum (SR) is limited. Therefore, Ca^{2+} keeps accumulating in the cytoplasm and eventually overloads [49]. In the insufficiency of oxygen, cellular respiration is converted to anaerobic glycolysis, which leads to aggregation of lactic acid, resulting in a decrease in pH within cells (to <7.0) [50]. The Na^{+}-H^{+} ion exchanger is activated by the accumulation of protons in the cytoplasm, then protons are extruded from the cell by exchanging with Na^{+} [51]. During ischemia, the function of $3Na^{+}$ -$2K^{+}$ -ATPase is terminated by the lack of ATP, which exacerbates intracellular Na + overload[17]. Meanwhile, the function of $3Na^{+}$-$2K^{+}$-ATPase is terminated by the lack of ATP, which means intracellular Na^{+} cannot be extruded outside. Na^{+} overloading is aggravated. In response, the cell attempts to expel Na + , which reverses the activation of the $2Na^{+}$ -Ca^{2+} ion exchanger, causing a large amount of extracellular Ca^{2+} to be transported into the cell, forming a Ca^{2+} overload [52]. The $2Na^{+}$-Ca^{2+} ion exchanger responds to high concentration of intracellular sodium ions. It performs a reverse function of squeezing Na^{+} out of cell and introducing Ca^{2+} into cytoplasm instead of extruding calcium under normal conditions. Finally, calcium overloading opens the prelude to cell death [53]. At the organ level, During this process, intracellular proteases are activated, causing destruction of myofibrils, resulting in excessive myocardial contraction and contracture necrosis [54].

When the blood supply is re-established, reperfusion triggers a series of events that further exacerbate tissue damage. During reperfusion, the electron transport chain is reactivated, producing reactive oxygen species (ROS) [55], which causes multiple damage to the cardiomyocytes[37]. First, ROS cause activation of the mitochondrial permeability transition pore (MPTP), leading to dysfunction of SR and subsequent myocardial reperfusion injury[37]. Activation of ROS is an important pathway that causes intracellular Ca^{2+} overload. Second, ROS destroys the cell membrane by lipid peroxidation, which causes the cardiomyocytes to lose their membrane integrity[37]. Third, ROS directly destroy DNA by an oxidation reaction[37].

Reperfusion and reactivation of the Na^+ -H^+ exchanger results in the elution of lactic acid, which causes a rapid recovery of physiological pH, thereby releasing inhibition of MPTP opening and cardiomyocyte contraction[56]. MPTP opening can also be induced by the recovery of mitochondrial membrane potential derived by calcium influx. Several hours after the onset of ROS release, inflammation aggravates in response to cytokines (such as IL-6, IL-8) and activated complements. Neutrophils, monocytes, macrophages, B-lymphocytes and $CD8^+$ T-cells accumulate in the infarcted myocardial tissue[37,57]. This pro-inflammation reaction to contribute to adverse post-MI left ventricular remodelling[56].

2.2 Variant cell death programs in ischemia/reperfusion injury

Cardiomyocytes suffer from apoptosis, necrosis and autophagy during I/R [2]. Necrosis and apoptosis are related to inflammation. Apoptosis is not involved in inflammatory response, it is the process of phagocytosis performed by macrophages or adjacent cells [2].

Necrosis is typically an unregulated cell death. Damage of plasma membrane and ATP depletion are two main features of necrosis. Necrotic cells and organelles, including the mitochondria, become swollen. However, the detailed mechanism of necrosis is not very clear. Only some studies have shown that cyclophilin D, death receptor, MPTP, Ca^{2+} and protease are involved, eventually leading to inflammation at the end of necrosis [58]. Some evidence suggests that necrosis can also be modulated, which is called programmed necrosis.

According to existing studies, apoptosis consists of at least two pathways: an extrinsic pathway and an intrinsic pathway[59-62]. The extrinsic pathway is performed using cell surface receptors and is characterized by being insensitive to bcl2 and involved in the activation of transduction signals derived from the tumor necrosis factor (TNF) receptor superfamily [62]. Extrinsic pathways are triggered by activation of cell surface death receptors (eg, Fas) by specific ligands[60]. Death domain-associated proteins (such as the Fas-associated death domain (FADD)) are then activated, opening the apoptotic cascade, and caspase-8 and caspase-3 activate and act. Caspase-8 cleaves only the BH3 protein and initiates a Bid-mediated exogenous pathway amplification. The truncated Bid (tBid) is transported to the

mitochondria and promotes mitochondrial apoptosis events[2]. This is an amplification that occurs in the mitochondria of the exogenous pathway. Within the mitochondria, the extrinsic pathway converges with the intrinsic pathway. The bcl-2 family of proteins controls the intrinsic pathway, Bax, only BH3 protein and Bak are involved[61]. Bax is a pro-apoptotic bcl2 family member protein. Cellular ATP depletion triggers the translocation of Bax, transducing death signals to mitochondria and SR[63]. Alternatively, only the BH3 protein converts the death signal in relatively specific stimulation. Both Bax and BH-3 only trigger mitochondrial apoptosis by altering the permeability of the mitochondrial outer membrane. Bak stimulates mitochondrial apoptors by interacting with Bax, including cytochrome C, APAF-1, c-FLIP and DEDs[2,64].

Autophagy is a highly conserved pro-survival mechanism that replenishes energy and nutrition under starvation[65]. During autophagy, cells decompose organelles, proteins, and lipids through lysosomal metabolism, thereby achieving material and energy cycling within the cell to generate energy and nutrient (amino acids and free fatty acids)[65,66]. The first step of autophagy is the autophagosome formation. Firstly, vesicles are formed by vesicle nucleation, involving the cytokines Beclin-1, UVRAG, Vps34 and IP3R. This is followed by the elongation of vesicles which is dependent on the Atg12 and Atg8 coupling pathway[67]. Then, the autophagolysosome is being assembled by fusing the autophagosome with the lysosome, resulting in lysosomal degradation[68]. There are two pathways responsible for autophagy: the mTOR pathway and the Beclin-1 pathway. The mTOR pathway acts differently between normoxia and hypoxia conditions[69]. Under sufficient blood supply, mTOR pathway suppresses autophagy by phosphorylating and deactivating Atg proteins. In contrast, parts of mTOR pathway class I complex are deactivated by PI3K-Akt signalling reduction when hypoxia occurs. Thus, autophagy is no longer supressed under hypoxic conditions[66]. Beclin-1 is a positive regulator of autophagy. It binds to the class III PI3K Vps34, Bcl-2 and Bcl-x_L, thereby facilitating autophagosome formation and relieving suppression of autophagy in hypoxia[2,70]. Although autophagy is known as one of the cell death programs, it reduces myocardial injury in hypoxia by degrading materials and nutrition to provide nutrients and energy for cell survival[71].

All three kinds of cell death can be seen in ischemia. This applies to after I/R in both clinical samples and animal model hearts. The signalling pathways for different death programs can be activated by ischemia/reperfusion.

