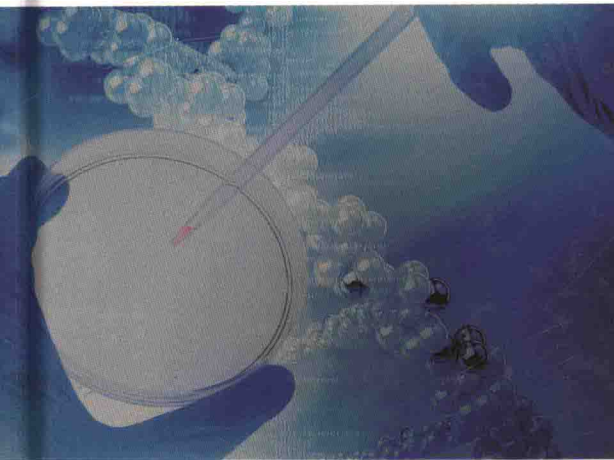


# C[REATIVE] CLINICAL BIOLOGY IN REGENERATIVE MEDICINE

**BRIDGING STEM CELLS  
AND FUTURE THERAPIES**



EDITORS

CHARLES C. HONG  
ADA S. AO  
JIJUN HAO

**WILEY**

# Chemical Biology in Regenerative Medicine

Bridging Stem Cells and Future Therapies

Edited by

CHARLES C. HONG

*Department of Medicine, Vanderbilt University, USA*

ADA S. AO

*Department of Medicine, Vanderbilt University, USA*

and

JIJUN HAO

*College of Veterinary Medicine, Western University of Health  
Sciences, USA*

WILEY

This edition first published 2014  
© 2014 John Wiley & Sons, Ltd

*Registered office*

John Wiley & Sons Ltd, The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, United Kingdom

For details of our global editorial offices, for customer services and for information about how to apply for permission to reuse the copyright material in this book please see our website at [www.wiley.com](http://www.wiley.com).

The right of the author to be identified as the author of this work has been asserted in accordance with the Copyright, Designs and Patents Act 1988.

All rights reserved. No part of this publication may be reproduced, stored in a retrieval system, or transmitted, in any form or by any means, electronic, mechanical, photocopying, recording or otherwise, except as permitted by the UK Copyright, Designs and Patents Act 1988, without the prior permission of the publisher.

Wiley also publishes its books in a variety of electronic formats. Some content that appears in print may not be available in electronic books.

Designations used by companies to distinguish their products are often claimed as trademarks. All brand names and product names used in this book are trade names, service marks, trademarks or registered trademarks of their respective owners. The publisher is not associated with any product or vendor mentioned in this book.

**Limit of Liability/Disclaimer of Warranty:** While the publisher and author have used their best efforts in preparing this book, they make no representations or warranties with respect to the accuracy or completeness of the contents of this book and specifically disclaim any implied warranties of merchantability or fitness for a particular purpose. It is sold on the understanding that the publisher is not engaged in rendering professional services and neither the publisher nor the author shall be liable for damages arising herefrom. If professional advice or other expert assistance is required, the services of a competent professional should be sought.

The advice and strategies contained herein may not be suitable for every situation. In view of ongoing research, equipment modifications, changes in governmental regulations, and the constant flow of information relating to the use of experimental reagents, equipment, and devices, the reader is urged to review and evaluate the information provided in the package insert or instructions for each chemical, piece of equipment, reagent, or device for, among other things, any changes in the instructions or indication of usage and for added warnings and precautions. The fact that an organization or Website is referred to in this work as a citation and/or a potential source of further information does not mean that the author or the publisher endorses the information the organization or Website may provide or recommendations it may make. Further, readers should be aware that Internet Websites listed in this work may have changed or disappeared between when this work was written and when it is read. No warranty may be created or extended by any promotional statements for this work. Neither the publisher nor the author shall be liable for any damages arising herefrom.

***Library of Congress Cataloging-in-Publication Data***

Chemical biology in regenerative medicine : bridging stem cells and future therapies / edited by  
Charles C. Hong, Ada S. Ao, and Jijun Hao.

p. : cm.

Includes bibliographical references and index.

