To mark the 60th birthday of *Circulation Research* (1953–2013), the editors have commissioned *Circulation Research Classics*, a series of commentaries highlighting seminal articles published in the Journal for the past 6 decades that have importantly shaped cardiovascular research. Written by leading experts, *Circulation Research Classics* are intended to describe the effect of these articles on the field by putting them in a historical perspective. The concept of classic is inextricably linked to time, a classic is something that maintains its value regardless of its age. Thus, an important consideration in selecting the articles to be highlighted is that they have stood the test of time, which is the most reliable indicator of the value of scientific work. By looking back at the illustrious past of *Circulation Research*, we hope to promote a deeper appreciation of the contributions of this Journal to the advancement of knowledge.

Classic Studies of Cultured Cardiac Myocyte Hypertrophy
Interview With a Transformer

Christopher C. Glembotski

***Circulation Research* Classics Summary**
This article is about three classic publications in *Circulation Research* from the laboratory of Dr Paul Simpson.

Differentiation of Rat Myocytes in Single Cell Cultures With and Without Proliferating Nonmyocardial Cells
Simpson, P. and Savion, S.
Circ Res. 1982;50:101–116

Myocyte Hypertrophy in Neonatal Rat Heart Cultures and Its Regulation by Serum and by Catecholamines
Simpson, P., McGrath, A., and Savion, S.
Circ Res. 1982;51:787–801

Stimulation of Hypertrophy of Cultured Neonatal Rat Heart Cells Through an α_1-Adrenergic Receptor and Induction of Beating Through an α_1- and β_1-Adrenergic Receptor Interaction
Simpson, P.
Circ Res. 1985;56;884–894

By way of these publications, Paul Simpson transformed how we think about the molecular and cellular mechanisms of cardiac myocyte hypertrophy.

Cardiac Hypertrophy 101

At my institution, I teach a course entitled The Molecular Basis of Heart Disease. Among the topics covered in the course is the cellular and molecular basis of pathological cardiac hypertrophy and heart failure. The reading list for the course includes many of what I believe to be classic articles on cardiac myocyte growth. Among the articles that have strongly influenced our understanding of the cellular and molecular basis of the pathology are 3 that were published by Dr Paul Simpson in *Circulation Research* in 1982[1,2] and 1985.[3] I became aware of these articles when they were published because my laboratory was examining the secretion of endocrine and paracrine factors by the heart, and our focus was on the peptide hormone, atrial natriuretic factor. In part, our studies required that we prepare separate cultures of rat atrial and ventricular myocytes, and that we maintain them in serum-free medium to test the effects of various adrenergic agonists on atrial natriuretic factor expression, post-translational processing, and secretion. Thankfully, the articles of Paul Simpson described with clarity and completeness how to not only isolate neonatal rat ventricular myocytes, but also how to maintain them in serum-free medium. Perhaps most remarkable to those of us studying mechanisms of cardiac myocyte function was the description of the growth effects of adrenergic agonists on cultured cardiac myocytes.

I decided to highlight these 3 articles in this installment of *Circulation Research* Classics because the techniques described in them are timeless mainstays in the technology toolbox of molecular cardiology research laboratories around the world, and because the results reported in them have had such great effect on the field. In addition to providing essential methodological detail, these articles report the results in a complete and convincing manner. Additional features of the articles that have contributed to their enduring, transformative nature include the following conclusions:

1. One can and must control the type, quantity, and density of the cells cultured from the neonatal rat heart to obtain valid, meaningful results.
2. The media components must be defined, and their composition and quality must be consistent.
3. The volume of media used in cultures can profoundly affect the results and, therefore, must be carefully considered and then made consistent.

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4. Experiments must be designed and performed with keen attention to detail and with a vision on the applicability of the results to the physiology and pathology under study.

Together, these classics have been cited >1300×. Moreover, the number of articles published subsequently in which the search phrase cultured neonatal rat cardiac myocytes appears has dramatically and continually increased from the mid 1980s, when these classics were published, to 2003, after which the citation rate has leveled (Figure 1). This citation pattern underscores the continued importance of the neonatal rat cultured heart cell model system in molecular cardiology research. I found that these articles serve as ideal starting points for teaching students about research on pathological cardiac hypertrophy and heart failure. In fact, they are the first 3 articles assigned in my class, the first section of which is entitled Cardiac Hypertrophy 101.