3. Linking ischemia resistance to genetic adaptions to high altitude

Environmental conditions substantially differ between low and high altitudes. Namely, oxygen concentration decreases at high altitude. For instance, at the Qinghai-Tibet Plateau at an altitude of about 4,000 meters, the oxygen concentration is only less than 40% of the sea level [4]. After numerous generations, human's life has adapted to high altitude environments through natural selection for random genetic mutations that confer improved survival in low oxygen conditions. Genetic adaptations that permit human life at high altitude might also afford protection to tissues undergoing severe hypoxia, such as the heart during myocardial infarction. The next chapter explores the link between ischemia resistance and genetic adaptions to high altitude.

3.1 Human genetic adaptations to life at high-altitude

Previous genetic studies have reported many genetic variations between people living on the plateau and in low-lying environments, prompting further investigations into the function outcomes of these adaptations. Expectedly, the large number of these genetic variations illustrates the complexity of human adaptation to low oxygen environments. Simonson and colleagues showed that *EGLN1* and *PPARA* were two specific positively selected genes in Tibetan population, which are related to decreasing hemoglobin phenotype[72]. These two genes showed significant haplotype differences between Tibetan population and the lowland populations. *EPAS1, HBB, HBG2, EP300, HMOX2*, and *TGFBR3* have highly differentiated genomic regions in Tibetan compared to these genes' DNA sequencing in Han Chinese in Beijing by XPCLR test ($P < 0.001$)[73].

Yi and colleagues sequenced exons of 50 unrelated Tibetan individuals and compared these sequences with Han Chinese populations, finding a single-nucleotide polymorphism (SNP) in *EPAS1* with a 78% difference between Tibetan and Han samples. They listed the top 34 genes with the greatest population branch statistic (PBS) value in the Tibetan population, including *SPP1*, *TMEM206*, *Clorf124*, and *DISC1* [74]. Yang and colleagues conducted a

genome-wide study of 3008 Tibetans and 7287 non-Tibetan individuals with East Asian descent, involving 7.3 million genotyping and estimated SNPs[4]. They found *MTHFR* and *EPAS1* are highly correlated to the haemoglobin concentrations and the metabolic pathway of folate and homocysteine ($P_{GWAS} < 1.5 \times 10^{-4}$) [4]. Most of these publications demonstrated *EGLN1* and *EPAS1* are highly associated to high altitude adaptation[4,73,75-77]. These two genes play dominant roles on HIF pathway. *BRINP3*, *NOS2*, and *TBX5*, which are not relevant to HIF pathway, are also reported to relevant to vascular health of high altitude population [78]. However, no direct evidence to show these gene adaptation to the phenotypes of heart tolerance to hypoxia. The biological verification of these genes function in

3.2 Connecting adaptations to high altitude and cellular ischemia protection

Genes associated with adaptations to high-altitude could provide insights into intrinsic protective mechanisms against hypoxia. Some different physiological differences in cardiovascular system between plateau living populations and lowland residents are reported, which reflect high-altitude populations make cardiovascular functional adaptations to hypoxia environment[79-82]. But none of these studies connect genetic adaptation significant genes with key phenotypes of heart adaptation to hypoxic conditions. Some genes are picked by researchers who focus on organ protection from hypoxia. *TMEM206*, a gene encoding a proton-activated Cl^- channel (PAC), was reported as one of the genes showed different exon sequencings between the Tibetan Plateau population and Han Chinese population[74]. PAC can only open in acidic conditions, such as acidosis[83,84]. Acidosis is an important cellular event during ischemia injury[85]. It is illustrated that suppression of *TMEM206* expression shows protect effect on the brain in a stroke model in mice. It means *TMEM206* may represent a potential therapeutic target for stroke and other acidosis-associated diseases[86]. If PAC exist in cardiomyocytes, the inhibitors of PAC potentially can protect cardiomyocytes from ischemia injury. ASIC1a inhibition results in protection during cerebral ischemia as well as in the heart based on current work in the Palpant laboratory [87]. *EPAS1* encodes hypoxia inducible factor 2α (HIF-2α), which was reported to have the most significant difference in past genome-wide screening of Tibetans vs Han Chinese [75,88]. Two studies of *EPAS1* showed that it played a role in myocardial ischemia. HIF-2α initiates the epithelial growth factor amphiregulin (AREG) expression in

cardiomyocytes, which enhances myocardial ischemia tolerance[89]. Tanaka and colleagues' study suggested HIF-2α regulates the expression of the adrenomedullin (AM) gene as a part of the response to hypoxia, which helped cardiomyocytes adapt to heart failure and hypoxia [90].

Many high altitude adaptation genes show expression in human left ventricular, human induced pluripotent stem cell cardiomyocytes, and mouse heart, which makes the functional investigation of these genes in cardioprotection possible[91-93]. These genes include ion channels (*TMEM206* and *TMEM38B*), cytokine (*SPP-1*, *EGLN1*/HIF-1, *EPAS1*/HIF-2α), transcription regulator (*MBNL1*, *TBX5*), membrane receptor (*EDNRA*, *PPARA*). They potentially disturb the process of ischemia/reperfusion, as this process initiates from mitochondrial to ion channel and delivery signalling to downstream pathway by various cytokines with transcription adjustment. Therefore, studying the ability of humans to adapt to high altitudes will help to elucidate the various mechanisms by which humans overcome the pressures of hypoxic supply, which are important for both lowland and highland populations.

4. Embryonic adaptation to cardiac hypoxia

In addition to the high-altitude environment, hypoxia *in utero* is another natural low oxygen condition. In the human body, oxygen pressure is also very different in different organs and tissues. The site of the largest partial pressure of oxygen is the alveolar arteriole. The partial pressure of oxygen here is 100 mm Hg. [3] Red blood cells transport oxygen to whole body tissues. The partial pressure of oxygen in different tissues varies from 20 mmHg to 40 mmHg. The partial pressure of oxygen in fetal arterial blood is approximately 20-30 mmHg, which is similar to the oxygen partial pressure in the physiological hypoxia state of adult tissues [3]. Remarkably, the fetal heart is more resistant to cell death caused by hypoxia than the adult heart[3]. On the one hand, the fetus changes the flow of blood, allowing more blood to flow from the surrounding tissue to important organs, to compensate for hypoxia, and to induce the conversion of hypoxia-dependent genes. On the other hand, more anaerobic energy metabolism pathways can be achieved in the fetus, improving the viability of the fetus in an anaerobic environment[94]. But this ability will be lost along with heart

development[95]. In addition, in terms of morphological physiology, the size of fetal cardiomyocytes, the histomorphosis and proliferation of myofibrils are different from those of adult cardiomyocytes[96,97]. Different expression of genes can be observed in fetal cardiomyocytes and adult cardiomyocytes[93]. Studies have shown that HIF-1α plays a central role in the hypoxia tolerance of embryonic hearts[95]. HIF-1α comprehensively knockout is consequent in suspend development before day E9 and fetal death before day E11[98,99]. Embryos observed to have significant cardiovascular abnormalities, including abnormal cardiac structures, aortic structures and abnormal remodeling of the cephalic vessels and mesenchymal cell death[98]. HIF-dependent signaling pathways directly activate related genes, causing aerobic respiration to turn into anaerobic respiration. The altered gene expression products include glucose transporter 1 (Glut1), aldolase A, enolase 1 (ENO1), lactate dehydroglucosidase A, and phosphoglycerate kinase 1 (PGK1) [3].