ISBN 978-1-118-34959-5 (cloth : alk. paper)

I. Hong, Charles C., 1967- editor of compilation. II. Hao, Jijun, editor of compilation.

III. Ao, Ada, editor of compilation.

[DNLM: 1. Stem Cells—chemistry. 2. Myocytes, Cardiac—chemistry. 3. Regenerative Medicine. QU 325]

QH588.S83

616.02'774—dc23

2014004305

A catalogue record for this book is available from the British Library.

ISBN: 9781118349595

Cover Image: © iStockphoto/faslooff, Sergey Nivens, luismmolina

Set in 10/12pt Times by Aptara Inc., New Delhi, India.

Printed and bound in Malaysia by Vivar Printing Sdn Bhd

# **Chemical Biology in Regenerative Medicine**

# List of Contributors

**Dikshya Bastakoty**, Department of Pathology, Vanderbilt University, USA

**Maria Borowski**, Department of Cell and Developmental Biology, University of Massachusetts Medical School, USA

**Jeffery B. Bylund**, Department of Medicine and Department of Pharmacology, Vanderbilt University, USA

**Robert B. Crochet**, Department of Biological Science, Louisiana State University, USA

**Kenneth J. Eilertsen**, NuPotential, Inc., USA and Pennington Biomedical Research Center, LSU System, USA

**Bryan A. Fioret**, Department of Medicine, Vanderbilt University, USA

**Ru Gao**, NuPotential, Inc., USA

**Federico González**, Developmental Biology Program, Memorial Sloan-Kettering Cancer Center, USA

**Jijun Hao**, College of Veterinary Medicine, Western University of Health Sciences, USA

**Antonis K. Hatzopoulos**, Department of Medicine, Vanderbilt University, USA

**Charles C. Hong**, Department of Medicine, Vanderbilt University, USA

**Danwei Huangfu**, Developmental Biology Program, Memorial Sloan-Kettering Cancer Center, USA

**Daqing Jin**, Department of Genetics, Fudan University School of Life Sciences, China

**Jeong-Do Kim**, NuPotential, Inc., USA

**Joseph C. Laning**, Research and Development, Provia Laboratories, LLC, USA

**Yong-Hwan Lee**, Department of Biological Science, Louisiana State University, USA

**Qiao Li**, Department of Genetics, Fudan University School of Life Sciences, China

**Simon Maltais**, Department of Cardiac Surgery, Vanderbilt University, USA

**Nikhil Munshi**, Internal Medicine, University of Texas Southwestern Medical Center, USA

**Young-Jae Nam**, Department of Medicine, Division of Cardiovascular Medicine, Vanderbilt University, USA

**David T. Paik**, Department of Medicine, Vanderbilt University, USA

**Jong S. Rim**, NuPotential, Inc., USA

**Sarika Saraswati**, Department of Pathology, Microbiology and Immunology, Vanderbilt University School of Medicine, USA

**Calvin C. Sheng**, Department of Cardiovascular Medicine, Vanderbilt University, USA

**Zhong-Dong Shi**, Developmental Biology Program, Memorial Sloan-Kettering Cancer Center, USA

**Devyn M. Smith**, Neusentis Research Unit, Pfizer Worldwide R&D, UK

**Kelly P. Smith**, Department of Cell and Developmental Biology, University of Massachusetts Medical School, USA

**Jaroslawn Staszkiwicz**, NuPotential, Inc., USA

**Hak-Joon Sung**, Department of Biomedical Engineering, School of Medicine, Vanderbilt University, USA

**Xintong Wang**, Department of Biomedical Engineering, Vanderbilt University, USA

**Pampee P. Young**, Department of Pathology, Microbiology and Immunology, Vanderbilt University, USA

**Angela L. Zachman**, Department of Biomedical Engineering, Vanderbilt University, USA

**Tao P. Zhong**, State Key Laboratory of Genetic Engineering, Fudan University School of Life Sciences, China; Department of Medicine, Vanderbilt University, USA

# Preface

The nascent field of regenerative medicine has advanced at a rapid pace, and clinical trials are ongoing to assess the safety and efficacy of stem cell-based therapies in indications that range from congestive heart failure to Crohn's disease. However, in general there is a lack of understanding of the specific mechanisms of action, and application of these new biologic products faces special challenges.

