Cellular models for fundamental and applied biomedical research

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Chapter 7

Summary and future perspectives

Summary

The general introduction of this thesis, **Chapter 1**, first describes the requirements of proper cell lines that need to be established for research models. Next, p53 and pocket proteins as main regulators in both senescence and quiescence are discussed. Finally, an overview is presented of the capacity of LT to immortalize cells via p53 and RB pathways bypassing senescence or inducing cell cycle reentry of arrested cells.

In **Chapter 2,** the impacts of heterochromatin proteins on premature senescence is investigated. It is shown that the level of heterochromatin protein 1α (HP1α) associated with the nuclear matrix is significantly increased in premature MEFs. Upon DNA damage, HP1α phosphorylation is significantly compromised leading to delayed formation of γ-H2AX foci. Knocking down of HP1α alleviates the delayed DNA damage response and accelerates senescence. Hence, HP1α is identified as a new molecular and mechanistic insight into heterochromatin mis-organization, delayed DNA damage response, and early senescence, providing a promising intervention target for both progeria and normal aging.

Chapter 3 describes a novel bipartite lentivirus vector to quantify cell-to-cell fusion by using the immortalized human myoblasts as a cell fusion model. To this purpose, immortalized human myoblasts are transduced either with a flippase-activatable *Gaussia princeps* luciferase (GpLuc) expression unit (acceptor cells) or with a recombinant gene encoding FLPe, a nuclear-targeted and molecularly evolved version of flippase (donor cells). The key advantage of this system is the replacement of *Photinus pyralis* luciferase (PpLuc) by GpLuc, which is a secretory protein allowing repeated analysis of the same cell culture with a much higher specific luciferase activity than PpLuc. In addition, to investigate whether the speed of FLPe in myotubes is limited due to the presence of the nuclear localization sequence (NLS), the ability of $FLPe^{NLS+}$ and an LV encoding an NLS-less version of FLPe ($FLPe^{NLS-)}$ to activate latent GpLuc genes are compared in myogenic fusion assays. The results show that both $FLPe^{NLS+}$ and $FLPe^{NLS-}$ activate the latent GpLuc gene but when the percentage of FLPe-expressing myoblasts is limiting, $FLPe^{NLS+}$ generally yields slightly higher signals than $FLPe^{NLS-}$. While at low acceptor-to-donor cell ratios, $FLPe^{NLS-}$ is usually superior. However, at high FLPe concentrations the presence of the NLS negatively affects reporter gene expression. This study develops a rapid and simple chemiluminescence assay for quantifying cell-to-cell fusion progression based on GpLuc.

In **Chapter 4**, a conditionally immortalized atrial myocytes (iAMs) line with preserved cardiomyogenic differentiation ability is established. Its proliferative and contractile phenotypes is controlled by a simple change in culture medium composition. Under proliferative conditions, the cells lose most of the properties typical for cardiomyocytes (CMCs) and proliferate with an average doubling time of 38 hour. Under differentiation conditions, the cells stop dividing and gradually reacquire a phenotype resembling that of primary atrial myocytes (pAMs) in gene expression profile, sarcomeric organization, contractile behavior and electrical properties and response to ion channel-modulating compounds. Following high-frequency electrical stimulation reentry is induced in monolayers of these cells resembling atrial fibrillation, which could be terminated by tertiapin treatment, just

like in pAMs monolayers. In addition, iAMs could readily engraft and undergo cardiomyogenic differentiation after injection in atrial tissue. In conclusion, by the controlled expansion and differentiation of iAMs, large numbers of functional CMCs were generated with properties far superior to any of the existing immortalized CMC lines. This cell line provides an attractive cardiac cellular model for disease modelling, drug screening, cell therapy, tissue engineering and fundamental research.

Chapter 5 describes the generation of monoclonal lines of conditionally immortalized brown preadipocytes (iBPAs) using a few mouse brown preadipocytes as starting material. These cells retain long-term proliferation as well as adipogenic capacity. Multilocular lipid droplets are abundantly formed and brown fat markers including uncoupling protein 1 (*Ucp1*) are highly expressed in brown fat cells differentiated from iBPAs. Furthermore, the differentiated cells respond to β3-adrenergic stimulation by increasing glycerol release and *Ucp1* expression. Taken together, iBPA-derived brown fat cells represent a functional easy-to-use model system for fundamental and applied research into BAT.