Cardiomyocytes, as the basic unit of heart contractile function, show structure changes during heart development and these changes are associated with heart hypoxia resistance. Among them, the important protein TnI constituting the myocardial fiber has two TnI subtypes whose development is regulated. The slow skeletal TnI (ssTnI) subtype is mainly expressed in fetal cardiomyocytes. This subtype gradually decreases as the heart develops. By adulthood, the expression of the cardiac TnI (cTnI) subtype in the myocardium is much higher than that of ssTnI [100]. TnI isoforms change during cardiomyocytes development, and this shift modifies acidosis sensitivity after ischemia[101].TnI isoforms conversion in cardiomyocytes lineage and development regulate acidosis sensitivity after ischemia[101]. TnI isoforms conversion during cardiomyocytes lineage and development regulate acidosis sensitivity after ischemia[101]. By replacing the alanine residue at cTnI164 in adults with histidine, the researchers converted it into a highly expressed ssTnI in immature cardiomyocytes, attempting to improve the adult rat cardiomyocytes using the unique biochemical structure of ssTnI[102,103]. Acidosis resistance. Subsequently, they performed calcium-sensitive titration experiments on bioengineered rat cardiomyocytes, demonstrating an increase in acidosis resistance *in vivo* [104]. Changes during development which make the hypoxia resistant ability disappear can be taken as potential therapeutic targets for cardioprotection from hypoxic injury.

5.Cardiac ischemia/reperfusion model based on iPSC-CMs and animals

I/R injury is the cellular reflection of myocardial infarction. Mimicking I/R injury on cardiomyocytes and *ex vivo/in vivo* hearts is a general method to study myocardial infarction pathobiology. Researchers keep testing various methods to induce I/R injury on cardiomyocytes, including neonatal rat cardiomyocytes, ESC-induced cardiomyocytes, and hiPSC-induced cardiomyocytes[47,105,106]. For a long time, researchers have relied heavily on non-smart people models for the early development of cardioprotective drugs. In fact, although many drugs have shown promise to reduce I/R damage in preclinical studies, these drugs have not shown benefit in large clinical trials. The use of animal models may be one of the reasons why these therapies have not been successfully forwarded to human clinical use. Thus, hiPSC-CMs, as human original cardiomyocytes, own overwhelming advantage than other cell lines for ischemia modelling. It is desirable to combine animal models of *ex vivo* or *in vivo* with human-derived cell models to provide a more reliable prediction of the effectiveness of therapies in future human clinical studies.

5.1 *In vitro* ischemia/reperfusion models based on iPSC-CM

Researchers use low oxygen and low glucose conditions to mimic the lack of blood supply *in vitro*. Human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs) are promising research tools for cardiovascular disease research. They share the whole genome with humans, providing a genetic similarity that avoids the problem of species comparison [107-109]. However, hiPSC-derived cardiomyocytes are similar to fetal phenotype in structure and function, which is dependent on glycolysis and therefore have partial ability to resist hypoxia[110]. Thus, researchers try to make metabolism maturation to achieve hypoxia sensitivity on hiPSC-CMs. White and his colleagues used modified media to obtain more mature hiPSC-CMs[109]. On this basis, they mimicked the I / R injury process and examined the sensitivity of cardiomyocytes to pH and physiological changes in glucose utilization. It is showed that low pH (pH 6.2) induced more severe cytotoxicity than normal pH (pH = 7.4) with significance in both standard medium and fatty acid treated medium. Glucose deprival also plays an important role in ischemic injury in this study. More LDH is released without glucose feeding during ischemia. The cardiomyocytes under pH 6.2 meanwhile without

glucose suffered worst ischemia injury with significance to other groups. Hypoxia condition was realized in 0% oxygen incubator in this study. Beside fatty acid treating, glucose replacement also accelerates iPSC-CMs maturation. Ward and colleagues established an oxygen stress model on iPSC-CMs on 25th day after differentiation with 6-hour 1% oxygen culture followed by 6-hour reperfusion in 10% oxygen incubator [107]. During ischemia, using galactose instead of glucose exacerbates I/R injury to shift the cells' metabolism from fetal-associated glycolysis to adult-associated mitochondrial respiration. Glucose group and galactose group performed similarly at 5% oxygen condition. At 1% oxygen, however, galactose cultured cardiomyocytes showed much worse injury than the glucose group. It is worth to be noticed that I/R injury happened in normal pH (pH 7.4) in this study[107]. However, ischemia model can also be achieved on iPSC-CM without intervention for maturation. hiPSC-CMs cultured in standard media (RMPI with B27) treated at 0% oxygen without glucose for 45 min following with reperfusion at normoxia with pO_2 up to 90% for 3 hours suffered ATP depletion, ROS increasing and calcium overloading. Apoptosis can be tested in this model[111]. The calcium management and electrophysiology of hiPSC-CMs changes under hypoxia and acidosis condition even without intervention for achieving maturation.

For I/R injury models, low oxygen concentration and glucose replacement are two necessary conditions for I/R injury model on hiPSC-CMs. However, metabolism maturation provides more similar energic dynamic intracellular environment to reduce hypoxia resistance. Studies focusing on how to make better ischemia models by improving iPSC-CM maturation are still limited, especially in a genetic perspective. Further work needs to be done to illustrate the links between iPSC-CMs maturation and ischemia resistant.

5.2 Animal models for I/R research

Currently, in the early stages of the development of therapies, researchers mainly tested the ability of candidate therapies to improve I/R damage through animal models. The animal model that usually simulates I/R injury is to block the left descending coronary artery (LAC) by suture for a certain period of time to form ischemia, so that the animal heart is completely or partially into hypoxia, and then the suture is released to form Reperfusion [18].