Four key areas in regenerative medicine are highlighted in this book: (i) cell-fate determination, (ii) regeneration, (iii) designer cells, and (iv) translation of cell-based therapies. The utility of stem cell-based therapies relies upon efficient manipulation of cell fates, not only to generate sufficient quantities of the desired cell type for transplantation but also to prevent tumor formation from residual, undifferentiated cells. The biologic mechanisms related to cell-fate determination will be presented in depth to provide the necessary background. While the focus of stem cell-based therapies has traditionally been on transplantation and engraftment, this is not the only paradigm. Instead, some therapies may stimulate regeneration in the host tissue through activation of endogenous stem-cell pools. In this volume, the use of mesenchymal stem cells (MSCs) in the context of wound healing and regeneration will be discussed. In addition, in recognition of the revolutionary advances in patient-derived induced pluripotent stem cells (iPSCs) and reprogramming of somatic cells, the issue of customizing iPSCs for research and clinical applications will be discussed. A myriad of technical and conceptual issues remain to be overcome before the full potential of iPSC technologies is realized, which will require an interdisciplinary approach. Thus, this book pays particular attention to the use of small molecules and biomaterials in addressing current challenges. Lastly, an overview of the practicalities related to translation of stem-cell therapies for clinical use will be provided.

This book is intended for those with interests ranging from basic developmental biology to stem-cell therapies. Biomedical researchers, clinicians, managers in biotechnology, and undergraduate and graduate students interested in stem cells and cell-based therapies will find this book useful in summarizing the most recent developments in stem-cell research.

The intrigue surrounding stem cell-based therapies lies not only in the treatment of chronic diseases but also in the potential to cure them. Therefore, regenerative medicine is rapidly moving to the forefront of many therapeutic areas, from cancer to heart disease. This book aims to summarize the collective knowledge of active researchers, illustrate current perspectives on the most pressing obstacles to clinical application, and serve as a guide to what lies ahead.

Charles C. Hong, MD, PhD  
Ada S. Ao, PhD  
Jijun Hao, PhD

# Contents

<i>List of Contributors</i>	xi
<i>Preface</i>	xiii
<b>1 Wnt Signaling in Regulation of Stem Cells</b>	<b>1</b>
<i>David T. Paik and Antonis K. Hatzopoulos</i>	
1.1 Overview of Wnt Signaling	1
1.2 Wnt Signaling in Embryonic Stem Cells	2
1.3 Wnt Signaling in Cardiovascular Progenitor Cells and Cardiomyocyte Differentiation	3
1.4 Wnt Signaling in Mesenchymal Stem Cells	5
1.5 Wnt Signaling in Hematopoiesis and Hematopoietic Stem Cells	7
1.6 Wnt Signaling in Neural Stem Cells	8
1.7 Wnt Signaling in Endothelial–Mesenchymal Transition	8
1.8 Conclusion	9
References	10
<b>2 Directed Cardiomyogenesis of Pluripotent Stem Cells</b>	<b>15</b>
<i>Jeffery B. Bylund and Antonis K. Hatzopoulos</i>	
2.1 Introduction	15
2.2 A Brief Review of Heart Development	16
2.2.1 Cellular and Morphological Movements	16
2.2.2 Molecular Events in Heart Development	19
2.2.2.1 Molecular Events of Mesoderm Derivation	19
2.2.2.2 Transcription Factors in Cardiac Development	20
2.2.2.3 Major Developmental Signaling Pathways in Cardiac Development	22
2.3 Introduction to Pluripotent Stem Cells	23
2.3.1 Unique Features of Pluripotent Stem Cells	23
2.3.2 Pluripotent Stem Cell Sources	24
2.3.3 Maintaining Pluripotency	24
2.4 Cardiomyocyte Differentiation	25
2.4.1 Inducing Differentiation	25
2.4.2 Directed Cardiomyogenesis	26
2.5 Conclusion	28
References	29