Chapter 6 shows an *in vitro* model for investigating the action potential (AP)-prolonging and associated proarrhythmic effects of Kv11.1 blockers established by neonatal rat ventricular myocyte cultures. In this study, a newly designed and synthesized compound LUF7244 were proved to fully prevent drug-induced proarrhythmic effects as an allosteric modulator. This is the first study to proof that allosteric modulation of I_{Kr} could protect against drug-induced arrhythmias *in vitro* by preventing potentially arrhythmogenic changes in AP characteristics. This study could therefore provide a rationale to counteract drug-induced ventricular arrhythmias by pharmacological combination therapy relying on allosteric modulation of Kv11.1 channel activity.

Future perspective

Inhibition of premature aging

Except the importance of HP1-mediated heterochromatin formation, heterochromatic gene silencing, chromatin remodelling and DNA damage response, HP1 involved in the repressive function of the retinoblastoma protein (pRB) by binding to the cyclin E promoter[1]. Besides, HP1 was found interacted with a number of other transcriptional repressors[2], indicated that it might have a role in inhibiting many other promoters. Thus, a more general, genome-wide function of HP1 in suppressing gene transcription should be considered in the future study. Rapamycin represses mTOR pathway, which in turn inhibits DNA damage response, and therefore slowing down cellular senescence[3,4]. In the accelerated aging model, mTOR signaling contributes to stem cell dysfunction that occurs in response to endogenous DNA damage, which can be rescued by rapamycin treatment[5]. In this scenario, it would be interesting to examine whether rapamycin could rescue defective DNA repair in *Zmpste24*-null cells and to find the relationship between abnormal prelamin A/ HP1 and mTOR in future study.

Improvement of the cell fusion assay

In **Chapter 3**, an rapid and simple assay to quantify the cell-to-cell fusion progression is developed. However, the use of FLP-FRT system to active the latent Gpluc gene delays the real monitoring time for hours. The sensitivity of the current assay could be improved by changing FLP-FRT system to the bioluminescence resonance energy transfer (BRET) by using two interacted proteins[6]. Besides, the prompter driving monitor genes expression can be optimized by changing a higher activity one.

Improvement of iAM1 differentiation

Various studies have shown that TGFβ critically involved in cardiac differentiation. TGFβ family members have been found to promote cardiomyogenic differentiation in embryonic stem cells (ESCs) and critically involve in the expression of cardiac-specific markers[7-11]. Besides, TGF-β1 significantly increases differentiation efficiency of human CMC progenitor cells into functional CMCs[12]. Apart from TGF-β1, factors such as neuregulin[13], follistatin-like 1[14] and insulin-like growth factor[15,16] have exhibited their cardioprotective effects which may further support the survival of the cells during differentiation. Although iAM1s spontaneously differentiate to functional CMCs in absence of dox with the properties very similar to that of primary AMs, these factors could be candidates to further improve the differentiation and maturation of iAM1s and this will be an interesting topic for future study.

Improvement of the model for drug screening

In **Chapter 6**, the use of neonatal rat ventricular CMC monolayer cultures and optical mapping to investigate compounds is a physiological screening, capable of determining their effects on action potential duration and pro-arrhythmic potential. However, this approach can be further improved by using conditional immortalized human ventricular myocytes. Although mammalian cardiac cell can predict drug responses and cardiotoxicity similar to human cardiac cells, a human system of course has the greater likelihood to mimic the responses in patients. Conditional

immortalized cardiac cells are able to rapidly divide and spontaneously differentiate, therefore they can generate large number of functional CMCs economically. Consequently, combination of these cells and voltage optical mapping can potentially be expanded to high throughput screening for developing drugs including allosteric modulators. In addition, changes in CMC electrophysiological properties upon drug treatment may display differently in a cell layer (2-dimension) than in the heart (3-dimension). The use of Langendorff technique may have greatly contributed to our current understanding of the electrophysiology on the whole heart.

Conditional immortalization in cardiac repair

Heart failure is a growing epidemic caused by the loss of CMCs. Various cell sources have been used for cardiac regeneration and repair, each with its own advantages and challenges.