An *ex vivo* I/R model named Langendorff system has been proven undoubtedly invaluable for cardiovascular research since established by Oscar Langendorff almost 200 years ago [112]. According to the original Langendorff system, the researchers replaced the original blood perfusion channel with aortic cannula to achieve cardiac perfusion. The perfusate flows through the aortic cannula through the aorta in the retrograde direction of normal blood flow, causing reverse pressure on the aortic valve, forcing the aortic valve to close, filling the aorta with a line of perfusate[113]. Then, perfusion buffer passes through the coronary vein through the vascular bed to the coronary sinus above the tricuspid space at the posterior wall of the atrium, through the right atrium drainage, avoiding the blood flow passage through the left ventricle, leaving the left ventricle dry and forming ischemia. In the Langendorff model, researchers can choose a retrograde perfusion of the heart at a constant or constant flow rate. Coronary flow, myocardial contractility/left ventricular systolic and diastolic function and electrocardiogram are the main parameters, which can be collected via the Langendorff system. Although this model was established in rat hearts, a wide range of mammalian species have been studied in this way, further adapting the system to mice, rats and rabbits, as wells as larger animals such as dogs, pigs, primates and even human hearts[114].

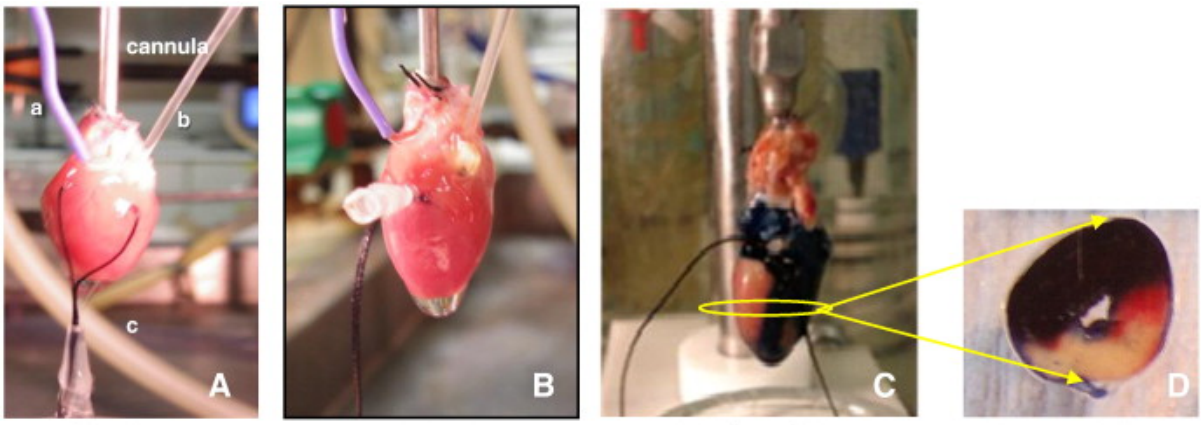


Fig. 3 The Langendorff system made using the rat heart[114]. The Langendorff system is divided into a stable phase (3A), an ischemic-reperfusion phase (3B), and an infarct zone staining marker (3C & 3D). The suture is first formed around the left anterior descending branch (LAD) to form a snare, which begins to infuse and forms a stable system. Then place the snare around the LAD and secure it. After a period of infusion, the release of the Evans

blue staining solution is injected into the heart along the bloodstream. Areas with an ischemic risk will not be stained. The heart was sectioned and stained with TTC. The viable myocardium will show pink and the infarcted myocardium will be light yellow.

The Langendorff heart preparation has now become an important tool for the study of ischemia-reperfusion therapy because it provides valuable cardiac physiology information. In this model, global ischemia can be achieved by completely stopping the flow of blood, and coronary flow can be attenuated to achieve low-flow ischemia. It can also be blocked by ischemia in the ischemic artery (usually the LDA) to induce ischemia. However, the ex vivo model cannot epitomize the overall physiology, such as the role of endocrine. *In vivo* models can be classified into small and large animal models. Because of economic efficiency and high adaptability, rodent animals (mouse, rat, and rabbit) are most widely used in I/R injury models. The problem is that there are many differences between the heart of the rodent and the human heart. Rodents have a higher heart rate and myosin exhibits different isoforms. Their heart basal metabolism is higher, and cardiac electrophysiology also has species uniqueness. For large animals, pigs are the closest analogs to the human body, and their morphology is similar in size to the human heart. Compared to the dog's circulatory system, the pig's circulatory system, like humans, has no protective coronary collateral blood flow; the pig's heart has no natural resistance to MI compared to the primate's heart [115].

Therefore, it is still very challenging to translate therapies work well in animal experiments into clinical therapy. On one hand, in most of the therapy test on animal model animal models, drug candidates are administrated into animals before ischemia. But prevent administration never happens in clinical. On the other hand, animal models do not recapitulate human specific physiology. Drug screening initiating from hiPSC-CMs models and following large animal models experiments may improve the accuracy of therapy targeting and reduce risk.

6. Summary

AMI is one of the premier causes of death globally. Currently, clinical treatment approaches for AMI do not protect cardiomyocytes from the stress of injury. Furthermore, reperfusion of coronary vessels is responsible for > 50% infarction area. It is therefore critical to develop novel therapeutics that address the sensitivity of cardiomyocytes to AMI. The low success rate of translating myocardial protection therapies to the clinic is in large part caused by the challenges of translating discoveries from pre-clinical models to clinical practice and the potential approaches for targeting the heart's sensitivity to hypoxia have been very limited.

We have taken an innovative approach to this fundamental question by reviewing relevant data on genetic adaptations to two kinds of natural hypoxia environments: high-altitude hypoxia and embryonic hypoxia. These data provide a rich resource to study novel mechanisms of genes that may play significant roles in the heart's natural adaptation to hypoxia. Our objective is to develop functional studies of these genes to identify candidates for myocardial protection. We will implement studies across diverse model systems from rodent *ex vivo* studies to *in vitro* human stem cell-derived heart muscle to establish a framework for functional validation and discovery. These insights will provide new knowledge into the genetic basis of cardiac sensitivity to hypoxia and provide opportunities for drug development for cardiac therapeutics to treat patients with AMI

7. Aims and Significance

Current therapies for AMI lack mechanisms to protect the myocardium, though cardiomyocytes are needed to be protected from injury both during ischemia and after reperfusion. Although significant researcher has been invested in developing drugs to protect cardiomyocyte from infarction, few drug candidates have gone to phase III clinical trials, and none have showed significant advantages compared with current therapies. We are seeking a new approach to discovering the molecular and cellular basis of the heart's injury response to infarction. While some drug candidates for cardiomyocyte protection target known pathways involved in ischemia/reperfusion; many genes we have identified are not studied to date and provide an enormous opportunity to establish novel mechanisms and therapeutic leads[116]. In this study, I will combine computational and

biological research approaches to find a path to discover mechanisms underlying cardiomyocyte sensitivity to ischemia/reperfusion injury using insights gained from the heart's natural adaptation to hypoxia.

Aim 1: Investigate key gene participating in hypoxia resistance of immature cardiomyocyte.