Background: Interview With a Transformer

In preparing for this article, I conducted an interview with Paul Simpson, during which I asked him why, in the early 1980s, he decided to examine the effects of adrenergic agonists on cultured cardiac myocytes? I also asked him what he expected to learn? He replied that because sympathetic tone was known to be increased in patients with ischemic heart disease[4] and because β-blockers were beneficial in such patients,[5] he thought that the adrenergic neurohormones, norepineprhine, and epinephrine might have deleterious effects on cardiac myocytes. Accordingly, he wanted to determine the mechanism of these effects using a cultured cell model system.

The decision of Simpson to use cultured cells was influenced partly by a research fellowship that he performed in the laboratory of Dr Marshall Nirenberg at the National Institutes of Health. Dr Nirenberg, who, along with Drs Robert Holley and H.G. Khorana earned the Nobel Prize in Physiology or Medicine in 1968 for pioneering studies on how the genetic code is translated into proteins.[6] Simpson told me that, at least as important as cell culture other techniques that he learned during that fellowship were the philosophies of Dr Nirenberg on mentorship and experimental design, which became foundations of Simpson's own illustrious independent research career at the University of California, San Francisco School of Medicine. Relevant to this article was Nirenberg's philosophy about being meticulous, and writing the methods of one's articles clearly enough that any investigator trying to reproduce the results could do so on the very first attempt.[7] Indeed, I found that the Methods sections of articles of Paul Simpson are so complete that I was able to replicate his method of culturing neonatal ventricular myocytes on the first attempt. I often refer others to those original articles as examples of how to write methods, as well as where to get the procedures to study hypertrophic growth of cardiac myocytes in culture.

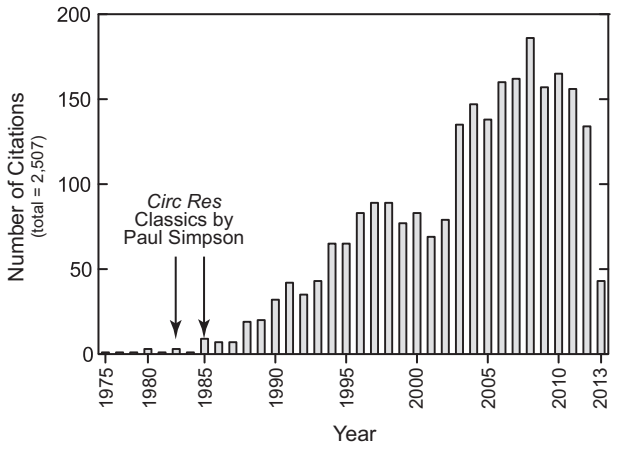

Figure 1. Cultured neonatal rat cardiac myocyte citation count. Shown is the number of citations from 1975 to 2013 (August), which is the result from a PubMed search using the phrase cultured neonatal rat cardiac myocytes.

Nirenberg et al had developed numerous cell culture methods to examine the molecular basis of cell function, including neuronal cell lines to study synapse formation. This taught Simpson that cell culture could be used to address mechanistic cell biology questions. However, because there were no available cardiac muscle cell lines, Simpson set out to learn how to culture primary myocytes during a several-month visit at the laboratory of Glen Langer at University of California, Los Angeles, where high-density neonatal rat ventricular myocyte cultures were being used to examine the mechanism of contractile calcium handling. According to Simpson, at the time, the rat was the experimental animal of choice, and neonatal rats were more cost-effective and easier to work with than adult rats. Mice were not as commonly used as experimental models as they are today. Interestingly, in a more recent study from Simpson's lab,[8] it was shown that neonatal mouse cardiac myocytes exhibit autonomous hypertrophic growth; so if he had used the mouse as the model system originally, he would not have made the discovery on which the rest of his research career was based. Moreover, Langer[9] believed that neonatal rat ventricular myocytes were more similar to myocytes of other species than adult rat myocytes. In addition to his keen attention to experimental detail, and to the care he took to select the appropriate model system is the talent of Paul Simpson for maintaining a broader vision of his research, and how it will contribute to improved treatments for heart failure. The vast breadth and acute depth of his vision, as well as his continued focus on developing better treatments and cures for heart failure, are evident in each of these *Circulation Research* Classics.