Human ESCs[17-22] and induced pluripotent stem cells (iPSCs)[23-25] derived CMCs have shown their ability to improve the cardiac function in damaged heart of animal models. However, generation of CMCs from ESCs or iPSCs yields heterogeneous populations of mostly immature CMCs as well as non-CMCs. Their relatively immaturity reduces their efficacy and endogenous integration. Also, the contamination of immature stem cells can induce to teratoma formation after the transplantation[26]. Besides, injection of CMCs derived from human ESCs in damaged myocardium has proarrhythmic risk[22]. Since proper alignment of cardiomyocytes play an important role in the heart function including the electric propagation and the contractile force[27], most likely the injected CMCs cannot align properly with the host CMCs which caused increased electric heterogeneity[28]. Moreover, delivery of high amount of donor cells into the damaged myocardium is very difficult. Cells transplanted in suspensions suffers from low engraftment and cell survival, since the cells may be squeezed out by the contractile force of the heart. Tissue engineering could help the alignment by using graft to guide the alignment of implanted CMCs, however, it is quite challenging to tightly adhere transplanted tissue on the contracting heart as well as subsequent vessel generation and blood supplement into the tissue.

Several groups have directly reprogramed fibroblasts into “induced cardiomyocyte-like cells” (iCMs) using cardiac transcription factors to improve cardiac function[29,30]. These studies provide proof-of-concept that the same cells that cause scar can be reprogramed to replenish the lost CMCs. While much questions remain to be addressed before clinical translation. At present, identification of fibroblast to iCM reprogramming relies on genetic lineage tracing approaches, which may cause false positive by cell fusion and the leakage of Cre activity. Currently, all *in vivo* reprogramming studies have used integrating viruses, which have the potential risk of oncogenesis. Besides, the reprogramming efficiency, maturity and integration of iCMs need to be further improved.

Many factors and pathways showed their functions on stimulation of CMC cell cycle re-entry which bring an attractive strategy to replenish CMCs lost in disease heart by inducing the re-entry of mature CMCs[31-34]. For instance, transgenic expression of Cyclin D2 significantly decreased the infarct size with effective cardiac regeneration[35]. miR-590 and miR-199a stimulated adult CMC proliferation and enhance myocardial recovery after myocardial infarction[36]. However, valid

measurements are required to monitor new CMC expansion as well as their therapeutic outcome. More insights about the molecular mechanisms and stronger pro-mitogenic genes of CMC proliferation need to achieve far more than presently available. Moreover, this strategy is largely limited by the oncogenic potential of pro-proliferative activity.

Another alternative cell source for cardiac repair could be the conditional immortalized cardiac cells. Conditional immortalized CMCs have the ability to amplify sufficiently *in vivo* which avoid the difficulties to delivery large number of cells into the heart. The proliferation of these cells is tightly and reversibly controlled by inducer that provides a protection from oncogenic risk and a repetitive repair upon multiple damage, respectively. Most important, immortalized CMCs could rapidly and synchronously differentiate to pure and mechanically and electrically mature CMCs, in the meantime forming functional couplings with neighbour CMCs from the host. The use of conditional immortalized CMCs in cardiac regeneration would obviate many of the difficulties faced by other cell types, such as immune-compatibility, oncogenic rick, enough cell expansion or delivery of a large number of cells, differentiation, and functional integration.

Future immortalization targets and their applications

Many animals such as rat, mice, dog, and primates are sacrificed to acquire cells from different organs for research purpose which is costly, laborious and not animal-friendly. Immortalization of cell lines from different animal spices and various organs will be an alternative way without sacrificing animals and with advantages.

Disease modeling is important for understanding the mechanisms and finding treatments of the disease. For this reason, models should be able to fully mimic the disease microenvironment. Conditional immortalization of the disease cells caused by the mutations of genes will generate large number of disease cells for development of acquired disease models and identifying new therapeutic treatments. Moreover, immortalization of disease cells from individual patient could be helpful to develop patient specific therapeutics targets.