Immature cardiomyocytes survive in embryonic hypoxia environment but with, with maturation during development, diverse changes in cell state result in increase sensitivity to hypoxia later in development. Few studies has been performed to explore the genetic and cellular basis of this development switch. We hypothesise that insights gleaned from analysis of changes in gene expression associated with developmental maturation can provide a vantage point into natural adaptation to hypoxia. In this aim, we will make use of human iPSCs undergoing a maturation time course to study gene expression changes during cardiomyocyte differentiation. These data will provide a basis for linking molecular changes to functional studies of cardiomyocyte sensitivity to hypoxia. The integration of these data will provide a basis to identify key genes related to hypoxia sensitivity, especially the gene helping immature cardiomyocytes resist to hypoxia during development.

Aim 2: Seek vital genes of heart natural adaptation to high-altitude hypoxia.

Many genes are known to be related to high-altitude hypoxia adaptation according to statistical genetics studies. Genetic variants have been identified as distinguishing people living in plateaux versus mainland populations from DNA sequencing of blood samples. However, there has been no systematic functional testing of genes identified in these studies to the functional biology of cardiac high-altitude adaptation. It is critical to determine how genetic adaptation in heart impact cardiac sensitivity to ischemia since the heart is one of the most hypoxia sensitive organs. But the difficulties to obtain heart samples make it impossible. To address this, we will combine computational and biological studies and check related gene expression in iPSC-CM, neonatal mouse heart and adult mouse heart based on previous studies in our group and published data. First, we will review and summarize publications about high-altitude adaptation genetic studies and establish a gene library for screening genes of cardiac genetic adaptation to hypoxia. Second, we will check selected genes' expression in 3 datasets, including iPSC-CM single cell sequencing data, CAGE sequencing of iPSC-CM, bulk RNA sequencing of both neonatal and

adult mouse hearts under normoxia or hypoxia conditions. Third, candidate genes from this narrowed list will be searched for having a putative link to cardiac biology or ischemia. Finally, we will take these genes forward for CRISPRi editing to establish genetic knockdown models in iPSCs. These cell lines will be utilized to study hypoxia models on both wild type hiPSC-CMs and CRISPRi cardiomyocytes. The sensitivities of different genotypes of cardiomyocyte will give answers to both single gene functions in hypoxia resistance and provide insights into how loss of function provides increased resistance or sensitivity to cardiac ischemia or acidosis.

Aim 3: Based on the genetic adaptation to natural hypoxia environment, discover and develop novel therapies to protect cardiomyocytes from ischemia injury for AMI patients. Currently, there are multiple problems blocking innovative development of cardiomyocyte therapeutics including lack of novel compounds and lack of innovative targets discovery. We will aim to find novel targets based on our genetic and functional modelling with the goal of utilizing substantial novel peptide resources in IMB for novel drug discovery. Genetics adaptations to natural hypoxia provide key strategies to seek novel targets for saving cardiomyocytes under hypoxia conditions. In this aim, genes passing the strict screening from the first two aims will be utilized for drug discovery with a specific focus on ion channels as receptors as ideal druggable targets. Stable cell lines of HEK-293 heterologous expressing target genes/protein will be established. We will seek specific agonist or antagonist of potential novel targets from IMB/QEDDI libraries for ion channels ligands. Auto-patch clamp (APC) and Fluorescence Imaging Plate Reader (FLIPR) will be recruited to realize high-throughput screening at the early stage. Promising candidates will be tested on animal models in the future. Besides traditional drug therapy, modulating gene expression to make cardiomyocytes similar to hypoxia resistant state can be a scenario for increasing the survival rate of AMI patients.

This project aims to establish a novel drug discovery system to seek cardioprotective candidates for AMI patients. Findings of this study will provide a novel potential druggable targets to protect cardiomyocytes from ischemia/reperfusion injury, which will open opportunities for drug discovery in this field. Cardioprotective drugs will benefit patients by reducing heart infarction size after AMI-onset, enlarging the treatment window and decreasing mortality.

8. Summary of Work to Date

AIM 1: Investigate key gene participating in hypoxia resistance of immature cardiomyocyte.

1. We finished RNA sequencing for 11 time points during the hi-PSCs differentiation to cardiomyocytes. Data analysis is on-going.

AIM 2: Seek vital genes of heart natural adaptation to high-altitude hypoxia.

1. We reviewed the literatures of high-altitude genetic adaptation and find genes which played important roles.
    1) 41 genes are involved and their expression in 3 sequencing datasets are checked (**Table 1.** The expression of gene candidates on hiPSC-CMs, mouse cardiomyocytes, and human left ventricular.)

Table 1. The expression of gene candidates on hiPSC-CMs, mouse cardiomyocytes, and human left ventricular.

	Human left ventricular	hiPSC-CMs	Mouse hearts		Human left ventricular	hiPSC-CMs	Mouse hearts
ACVR1B	✓	✓	✓	*MBNL1*	✓	✓	✓
ARNT2	✓	✓	✓	*MDH1B*	✗	✓	✗
ASIC1	✓	✓	✓	*MKL1*	✓	✓	✓
DISC1	✗	✓	✓	*NAGLU*	✓	✓	✓
DST	✓	✓	✓	*NOS2*	✗	✗	✓
EDNRA	✓	✓	✓	*NOS3*	✓	✓	✓
EDNRB	✓	✓	✓	*OR10X1*	✗	✗	✗
EGLN1	✓	✓	✓	*OR6Y1*	✗	✗	✗
EPAS1	✓	✓	✓	*OTX1*	✗	✓	✗
FABP3	✓	✓	✓	*PKLR*	✗	✓	✗
FAM98C	✓	✓	✓	*PPARA*	✓	✓	✓
FANCA	✗	✓	✓	*PRKAA1*	✓	✓	✓
FXYD6	✓	✓	✓	*PSME2*	✓	✓	✓
HBB	✓	✓	✓	*RFX3*	✗	✓	✓

HBG2	✗	✓	✗	SLACK	✓	✓	✓
HIST1H2BE	✗	✓	✓	SPP1	✓	✓	✓
HIST1H3C	✗	✗	✓	TBX5	✓	✓	✓
HIST1H4B	✗	✓	✓	TMEM164	✓	✓	✓
IFI27L1	✓	✓	✗	TMEM206	✓	✓	✓
KLF2	✓	✓	✓	TMEM38B	✓	✓	✓
KRTAP21-2	✗	✗	✗	TTLL3	✓	✓	✓
LRRC3B	✗	✗	✓	VAV3	✗	✓	✓

Gene name: they will be explored
Gene name: they will be excluded
Gene name: they will be as backups

2) We narrowed down this gene library by integrating their connection to ischemia or heart diseases (**Table 2.** Gene candidates from high-altitude adaptation expressing differently between Sham and MI mouse hearts and **Table 3.** Connections between gene candidates and ischemia protect)

Table 2. Gene candidates from high-altitude adaptation expressing differently between Sham and MI mouse hearts