Article 1: The Model System

In the first classic, published in *Circulation Research* in 1982, Paul Simpson and Shoshana Savion wanted to establish a model system with which they could study the mechanisms of the effects of catecholamines on cardiac myocyte viability and function in the absence of hemodynamic influence. With these goals in mind, they described a detailed method for preparing cultures from neonatal rat ventricles that, in contrast to previous reports, were relatively free of nonmyocardial cells, in which the myocardial cells were spaced far enough apart so as not to make contact with each other.[1] They reasoned that, to examine the effects of catecholamines on myocardial cells, the nonmyocardial cells, which continued to divide in

culture, would influence the function of myocardial cells that did not divide in culture. Therefore, Simpson and Savion used techniques to arrest nonmyocardial cell proliferation, and then demonstrated that the remaining myocardial cells, cultured at isolated cell densities, did not proliferate and they exhibited the hallmarks of cardiac myocytes, that is, striated ultrastructure, as well as increased beating rate in response to the adrenergic agonist, isoproterenol. Interestingly, the myocyte-specific features decreased if proliferation of nonmyocardial cells was not inhibited. These results led to the hypothesis that "...nonmyocardial cells can alter myocardial cell differentiation...." This visionary concept was supported by earlier studies, which indicated that myocytes may release substances that enhance their own survival.[10] Accordingly, the results in this classic served as the underpinning of many subsequent studies of paracrine signaling substances in the heart, which are now known to be important in essentially all aspects of heart function examined to date.[11,12]

Article 2: An Unexpected Result Forms the Basis for a Career

In the second classic, also published in *Circulation Research* in 1982, Paul Simpson, Ann McGrath, and Shoshana Savion used their newly developed culture system to address the hypothesis of Simpson that adrenergic neurohormones would decrease viability of isolated cardiac myocytes.[2] In our interview, Paul Simpson said that at that time, it was the era of infarct size reduction, and he wanted to study how ischemia damages the heart, postulating that catecholamines were contributors to the damage. However, instead of damaging the myocytes, he was surprised to see that adrenergic agonists did not have deleterious effects but, instead, they increased myocyte size and contractility. This unexpected result set the stage for Simpson's research career that has been devoted mostly to pursuing the mechanism of catecholamine-induced cardiac hypertrophy.

In this landmark article, light microscopy was used in a resourceful way to measure myocyte volume that ranged from ≈500 to 3000 μm^3/cell, depending on the growth conditions. Myocardial cell surface area, which ranged from ≈500 to 4000 μm^2/cell, and protein, ranging from ≈500 to 1500 pg/cell, were also quantified, as was myocardial cell number, which did not increase, regardless of the medium conditions. Accordingly, this article used 3 different quantitative measurements of cell size to demonstrate that neurohumoral substances increase myocardial cell hypertrophy in the absence of hemodynamic strain.

At that time, it was widely thought that in the pathological heart, hemodynamic strain of the myocardium alone was responsible for cardiac myocyte hypertrophy.[13] However, results of Paul Simpson shifted thinking in the field toward the concept that neurohormonal substances contribute to cardiac hypertrophy.[14] This paradigm shift has not only withstood the test of time, but transformed how we think about cardiac hypertrophy. As a result, numerous subsequent studies have used the neonatal rat ventricular myocyte model system to show that neurohormonal substances, in addition to norepineprhine and epinephrine, act in an endocrine/paracrine manner to regulate cardiac myocyte hypertrophy.[15–19] In fact, experiments with cultured heart cells grown on substrates that allow them to be stretched, which mimics hemodynamically induced strain, in vivo, have shown that the stretch increases the synthesis and secretion of signaling substances, such as angiotensin II and endothelin, which act in a paracrine manner to induce hypertrophy of cultured cardiac myocytes.[20–22]