<b>3</b>	<b>Chemical Genetics in Cardiomyocyte Generation</b>	<b>35</b>
	<i>Daqing Jin, Qiao Li, and Tao P. Zhong</i>	
3.1	Introduction	35
3.2	iPSC Generation	36
3.3	The Chemical Genetics Approach in iPSC Generation	37
3.4	Heart Regeneration	40
3.5	The Chemical Genetics Approach in Heart Regeneration	42
3.6	Cardiac Cell Transdifferentiation	43
3.7	Conclusion	44
	Acknowledgements	44
	References	44
<b>4</b>	<b>Challenges and New Directions for Cardiac Reprogramming</b>	<b>49</b>
	<i>Young-Jae Nam and Nikhil Munshi</i>	
4.1	Introduction	49
4.2	Strategies for Heart Repair	49
4.3	Direct Reprogramming Approaches	50
4.4	Current Challenges	53
4.5	Conclusion	56
	Acknowledgements	56
	References	56
<b>5</b>	<b>Comparative Analysis of Adult Stem Cell Niches</b>	<b>59</b>
	<i>Bryan A. Fioret and Antonis K. Hatzopoulos</i>	
5.1	Adult Stem Cells	59
5.2	Adult Stem Cell Niches	60
5.3	The Hair Follicle Stem Cell (HFSC) Niche	61
5.4	The Intestinal Stem Cell (ISC) Niche	63
5.5	The Hematopoietic Stem Cell (HSC) Niche	66
5.5.1	Endosteal Niche	66
5.5.2	Vascular Niche	67
5.5.3	Progeny "Niche"	68
5.6	The Neural Stem Cell (NSC) Niche	68
5.6.1	V-SVZ Niche	69
5.6.2	SGZ Niche	70
5.7	A Comparison between Tissue-Specific Adult Stem Cell Niches	71
5.8	Future Challenges	73
	Acknowledgements	73
	References	73
<b>6</b>	<b>Chemicals and Stem Cells in the Promotion of Regeneration</b>	<b>77</b>
	<i>Dikshya Bastakoty, Sarika Saraswati, and Pampee P. Young</i>	
6.1	Introduction	77
6.2	Biologics in Regenerative Medicine	78
6.2.1	Growth Factors and Pro-Angiogenic Agents	78

6.2.2	Immune-Modulatory Therapies	79
6.2.3	Extracellular Matrix-Based Approaches	79
6.3	Chemicals and Biomaterials for Healing	79
6.3.1	Small Molecules	80
6.3.2	Biomaterial Scaffold and Sustained Delivery	81
6.4	Stem-Cell Therapy	81
6.4.1	Chemical Manipulation of Stem Cells in Regeneration	82
6.4.2	Embryonic Stem Cells (ESCs)	82
6.4.2.1	Small Molecules for the Culture and Maintenance of ESCs	82
6.4.2.2	Small Molecules for ESC Differentiation	83
6.4.3	Induced Pluripotent Stem Cells (iPSCs)	84
6.4.3.1	Generation of iPSCs	84
6.4.3.2	Small Molecules that Affect iPSC Epigenomes	84
6.4.3.3	Small Molecules that Affect iPSC Signaling Pathways	84
6.4.4	Mesenchymal Stem Cells (MSCs)	85
6.4.4.1	Properties of MSCs	85
6.4.4.2	Small Molecules that affect MSC Differentiation	85
6.4.4.3	Biopolymers that affect MSC Biology	86
6.4.5	Hematopoietic Stem Cells (HSCs)	86
6.5	Conclusion	87
	References	88
<b>7</b>	<b>Chemically Induced Pluripotent Stem Cells (CiPSCs): A Potential Chemical Biological Breakthrough in Reprogramming?</b>	<b>95</b>
	<i>Calvin C. Sheng, Jijun Hao, and Charles C. Hong</i>	
7.1	Searching for the “Perfect” Platform	95
7.2	Defining the Advantages of Small Molecules in Reprogramming	96
7.3	Understanding the Disadvantages of Using Small Molecules	97
7.4	Breakthrough: The CiPSC Paradigm	97
7.5	Conclusion	101
	References	101
<b>8</b>	<b>An Introduction to Cellular Reprogramming: The Plasticity of Cell Fates and Identities</b>	<b>103</b>
	<i>Kelly P. Smith, Maria Borowski, and Joseph C. Laning</i>	
8.1	Defining Cell Potency	104
8.2	Types of Pluripotent Cell	105
8.2.1	Isolated Cell Types	105
8.2.1.1	Embryonal Carcinoma Cells	105
8.2.1.2	Embryonic Stem Cells	105
8.2.1.3	Embryonic Germ Cells	105