Gene name		Expression in adult mouse heart
ACVR1B	Sham	36.0675
	MI	45.4248
ARNT2	Sham	0.3153
	MI	1.2297
ASIC1	Sham	6.2940
	MI	6.5874
DISC1	Sham	2.5093
	MI	2.8625
DST	Sham	81.9754
	MI	94.4767
EDNRA	Sham	61.5137
	MI	63.1431
EDNRB	Sham	17.8946
	MI	23.8427
EGLN1	Sham	677.2454
	MI	509.1340
EPAS1	Sham	227.9770
	MI	188.8457
FABP3	Sham	1326.6295
	MI	1247.6475
FANCA	Sham	6.8144
	MI	5.9495
FXYD6	Sham	76.3393
	MI	101.6419
HIST1H2BE	Sham	12.2169
	MI	12.4236
HIST1H4B	Sham	0.5627
	MI	3.3797
KLF2	Sham	53.6239
	MI	47.7335
MBNL1	Sham	288.0638
	MI	214.4716

Gene name		Expression in adult mouse heart
MKL1	Sham	12.4763
	MI	13.9715
NAGLU	Sham	23.9519
	MI	36.3229
NOS3	Sham	34.0702
	MI	30.4213
PPARA	Sham	125.4994
	MI	97.3554
PRKAA1	Sham	24.6196
	MI	23.2281
PSME2	Sham	11.0015
	MI	12.2812
RFX3	Sham	2.6301
	MI	3.1087
SLACK	Sham	5.6257
	MI	0.6716
SPP1	Sham	0.1602
	MI	240.6666
TBX5	Sham	37.3223
	MI	42.3890
TMEM164	Sham	107.9990
	MI	176.5864
TMEM206	Sham	5.6257
	MI	7.3314
TMEM38B	Sham	41.1474
	MI	44.7674
TTLL3	Sham	2.3407
	MI	2.0767
VAV3	Sham	5.6298
	MI	6.8184

 # Gene name: expressed differently between Sham group and MI group
 # Gene name: expressed similarly in Sham group and MI group

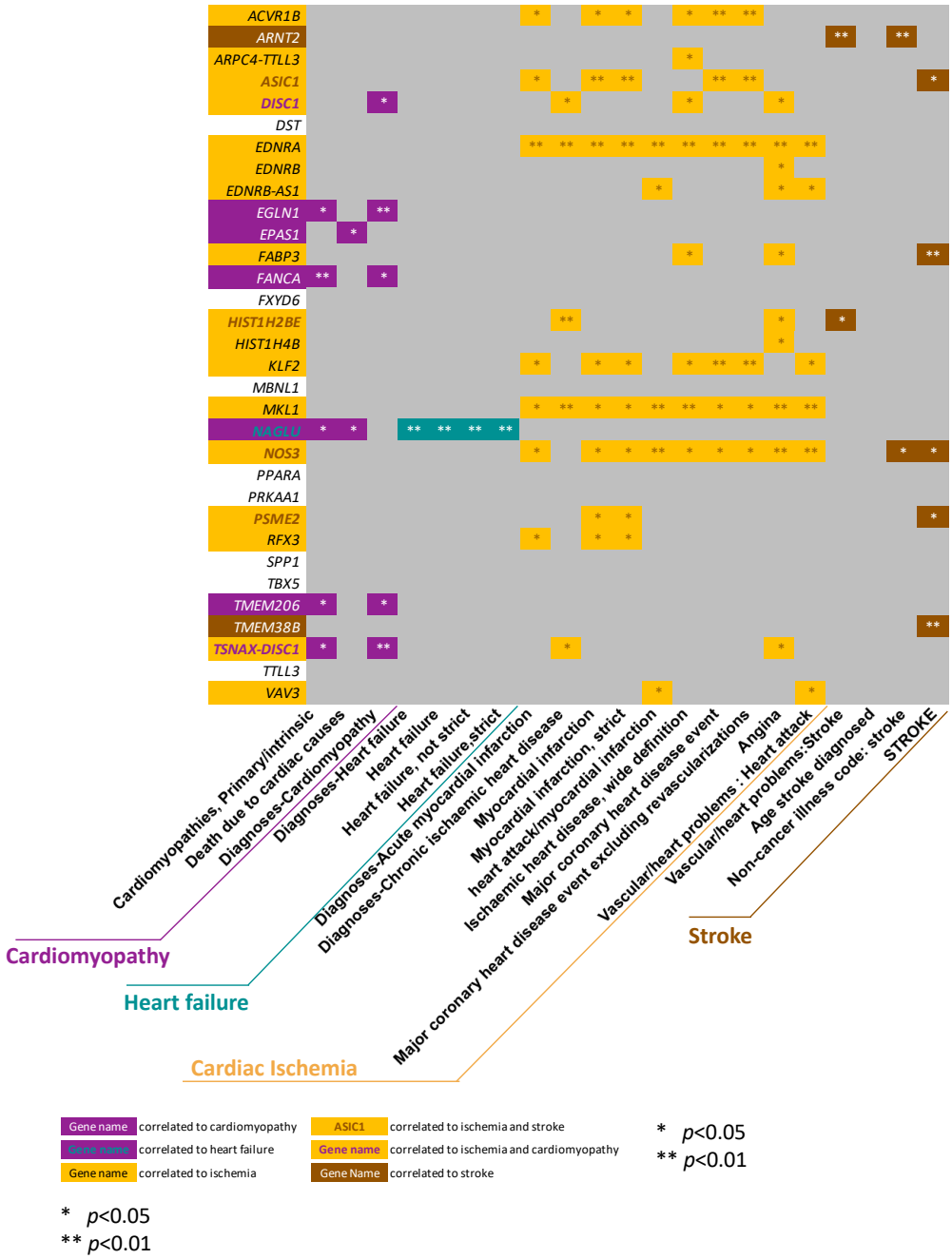
Table 3. Connections between gene candidates and ischemia protect

Gene name	Connection to hypoxia	Membrane channels
ACVR1B[117]	- Upregulated in hypoxic primary monocytes.	YES
ARNT2[118]	- *ARNT2* forms functional HIF complexes; - ARNT is part of the HIF pathway, which mediates adaptive responses to help cells survival under hypoxia; - plays a role in zebrafish heart development	NO
ASIC1[119]	- participates in ischemic brain injury; - inhibitors of ASIC1a shows cardioprotection under the acidosis condition (study in our group).	YES
DISC1[120]	- an intracellular scaffolding molecule; - DISC1 stability is changed in hypoxic conditions.	NO
DST[121,122]	- long term hypoxia treatment on human primary macrophages decreases *DST* transcription indirectly; - situating dystonin in cardiac muscle fibers affects the intricate structure of the sarcomere.	NO
EDNRA[123]	- the endothelin A receptor is encoded by *EDNRA*; - activation of endothelin A receptors protect rat hearts from hypoxia injury; - the activation of endothelin 1 signalling pathway has been shown to exert cardioprotection against chronic intermittent hypoxia.	NO
EDNRB[118,124]	- suppression of *EDNRB* expression improve the hypoxia resistance of mouse hearts.	NO
EGLN1[125]	- encodes a hydroxylase, which catalyses the post-translational formation of 4-hydroxyproline in HIF-α.	NO