In cell therapy, the transplanting of patient specific immortalized cells to the damage tissues may eliminate the chances of immune rejection. Similar for the gene therapy, patient specific cells can be genic corrected and amplified by immortalization in the laboratory and then transplanted to one's own body, hence there is no risk of immune rejection. Cell sheet-based tissue engineering is a new generational cell-based regenerative medicine which requires huge amount of functional cells. The conditional immortalization is an attractive cell sources for tissue engineering because it is able to provide pure, abundant, and fully functional cells by using only a few cells as start material.

Cardiac toxicity is a big safety concern for drug development[37]. Immortalized cardiac cell line has shown its globally and efficiently respond to drug induce hazard in **Chapter 4**. Actually, immortalization of human ventricular myocyte would be the ideal cellular model for drug screening upon cardiac safety issue. Moreover, conditional immortalization has the potential to provide a plentiful source of patient-derived cells to screen or test experimental drugs for disease treatment.

Human cell lines have the ability to produce proteins with full human post-translational modifications (PTMs; most notably glycosylation) same to those synthesized naturally in humans, which are a frequently used expression system for biopharmaceutical manufacturing[38]. On one hand, the expression of oncogenes is required for cell lines to grow rapidly to achieve a high cell density and synthesize concentrated protein products. On the other hand, the possibility of transmitting oncogenes from the cell lines to human body raises safety concerns. In this case, conditional immortalization therefore provide an extra layer to protect from this issue.

Moreover, conditional immortalized cell lines provide an attractive new model system for fundamental research. For instance, CMC proliferation and re-differentiation is a potential therapeutic target for myocardial regeneration and repair. However, little is known, on the fundamental mechanisms underlying cell cycle re-entry, proliferation and re-differentiation of CMCs. Given the highly attractive feature of the iAM1s that they spontaneously undergo cardiomyogenic differentiation in the absence of dox and fully differentiated iAM1s resume cell dividing ability in the presence of dox both in a highly synchronized way, this homogenies population provides an useful model to identify the crucial factors involved in cardiac differentiation and de-differentiation (**Figure 1**).