8.2.2	Reprogrammed Cell Types	106
8.2.2.1	Cell-Fusion Hybrids	106
8.2.2.2	Somatic Cell Nuclear Transfer Cells	106
8.2.2.3	Induced Pluripotent Stem Cells	106
8.3	Defining Pluripotency	107
8.4	The Molecular Basis of Pluripotency	108
8.5	Cellular Reprogramming: Altering the Epigenetic State	110
8.6	Cellular Reprogramming: Primary Regulatory Pathways	111
8.6.1	Temporal and Stoichiometric Considerations	113
8.6.2	Target Cell Type	113
8.7	Reprogramming Methods	114
8.7.1	Viral-Driven	114
8.7.2	Nucleic Acid/Episomal-Driven	115
8.7.3	mRNA-Driven	117
8.7.4	miRNA-Driven	117
8.7.5	Protein-Driven	118
8.7.6	External Factors/Enhancers	118
8.7.7	Direct Reprogramming	120
8.8	Applications and Future Trends	121
8.8.1	Moving Toward Clinical Applications for Cellular Reprogramming	121
8.8.2	The Merging of Stem Cells and New Methods of Genetic Engineering	125
8.8.3	Efficiency, Expense, and Safety	125
8.8.4	Developing Standards	126
8.9	Conclusion	127
	References	127
<b>9</b>	<b>Chemicals Facilitating Reprogramming</b>	<b>141</b>
	<i>Zhong-Dong Shi, Federico González, and Danwei Huangfu</i>	
9.1	Introduction	141
9.2	Chemicals Modulating Epigenetic Barriers	145
9.2.1	Histone Deacetylase Inhibitors	146
9.2.2	Histone Methyltransferase Inhibitor and Demethylase Inhibitor	147
9.2.3	DNA Methyltransferase Inhibitors	149
9.3	Chemicals Targeting Signaling Pathways	150
9.3.1	TGF $\beta$ Signaling Inhibitors	150
9.3.2	Wnt Signaling and GSK3 Inhibitors	151
9.3.3	Other Kinase Inhibitors and Activators	152
9.3.4	Cell Senescence Alleviators	153
9.4	Chemicals Promoting Lineage Reprogramming	154
9.5	Conclusion	155
	References	156

<b>10 Chemicals Facilitating Reprogramming: Targeting the SAM Binding Site to Identify Novel Methyltransferase Inhibitors</b>	<b>163</b>
<i>Jeong-Do Kim, Jong S. Rim, Robert B. Crochet, Yong-Hwan Lee, Jaroslaw Staszkiwicz, Ru Gao, and Kenneth J. Eilertsen</i>	
10.1 Introduction	163
10.2 DNA Methyltransferases, Inhibition, and Reprogramming	164
10.3 DNMT Inhibitors	164
10.4 Histone Methyltransferases, Inhibition, and Reprogramming	167
10.5 Inhibitors of Lysine Methyltransferases	168
10.6 Identification of DNMT1 Inhibitor Candidates Using Virtual Screening	169
10.6.1 Functional Screening Using a DNMT1 Activity Assay	169
10.7 Targeting the SAM Binding Site to Identify Novel HMT Inhibitors	171
10.7.1 SAM Competitive Assay	173
10.7.2 SAM Binding Site is Unique and Selective across Multiple Epigenetic Targets	173
10.8 Conclusion	177
References	177
<b>11 Biomaterials for Directed Differentiation</b>	<b>181</b>
<i>Xintong Wang, Angela L. Zachman, Simon Maltais, and Hak-Joon Sung</i>	
11.1 Introduction	182
11.2 Natural Biomaterials	183
11.2.1 ECM-Derived Materials	183
11.2.1.1 Matrigel	183
11.2.1.2 Fibrin	184
11.2.1.3 Collagen	185
11.2.1.4 Laminin	187
11.2.2 Non-ECM-Derived Materials	188
11.2.2.1 Chitosan	188
11.3 Synthetic Biomaterials	189
11.3.1 Polyesters	189
11.3.1.1 Poly(Lactic Acid) and Poly(Glycolic Acid) Copolymers	189
11.3.1.2 Poly( $\epsilon$ -Caprolactone)	192
11.3.2 Polyethylene Glycol	194
11.4 Conclusion	195
References	196
<b>12 Practicalities to Translation from the Clinic to the Market</b>	<b>203</b>
<i>Devyn M. Smith</i>	
12.1 Introduction	203
12.2 Commercialization Comparison with Small Molecules, Medical Devices, and Biologics	204