EPAS1[76]	- active under hypoxic conditions; - a key player in heart development.	NO
FABP3[126]	- *FABP3* overexpression enhances mesenchymal stem cell proliferation survival in hypoxia	NO
FANCA[127]	- The FANCA gene encodes a protein that is involved in a cell process known as the fanconi anemia (FA) pathway. - Hypoxia disrupts the FA pathway in cancer.	NO
FXYD6[128]	- overexpression > 2 folds under hypoxia in human bone marrow-derived stem cell.	YES
HIST1H2BE[129]	- corelated to hypoxia stimulation during neuronal differentiation.	NO
HIST1H4B	N.A.	NO
KLF2[130,131]	- induced expression during hypoxia in human endothelial cells - inhibits hypoxia-inducible factor 1 alpha expression	NO
MBNL1[132,133]	- upregulating of *MKL1* protects sevoflurane-pretreated mice against I/R injury after total knee arthroplasty; - related to heart development.	NO
MKL1[134,135]	- transcription factor of myocardin which is a key regulator of smooth muscle cell differentiation; - the protein encoded by *MKL1* activates ET-1 transcription in response to hypoxia in endothelial cells;	NO
NAGLU	N.A.	NO
NOS3[136,137]	- transcript of *NOS3* destabilizes after hypoxia treated (on cellular level); - a NOS3 gene SNP rs2070744 was high corelated to hypoxic-ischemic encephalopathy in Chinese Han population.	NO
PSME2[138]	- participates in HIF regulation indirectly.	NO
SLACK[139-141]	- predicted to play a role in hypoxia (when both Na^+ and H^+ intracellular concentrations are increasing)	YES

SPP1[142]	- encodes osteopontin (OPN), a member of the matricellular protein family; - expression low in adult cardiomyocytes but high in immature cardiomyocytes.	NO
TTLL3[143]	- expresses differently in bone marrow-derived VSELs exposed to intermittent hypoxia.	NO
RFX3	N.A.	NO
PPARA[144,145]	- down regulated by HIF1 during hypoxia; - has potential cardioprotection in rat experiment.	NO
PRKAA1[146]	- encodes the alpha1 catalytic subunit of AMPK; - is important to the transcriptional activity of HIF pathway; - involved in physiological responses to hypoxia and pregnancy.	NO
TBX5[78]	- expresses most in the left ventricle and the aorta; - SNAPs of *TBX5* are correlated to the left ventricle of the heart symptoms.	NO
TMEM206[86]	- suppression of *TMEM206* protect brain from ischemia injury in mouse MCAO model.	YES
TMEM38B	N.A.	YES
VAV3[117,147]	- expression decreases in human primary monocytes after hypoxia modification; - the protein encoded by *VAV3* induces GTPase activity and is involved in angiogenesis in response to hypoxia.	NO

3) Find related heart or ischemia diseases from UK Biobank data to high-altitude adaptation genes by GWAS analysis (**Fig.4** GWAS analysis of UK Biobank dataset for high-altitude adaptation genes.).



* p<0.05
** p<0.01

Fig. 4 GWAS analysis of UK Biobank dataset for high-altitude adaptation genes. In this analysis, we grouped genes into 6 groups according to their correlations to phenotypes. *ACVR1B*, *ARPC4-TTLL3*, *EDNRA*, *EDNRB*, *FABP3*, *HIST1H4B*, *KLF2*, *MKL1*, *RFX3* and *VAV3* are correlated to ischemic phenotypes; *ARNT2* and *TMEM38B* are related to stroke; *EGLN1*, *EPAS1* and *TMEM206* are associated with cardiomyopathy; *ASIC1a*, *DISC1*, *TSNAX-DISC1* and *NAGLU* are related to multiple diseases relevant phenotypes.

4) We keep the following genes in our candidate library for cardioprotection from hypoxia injury: *ACVR1B*, *ARNT2*, *EDNRA*, *EDNRB*, *EPAS1*, *FABP3*, *FXYD6*,

IFI27L1, *KLF2*, *MBNL1*, *MKL1*, *NAGLU*, *NOS3*, *PPARA*, *PSME2*, *SLACK*, *SPP1*, *TMEM206*, *TMEM38B*. These genes expressing in human cardiomyocytes have a relatively big change when hearts suffer hypoxia or show significant correlation with cardiac/ischemia diseases.

5) Take *TMEM206* as a promising candidate, conduct primary biological verification. We tested universal chloride current blocker, 4,4'-Diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS), on hiPSC-CM acidosis-hypoxia model and found DIDS protected cardiomyocytes from hypoxia injury at pH 5.0 (**Fig.5** Chloride current blockers reduced acidosis injury on hiPSC-CMs). Control groups were treated with 0.2% DMSO in HBSS during hypoxia; 80 μM groups were treated with 80 μM in HBSS during hypoxia; pre-treat 80 μM groups are based on 80 μM groups with 2 h treatment of 80 μM DIDS in media before hypoxia. The same as 160 μM groups and pre-160 μM groups.

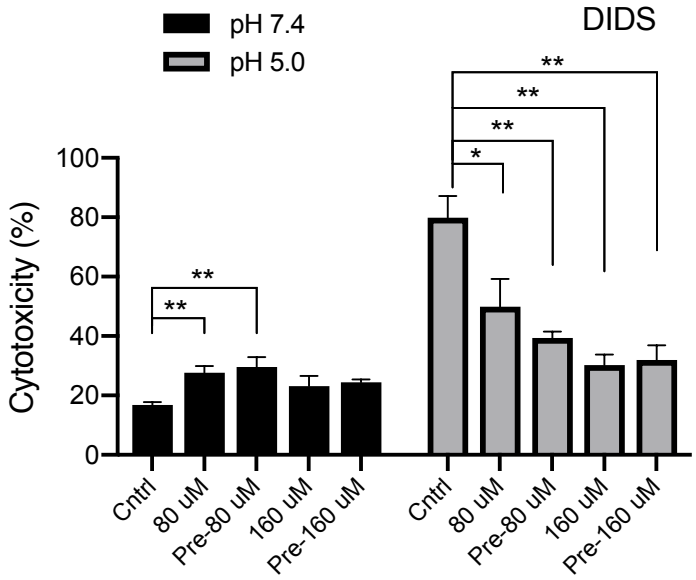
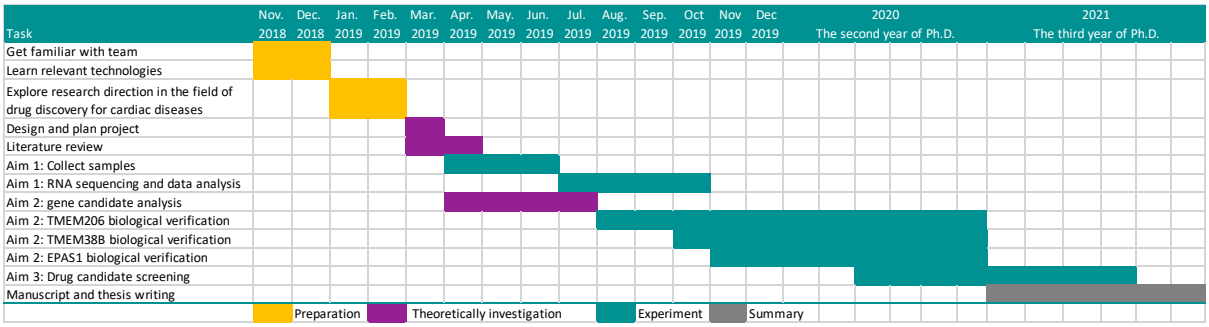