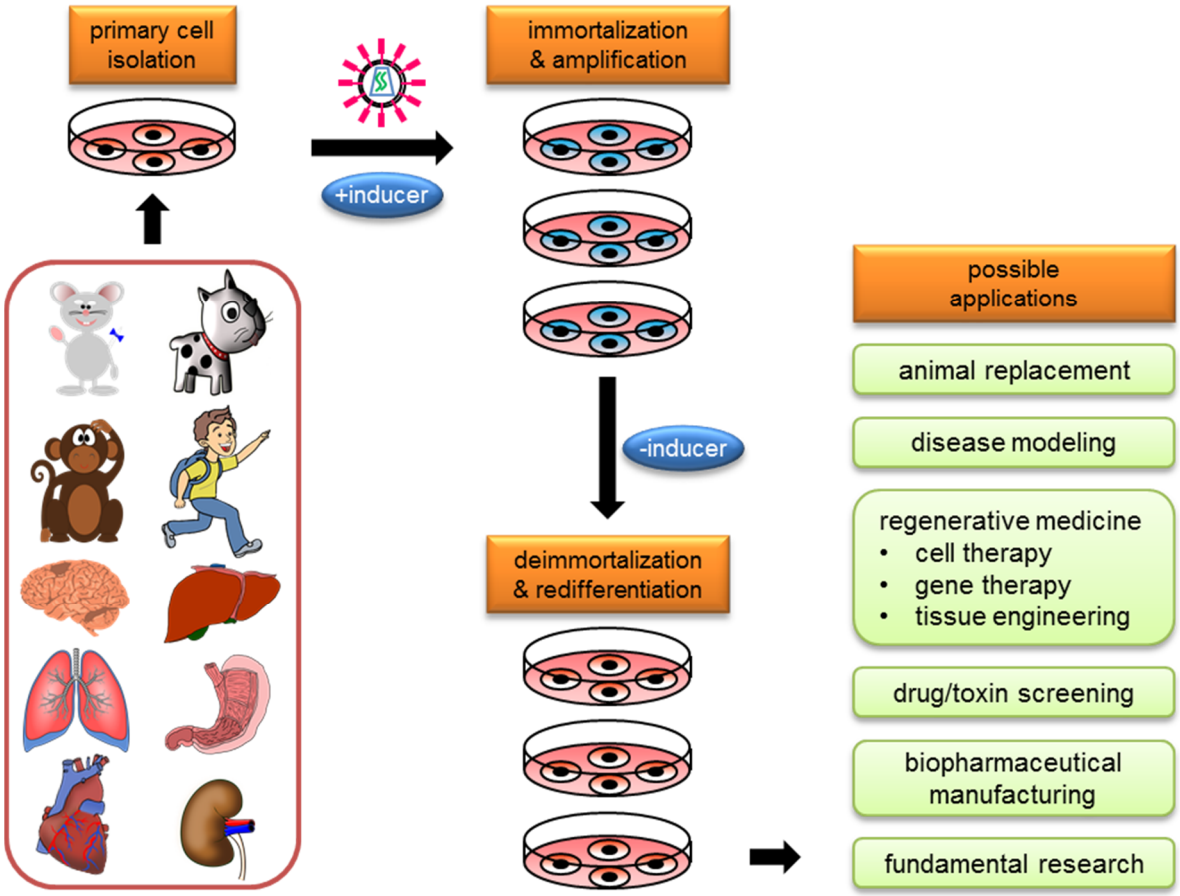

Figure 1. Future immortalization targets and their possible applications. Cell of interesting isolated from different organs of species are used for the reversible immortalization, amplification, re-differentiation and subsequent application.

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Samenvatting

In de algemene introductie van dit proefschrift, **Hoofdstuk 1**,worden de voordelen van cellijnen als onderzoeksmodellen beschreven. Vervolgens worden de rollen van p53 en de "pocket proteins" als belangrijke regelaars van zowel "senescence" als "quiescence" bediscussieerd. Tenslotte wordt er een overzicht gegeven over het vermogen van het "simian virus 40" LT eiwit om cellen te immortaliseren via de p53 en pRb signaaltransductieroutes, waardoor "senescence" wordt omzeild en in slapende cellen de celcyclus wordt gereactiveerd.

In **Hoofdstuk 2** wordt het effect van heterochromatine eiwitten op "premature senescence" onderzocht. In dit hoofdstuk wordt aangetoond dat de eiwitexpressie van aan de kernmatrix gebonden heterochromatine eiwit 1α (HP1α) significant verhoogd is in premature MEFs. Wanneer er DNA schade optreedt, leidt dit tot een significante verstoring in de fosforylering van HP1α, wat zorgt voor een vertraging in de formatie van γ-H2AX foci.

Hoofdstuk 3 beschrijft een nieuw tweedelig lentiviraal vectorsysteem om fusie tussen cellen te kwantificeren door gebruik te maken van humane myoblasten als celfusie model. Voor dit doeleinde werden geïmmortaliseerde humane myoblasten getransduceerd met een flippase-activeerbaar *Gaussia princeps* luciferase (GpLuc) expressiemodule (acceptorcellen) of met een recombinant gen coderend voor FLPe, een naar de kern getransporteerde en moleculair geëvolueerde versie van flippase (donor cel). Het belangrijkste voordeel van dit systeem is het gebruik van GpLuc in plaats van *Photinus pyralis* luciferase (PpLuc). GpLuc heeft een veel hogere luciferase activiteit dan PpLuc en wordt bovendien uitgescheiden waardoor dezelfde celkweken meerdere keren geanalyseerd kunnen worden. Daarnaast hebben we onderzocht of de enzymatische activiteit van FLPe in myotubes gelimiteerd is door de aanwezigheid van een kernlokalisatiesignaal (NLS). In een myogene fusie assay werden wild-type $FLPe^{NLS+}$ en een NLS-loze versie van FLPe ($FLPe^{NLS-}$) gebruikt om latent GpLuc te activeren. De resultaten laten zien dat zowel $FLPe^{NLS+}$ als $FLPe^{NLS-}$ het latente GpLuc gen activeren. Maar wanneer het percentage FLPe-expresserende myoblasten gelimiteerd is, wordt er in het algemeen een iets hoger signaal gedetecteerd in $FLPe^{NLS+}$ ten opzichte van $FLPe^{NLS-}$ myoblasten, terwijl bij lage acceptor-donor cel ratio's meestal $FLPe^{NLS-}$ beter functioneert. Echter, bij hoge FLPe concentraties heeft de aanwezigheid van de NLS een negatieve werking op de reporter genexpressie. Deze studie laat een snelle en simpele chemiluminiscentie assay zien voor het kwantificeren van fusie tussen cellen gebaseerd op GpLuc.