Article 3: The Beginnings of a Mechanism

In the third, and perhaps highest impact *Circulation Research* article of these classics, Paul Simpson published a series of experiments in which he demonstrated that cardiac myocyte growth and beating can be regulated independently through separate cellular pathways. There are numerous methodological and experimental features of note in this article, but one of particular importance was the use of labeling of cultured cell protein with radioactive amino acids, which provided a more sensitive and reproducible method of determining changes in protein than measuring total protein per culture. The clarity and completeness with which Simpson described and validated this technique is remarkable. As a result, this technique has become a mainstay in molecular cardiology laboratories around the world. Simpson used the radiolabeling technique and a detailed pharmacological approach to demonstrate that catecholamine-induced cardiac myocyte hypertrophy is mediated through the α_1-adrenergic receptor, which extended the findings of his earlier publication in the *Journal of Clinical Investigation*.[23] Moreover, in this *Circulation Research* Classic, Simpson showed that the frequency of myocyte contraction was determined by the combined effects of norepineprhine on α_1- and β_1-adrenergic receptors. This article concluded with a figure that combined the results of all 3 classics to describe a hypothetical mechanism by which norepineprhine regulates cardiac myocyte hypertrophy and contraction (Figure 2A). Thus, the initial step of the molecular mechanism by which adrenergic agonists affect cardiac myocyte hypertrophy had been established.

30 Years Later

Since the publication of these classics 30 years ago, many studies have probed deeper into characterizing the α_1-adrenergic receptors associated with cardiac myocytes, as well as the intracellular events responsible for α_1-adrenergic receptor–mediated cardiac myocyte growth. These studies have revealed that there are multiple forms of α_1-adrenergic receptors, and that cardiac myocytes express mainly the α1A and α1B subtypes.[19,24] However, unexpectedly, it was found that a majority of α_1-adrenergic receptors are located on the nuclear membrane and not on the sarcolemma. This finding suggests that catecholamines, which can be taken up by cardiac myocytes, may exert their signaling effects mainly through binding to receptors located on the nuclear envelope[25] (Figure 2B, 1). The localization of other G-protein–coupled receptors, such as angiotensin II and endothelin, to the nuclear membrane of cardiac myocytes suggests that this theme of signal transduction in the heart may be of widespread importance in cardiac function.[26] The α1A subtype is coupled primarily to the G-protein,