Fig. 5 Chloride current blockers reduced acidosis injury on hiPSC-CMs. We executed LDH test on hiPSC-CMs after 18h hypoxia treatment (5% O_2) in pH 7.4 and 5.0 HBSS. In pH 7.4, 80 μM groups and pre-80 μM groups, DIDS showed cytotoxicity on hiPSC-CM when compared with control (** $p < 0.01$). In pH 5.0, both 160 μM and 80 μM DIDS showed protect effects on hiPSC-CMs from acidosis injury significantly (* $p < 0.05$, ** $p < 0.01$).

9. The Research Plan and Timeline

In this project, we plan to complete at least 3 gene candidates primary biological verification within half a year. Then, we will choose the best gene to do further exploration, such as animal experiments. After confirmation of this gene's function, we will set this gene as a potential target and establish a high-throughput method to discover novel ligands for the potential druggable target.



10. Skills and Resources

Skills	
Biological technologies	- hiPSC culture and cardiomyocytes differentiation
	- CRISPRi cell line establishment
	- Hypoxia model on hiPSC-CMs
	- Langendorff system on rat hearts
	- Intracellular calcium dynamic measurement on iPSC-CMs in a high-throughput method
Bioinformatic skills	- Bulk RNA sequencing data normalization and analysis
Resources	
Equipments	- Tissue culture system
	- RNA sequencing system
	- Fluorescence Imaging Plate Reader
	- Auto-patch clamp platform
Compound library	- Peptides
	- Novel small moleculars

11. Current and Planned Publications

We plan to publish 2-4 papers in this project. Firstly, the genetic dynamic of hiPSC-CM differentiation and cardiomyocyte maturation will form an independent paper. Second, if TMEM206 knock-out can protect cardiomyocyte from hypoxia injury, this will be a publication with the exploration of chloride current's function in cardiomyocyte ischemia (No one covered this topic before). Third paper depends on the results of TMEM38B and

EPAS1 studies. The last paper will be about drug candidate discovery, which depends on the results of Aim 3.

12. Supplemental material for literature review

Table S1. Failed clinical trials for cardiovascular AMI drug candidates

Drug	Start	Complete	Stage	Main outcomes	Identifier
colchicine	2015	-	Phase 4	Withdrawn recruitment	NCT02995512
erythropoietin	2008	2012	Phase 2	Single high-dose EPO administered timely after successful reperfusion in patients with STEMI did not reduce infarct size at 3-month follow-up.	NCT00648089
Prolastin C	2013	2018	Phase 1 & 2	Prolastin C shortens the duration of the I/R injury in STEMI patients.	NCT01936896
Abciximab	2008	2017	Phase 3	STEMI patients receiving abciximab in ambulance did not improve either STR or TIMI flow rate after PCI. But it showed benefits on side endpoints.	NCT00638638
Tocilizumab	2015	2017	N.A.	Tocilizumab is supposed to protect cardiomyocytes via suppress IL-6. But outcomes showed tocilizumab cannot improve CRP performance compared with control group, though it was safe and safe and well tolerated	NCT02419937

Octagam (IVIG)	2007	2014	Phase 3	Octagam (IVIG) failed to reduce left ventricular remodeling in patients with myocardial dysfunction during hospitalization after acute myocardial infarction.	NCT00430885
Ciclosporin A	2006	2007	Phase 2 & 3	Ciclosporin A persistently reduced infarct size at 6 months follow-up.	NCT00403728
Ciclosporin A	2012	2015	Phase 3	A single intravenous Ciclosporin A bolus before primary PCI didn't change ST-segment resolution or hs-cTnT, and did not improve clinical outcomes or LV remodeling up to 6 months.	
Nicorandil	2015	2018	Phase 3	No result available	NCT02449070
colchicine	2017	2022	Phase 2	Recruiting. For left ventricular remodeling treatment in AMI.	NCT03156816
Methotrexate	2012	2017	Phase 2	Failed to reduce infarction size and worsened LVEF at 3 months.	NCT01741558
Morphine chlorhydrate	2010	2017	Phase 3	Intracoronary morphine at reperfusion reduce infarct size or improve left ventricular systolic function in patients with STEMI without significance.	NCT01186445
Melatonin	2008	2016	Phase 2	No result available	NCT00640094

Morphine	2015	2019	Phase 4	No result available	NCT02627950
Exenatide	2015	2019	Phase 3	No result available	NCT02404376
Anakinra	2013	2019	Phase 2 & 3	No result available	NCT01950299
ATH3G10	2019	2020	Phase 2	Recruiting. For patient with STEMI.	NCT03991143

13. Abbreviations

Full form	**Abbrev.**
enolase 1	ENO1
fanconi anemia	FA
Fas-associated death domain	FADD
FADD-like interleukin-1-β-converting enzyme	FLICE
Fluorescence Imaging Plate Reader	FLIPR
First Medical Contact	FMC
glucose insulin potassium	GIK
glucose transporter 1	Glut1
heart failure	HF
hypoxia inducible factor	HIF
hypoxia inducible factor 2α	HIF-2α
Ischemia/reperfusion	I/R
ischaemic heart disease	IHD
left anterior descending artery	LAD
left descending coronary artery	LCA
myocardial infarction	MI
mitochondrial permeability transition pore	MPTP
methylenetetrahydrofolate reductase	MTHFR
non-ST segment elevation myocardial infarction	NSTEMI
osteopontin	OPN
proton-activated Cl− channel	PAC
percutaneous coronary intervention	PCI
phosphoglycerate kinase 1	PGK1
reactive oxygen species	ROS
single-nucleotide polymorphism	SNP
sarcoplasmic reticulum	SR
ST segment elevation myocardial infarction	STEMI
tumor necrosis factor	TNF

triphenyltetrazolium chloride	TTC
anti-ultraviolet radiation-related genes	UVRAG
vesicle protein sorting 34	Vps34

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