In **Hoofdstuk 4** wordt een conditioneel geïmmortaliseerde atriale myocyten (iAM) cellijn met geconserveerd cardiomyogene differentiatie eigenschappen gemaakt waarvan het fenotype (proliferatief of contractiel) kan worden gecontroleerd door een simpele verandering in de compositie van het kweekmedium. Onder proliferatieve condities verliezen de cellen het merendeel van hun eigenschappen die typische zijn voor cardiomyocyten (CMCs) en verdubbelen zich gemiddeld elke 38 uur. Onder differentiatiecondities stoppen de cellen met delen en verkrijgen geleidelijk aan een fenotype dat lijkt op het fenotype van primaire atriale myocyten (pAM) voor wat betreft genexpressieprofiel, sarcomere structuur, contractiel gedrag, elektrische eigenschappen en effecten van ionkanaal-modulerende stoffen. Hoog frequente

elektrische stimulatie induceerde rotors (zogenaamde elektrische stormen) in iAM monolagen lijkend op boezemfibrilleren welke beëindigd konden worden door toediening van tertiapin net zoals in monolagen van pAMs. Daarnaast bleken iAMs zich na injectie in boezemweefsel te kunnen differentiëren in CMCs die niet te onderscheiden waren van de omliggende endogene hartspiercellen. Concluderend kan dus worden gesteld dat met behulp van iAMs, grote hoeveelheden functionele CMCs geproduceerd kunnen worden via gecontroleerde proliferatie en differentiatie. De eigenschappen van deze hartspiercellen zijn superieur aan die van alle bestaande cellijnen van geïmmortaliseerde CMCs. De iAM cellijn is daardoor een aantrekkelijk cellulair modelsysteem voor het nabootsen van hartziekten, drug screening, celtherapie, "tissue engineering", en fundamenteel onderzoek.

Hoofdstuk 5 beschrijft de creatie van monoclonale cellijnen van conditioneel geïmmortaliseerde bruine pre-adipocyten (iBPAs) met enkele bruine pre-adipocyten uit muizen als startmateriaal. De iBPAs behouden langdurig zowel proliferatieve als adipogene eigenschappen. Multiloculaire vetdruppeltjes worden rijkelijk gevormd en markers van bruin vetweefsel, inclusief uncoupling protein 1 (Ucp1), komen sterk tot expressie in gedifferentieerde iBPAs. Daarnaast reageren deze cellen op β3-adrenergische stimulatie door een verhoogde glycerolproductie en een verhoogde expressie van Ucp1. iBPA-afgeleide bruine vetcellen vertegenwoordigen dus een functioneel "easy-to-use model" voor fundamentele en toegepaste wetenschap naar bruin vet.

In **Hoofdstuk 6** wordt een kweekmodel gebaseerd op neonatale ventriculaire myocyten van de rat beschreven om verlenging van de actiepotentiaal (AP) door zogenaamde $K_v11.1$ blokkers en de hiermee geassocieerde pro-aritmische effecten onderzoeken. Deze studie bewijst dat een nieuw ontworpen en gesynthetiseerde stof (LUF7244) pro-aritmische effecten volledig kan voorkomen via allosterische modulatie. Dit is de eerste studie die aantoont dat allosterische regulatie van I_{Kr} *in vitro* bescherming biedt tegen drug-geïnduceerde aritmiën door potentiele aritmogene veranderingen in de AP karakteristieken te voorkomen. Deze studie zou als basis kunnen dienen voor de ontwikkeling van farmacologische combinatietherapieën ter bestrijding van drug-geïnduceerde ventriculaire aritmiën. Effectieve geneesmiddelen die door hun remmende werking op het $K_v11.1$ ionkanaal en het hiermee gepaard gaande risico op plotselinge hartdood momenteel niet kunnen worden toegepast zouden dan alsnog kunnen worden ingezet.