12.3	Historical Review and Case Studies	205
12.3.1	Dermagraft	205
12.3.2	Provenge	206
12.4	Commercialization Challenges and How to Overcome Them	209
12.5	Translation from the Bench to the Clinic: Key Considerations	209
12.6	Conclusion	213
	References	214
	<i><b>Index</b></i>	<b>217</b>

# 1

## Wnt Signaling in Regulation of Stem Cells

*David T. Paik and Antonis K. Hatzopoulos*  
*Department of Medicine, Vanderbilt University, USA*

### 1.1 Overview of Wnt Signaling

The Wnt signaling pathway is classically divided into so-called canonical and noncanonical branches based on the activation of specific intracellular components. Canonical Wnt signaling is activated when Wnt ligands bind to the Frizzled (Fzd) family of 7-transmembrane domain receptors and co-receptors, such as low-density lipoprotein receptor-related protein (LRP) 5/6, Ryk, and Ror2 [1–4]. This disrupts the formation of the  $\beta$ -catenin destruction complex, which consists of the scaffolding protein Axin, the Adenomatous polyposis coli (APC) protein, Dishevelled (Dsh), casein kinase I $\alpha$  (CK-I $\alpha$ ), and glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ). The dissociation of the destruction complex leads to stabilization of cytoplasmic  $\beta$ -catenin, which translocates to the nucleus to interact with TCF/LEF transcription factors and initiate transcription of canonical Wnt signaling target genes, such as *c-Myc*, *Axin2*, and *Snail* [5–7]. When canonical Wnt signaling is turned off, the destruction complex phosphorylates  $\beta$ -catenin for ubiquitin-mediated proteosomal degradation [8].

Noncanonical Wnt signaling pathways are  $\beta$ -catenin-independent and are mediated through other intracellular proteins [1–4, 9, 10]. In the Wnt/JNK pathway, binding of Wnt to Fzd receptors activates small-GTPases, RhoA, and Rac through recruitment of Dsh, which thereby activate Rho kinase and c-Jun N-terminal kinases (JNK). In the Wnt/Ca<sup>2+</sup> pathway, binding of Wnt to Fzd receptors increases intracellular Ca<sup>2+</sup> levels, activating

calcium/calmodulin-dependent kinase (CaMK) II, protein kinase C (PKC), and the protein phosphatase calcineurin (CaCN) to trigger dephosphorylation of NF-AT transcription factors. Thus activated NF-AT transcription factors translocate to the nucleus to stimulate transcription of their target genes [11]. Intriguingly, noncanonical Wnt signaling has been shown to inhibit canonical Wnt signaling in various mechanisms [10, 12, 13].

To date, 19 Wnt ligands and 10 Fzd receptors have been identified. The 19 Wnt genes fall into 12 conserved Wnt subfamilies, which exist in most mammalian genomes, including the human genome [14]. Different combinations of individual Wnt ligands, receptors, and co-receptors allow differential activation of  $\beta$ -catenin-dependent/canonical Wnt signaling,  $\beta$ Wnt signaling, ligands, receptors, and/or Wnt signaling in a cellular context-dependent manner.

In the past 2 decades, the connection between Wnt signaling and human disease has been well established. Numerous components of the pathway have been implicated in cancer, obesity, osteoporosis, diabetes, and cardiovascular diseases [15]. Interestingly, Wnt signaling components are also critical regulators of stem and progenitor cells in various organs and tissues. A thorough understanding of the Wnt signaling pathway in the regulation of stem cells will be instrumental in translating the potential of stem cells to effective therapeutic solutions for human degenerative diseases or to the restoration of organ function after injury.

## 1.2 Wnt Signaling in Embryonic Stem Cells

Wnt signaling has been implicated in the maintenance of the pluripotency and differentiation potential of embryonic stem cells (ESCs). A number of studies have demonstrated that individual Wnt ligands can stimulate self-renewal of ESCs [16–18]. Activation of the canonical Wnt pathway complements the LIF/JAK-STAT pathway via upregulation of the Stat3 gene to inhibit ESC differentiation [16]. Paracrine and autocrine Wnt signaling is essential not only for self-renewal of mESCs but also to inhibit differentiation into epiblast stem cells (epiSCs) [7]. Accordingly, mutations in the  $\beta$ n of the *io* destruction complex APC protein increase  $\beta$ -catenin levels, diminishing the differentiation capacity of mouse ESCs (mESCs) into the three germ layers [19].