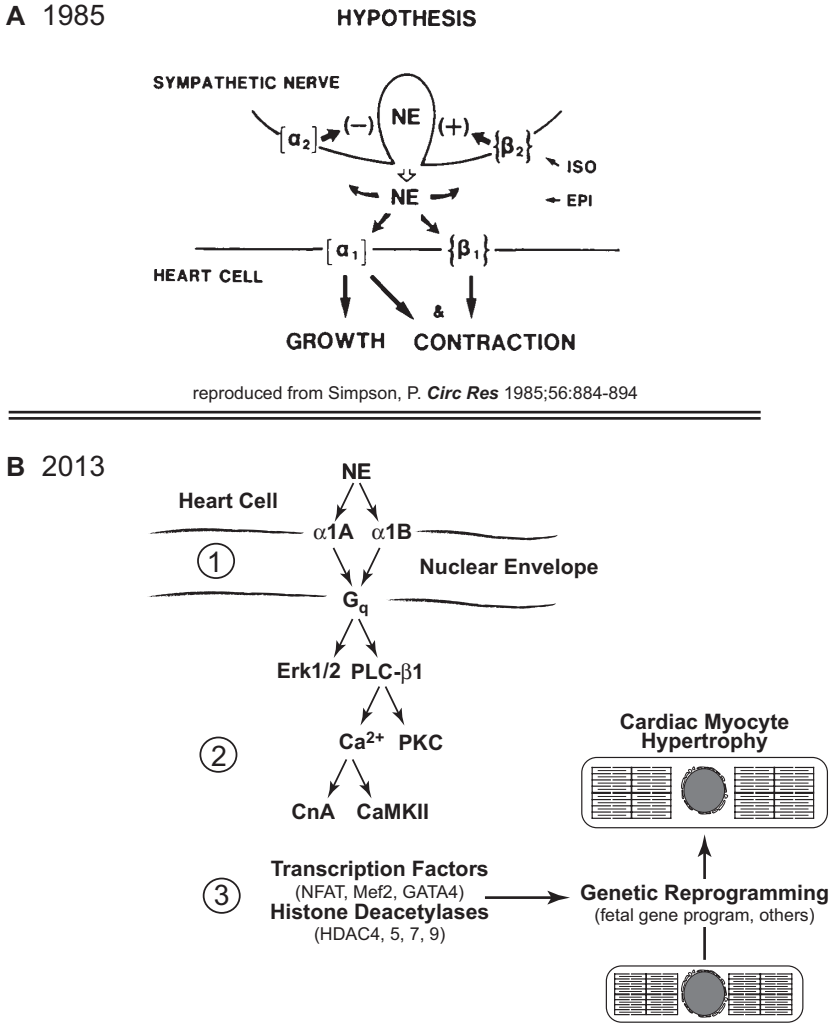

Figure 2. Hypothetical mechanism of norepinephrine action on cardiac myocyte function. A, 1985: the hypothesis from Simpson, P. *Circ. Res.* 1985;56:884–894 showing the actions of norepinephrine (NE), epinephrine (EPI), and isoproterenol (ISO) on α2 and β2-adrenergic receptors associated with sympathetic nerve terminals and on α1 and β1-adrenergic receptors associated with heart cells. **B**, 2013: summary showing some of the major signaling processes that, as of 2013, have been found to contribute to cultured cardiac myocyte hypertrophy in response to NE. CaMKII indicates calcium/calmodulin kinase II; CnA, calcineurinA; Erk1/2, extracellular regulated kinase 1/2; NFAT, nuclear factor of activated T cells; PKC, protein kinase C; and PLC-β1, phospholipase-β1.

G_q, whereas the α1B couples to G_q and G_i. Numerous studies, including some that used genetically modified mouse models in which the α subunit of G_q was overexpressed or deleted, have shown that this G-protein is a key mediator if cardiac myocyte hypertrophy in response to α_1-adrenergic receptor agonists, as well as other paracrine signaling substances, such as angiotensin II and endothelin.[27–29] G_i has also been shown to couple α_1-adrenergic receptors to cardiac hypertrophy.[30] Although, numerous signaling pathways are known to be regulated by G_q, among the predominant G_q-regulated signaling molecules involved in cardiac myocyte hypertrophy are the mitogen activated protein kinase, extracellular regulated kinase 1/2, the nuclear factor of activated T cells phosphatase, calcineurin, protein kinase C, and calcium/calmodulin kinase II (Figure 2B, 2). Together, these signaling molecules alter the levels and activities of transcription factors and histone deactylases,[16–18] which change the cardiac myocyte gene program in ways that lead to hypertrophic growth (Figure 2B, 3). Interestingly, genes normally expressed only in the fetal heart are often upregulated during cardiac pathologies, including hypertrophy.[31] Although members of this fetal gene program are commonly used as markers of pathological cardiac hypertrophy, the roles of the proteins encoded by these genes in exacerbating or moderating the disease are not clear.[32] A recent article, published in *Circulation Research* by Javier Lopez et al, working in the laboratory of Paul Simpson, described and thoroughly validated a flow cytometry approach, which was used to address the relationship between fetal gene induction and cardiac myocyte hypertrophy in mice subjected to pressure overload.[33] It was found that the fetal gene, β-myosin heavy chain, was expressed in minor subpopulation of smaller, nonhypertrophic cardiac myocytes but was not found in any of the cardiac myocytes that had hypertrophied. This remarkable finding challenges the long-standing belief that fetal genes are indicative of hypertrophy. Given the impact that this study has already made on the field, it is probable that it, too, will become a *Circulation Research* Classic in the future.

Reading Assignment

The *Circulation Research* Classics highlighted in this article were transformational, in that they contributed significantly to our current understanding of the molecular mechanisms governing hypertrophic growth of cardiac myocytes. I invite you to join me and the students in my class as we read and enjoy the original scripts that had such a widespread effect on how we think about, study and, eventually, how we will cure pathological cardiac hypertrophy and heart failure. And when you

do, think about them, not only as historical accounts of how the research was done but also as exemplars of how research should be done, and how a 30-year-old article in *Circulation Research* can impact the direction and quality of articles published in the journal today.

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Disclosures

None.

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KEY WORDS: adrenergic agents ■ alpha1-adrenergic receptor ■ cultured cardiac myocytes ■ heart failure ■ hypertrophy ■ muscle cells

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