In contrast, whether Wnt signaling promotes self-renewal or differentiation of human ESCs (hESCs) has been rather controversial. In the presence of supportive feeder cells or a conditioned medium (CM) rich in factors preventing differentiation, Wnt3a enhanced self-renewing proliferation of undifferentiated hESC H1 cells. In the absence of CM, however, activation of Wnt signaling accelerated both proliferation and differentiation of hESCs [20]. The canonical Wnt ligand Wnt1 displayed the same effects on hESCs as Wnt3a, while the noncanonical Wnt ligand Wnt5a did not affect the proliferation of hESCs, indicating that  $\beta$ -catenin-dependent canonical Wnt activation is responsible for enhanced hESC proliferation [21, 22]. Activation of the canonical Wnt pathway by 6-bromoindirubin-3'-oxime (BIO), a specific inhibitor of glycogen synthase kinase 3 (GSK3), produced comparable results in maintaining the undifferentiated phenotype of hESCs marked by sustained expression of the pluripotent transcription factors OCT3/4 (POU5F1), REX1, and NANOG and by prevention of the epithelial–mesenchymal transition (EMT) of hESCs [23, 24]. In complementary fashion, addition of the Wnt inhibitors Sfrp-1, Sfrp-2, and Sfrp-4, singly or in combination, promoted differentiation of hESCs [22].

However, other studies have reported that canonical Wnt activation disrupted hESC self-renewal and promoted differentiation. Specifically, conditional activation of stabilized  $\beta$ -catenin in KhES-1 and KhES-3 lines resulted in downregulation of the pluripotent markers NANOG, SOX2, and POU5F1 and upregulation of the mesodermal marker *T Brachyury*, followed by induction of the ventral mesodermal and endothelial marker KDR (VEGFR2) and the early cardiac marker NKX2.5 [25].

Experimental evidence also suggests that canonical Wnt signaling interacts with Activin/Nodal and bone morphogenetic protein (BMP) signaling pathways to specify differentiation lineages in hESCs. Canonical Wnt activation induced Activin/Nodal and BMP signaling to promote posterior Primitive Streak (PS) and mesoderm differentiation of hESCs. Synergistic interaction between Wnt and Activin/Nodal pathways was shown to be required for anterior PS and endoderm specification, while BMP and MAPK signaling antagonized it [25]. Recent studies have also shown that hESCs treated with the Wnt inhibitor IWP are maintained as pluripotent, with the ability to differentiate into neural cells [26]. Furthermore, Wnt3a-treated hESCs acquired PS-like characteristics and differentiated into mesodermal and endodermal cells [26].

It is noteworthy that the effects of Wnt3a on hESCs and mESCs differ significantly. In mESCs, Wnt3a prevented progression of mESCs to EpiSCs, while in hESCs Wnt3a facilitated their differentiation into mesodermal and endodermal lineages [7, 26]. In another study, the pluripotent marker OCT4 in hESCs was shown to repress  $\beta$ -catenin during self-renewal, whereas knockdown of OCT4 activated canonical Wnt signaling [27]. In support of these studies, Wnt1-treated hESCs displayed induced differentiation to hemogenic endothelial cells, while treatment with the Wnt inhibitor Dkk1 reduced this differentiation potential [28].

The apparently contradictory reports of Wnt signaling in hESC self-renewal and differentiation may be due to the epiblast origin of the various hESC lines tested [25]. It has also been proposed that the effects of Wnt signaling on hESCs are highly sensitive to the level of Wnt activation [26, 27]. Therefore, it is likely that apparently contradictory results regarding the role of Wnt signaling in human ESC pluripotency, proliferation, and differentiation reflect the heterogeneity of the corresponding lines and their sensitivity to canonical Wnt signaling levels.

### 1.3 Wnt Signaling in Cardiovascular Progenitor Cells and Cardiomyocyte Differentiation

Wnt signaling also plays important roles in the maintenance and expansion of cardiovascular progenitor cells and in their differentiation into endothelial and cardiomyocyte lineages [2, 10, 11]. The mammalian heart is one of the first organs to form during embryogenesis and Wnt signaling has been implicated in all phases of cardiogenesis. Initially, canonical Wnt signaling is necessary for the formation of mesodermal progenitor cells, but it must then be suppressed in order for mesoderm progenitors to yield cardiac progenitor cells (CPCs) [11]. Subsequently, noncanonical Wnt signaling is necessary for the specification of CPCs. Wnt5a, a noncanonical Wnt activator, is upregulated by the mesoderm-specific transcription factor *Mesp-1* to promote formation of CPCs [29]. Other studies have shown that intrinsic Wnt2 expression in mouse ES cells is essential for efficient cardiomyocyte



differentiation and that exogenous Wnt2 promotes cardiomyocyte differentiation. Interestingly, Wnt2 induced cardiogenesis through activation of the noncanonical JNK/AP-1 pathway [30]. After CPC specification, canonical Wnt signaling activation stimulates proliferation of Isl1<sup>+</sup> CPCs, whereas subsequent Dkk1 inhibition of canonical Wnt signaling and noncanonical Wnt activation by Wnt11 are required for cardiomyocyte differentiation [31, 32].

The role of Wnt signaling and the effects of Wnt signaling manipulation during heart development have been well documented in various animal models. In mouse embryos, deletion of  $\beta$ -catenin in the definitive endoderm led to formation of multiple hearts along the anterior–posterior (A/P) axis, as one of the earliest pieces of evidence to implicate Wnt signaling in the endoderm in the induction of precardiac mesoderm [33]. Notably, the supernumerary hearts followed the ectopic expression patterns of BMP2. Ectopic cardiac tissue also formed in zebrafish embryos with overexpression of the BMP antagonist Gremlin2, while Gremlin2 morphants exhibited a rise in the intracellular levels of  $\beta$ -catenin, suggesting a crosstalk between Wnt and BMP signaling during cardiac development [33, 34]. In chick embryos, Crescent, a Frizzled-related protein that inhibits Wnt8c, is expressed in the anterior endoderm during gastrulation, while Wnt3a and Wnt8c expression is localized to the primitive streak and posterior lateral plate. Dkk1 induces cardiac gene expression in the posterior lateral plate mesoderm, while ectopic Wnt activation in the anterior mesoderm inhibits cardiac formation. Therefore, inhibition of Wnt signaling in chick embryos promoted heart formation in the anterior lateral mesoderm, supporting a model that cardiogenesis initially requires high levels of BMP but low canonical Wnt activity [35]. In *Xenopus* embryos, Wnt6 was found to be expressed in tissues close to and inside the developing heart and to be required to restrict heart development, since the absence of Wnt6 led to an abnormally large heart [36]. Intriguingly, Wnt6 is not required during the gastrulation stage but rather in the later stages of organogenesis that precede the differentiation of cardiogenic mesoderm into myocardium. Overexpression of Wnt6 reflected such a phenotype through the activation of  $\beta$ -catenin-dependent/canonical Wnt signaling, which repressed cardiogenic transcription factors such as Gata6 and Mlc2. Sfrp-1, an endogenous Wnt inhibitor, is strongly induced in differentiating cardiomyocytes and participates in a negative-feedback regulatory loop of Wnt signaling in regulating the cell fate of cardiac mesoderm [37].

Later studies reinforced a biphasic role for canonical Wnt signaling in cardiac specification in zebrafish and mice. For example, it appears that  $\beta$ -catenin-dependent Wnt signaling before gastrulation promotes cardiac differentiation while inhibiting heart formation during gastrulation [38]. Early treatment of mESCs with Wnt3a induced mesoderm specification, which activated a feedback loop that subsequently repressed the Wnt pathway, which in turn increased cardiac differentiation. Late activation of  $\beta$ -catenin in mESCs, on the other hand, repressed cardiac differentiation. Overexpression of the noncanonical Wnt ligand Wnt11, which may inhibit canonical Wnt signaling, promoted cardiac differentiation during early stages but repressed it in later ones. Moreover, canonical Wnt signaling in the early stages of embryoid body (EB) formation induced cardiac differentiation but suppressed hematopoietic and vascular cell lineages. Activation of canonical Wnt signaling in the later stages of EB formation, however, inhibited cardiac formation and induced expression of hematopoietic and vascular genes through the suppression of BMP signaling [39]. Such results highlight the strict temporal and spatial requirement of Wnt signaling