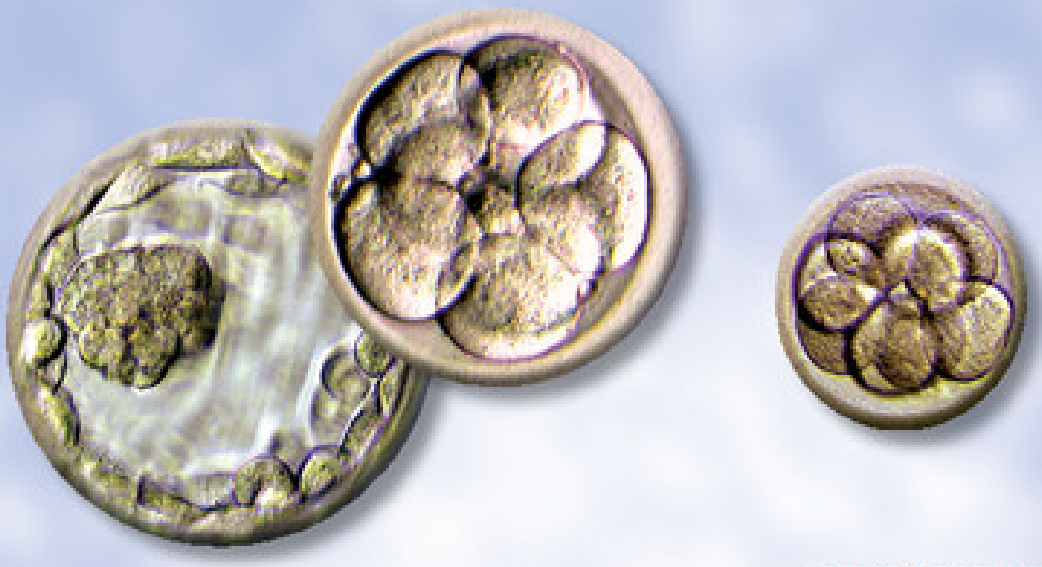

SECOND EDITION

BIOLOGY AND PATHOLOGY OF THE OOCYTE

Role in Fertility, Medicine, and Nuclear Reprogramming

EDITED BY:
ALAN TROUNSON
ROGER GOSDEN
URSULA EICHENLAUB-RITTER

CAMBRIDGE Medicine

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Role in Fertility, Medicine, and Nuclear Reprogramming

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Edited by

Alan Trounson

Californian Institute for Regenerative Medicine, San Francisco, CA, USA

Roger Gosden

Center for Reproductive Medicine and Infertility, Weill Medical College of Cornell University, New York, NY, USA

Ursula Eichenlaub-Ritter

Faculty of Biology, Institute of Gene Technology/Microbiology, University of Bielefeld, Bielefeld, Germany

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In memory of Bob Edwards (1925–2013), mentor,
colleague, friend

Contents

Section 5 – Pathology

Section 6 – Technology and clinical medicine

Contributor affiliations

Dasari Amarnath
Fels Institute for Cancer Research and Molecular Biology, Temple University School of Medicine, Philadelphia, PA, USA

Ellen Anckaert
Follicle Biology Laboratory, UZ Brussel, Brussels, Belgium

Valerie L. Baker
Department of Obstetrics and Gynecology, Reproductive Endocrinology and Infertility, Stanford School of Medicine, Palo Alto, CA, USA

Natalie Barbey
Infertility Center of St. Louis, St. Luke's Hospital, St. Louis, MO, USA

Tiziana A. L. Brevini
Laboratory of Biomedical Embryology, Centre for Stem Cell Research UNISTEM, Università degli Studi di Milano, Milan, Italy

Frank Broekmans
Department of Reproductive Medicine and Gynecology, University Medical Center, Utrecht, the Netherlands

Anne Grete Byskov
Laboratory of Reproductive Biology, Juliane Marie Center, Rigshospitalet, Copenhagen, Denmark

John Carroll
Department of Anatomy and Developmental Biology, School of Biomedical Sciences, Monash University, Melbourne, Australia

Rita P. Cervera
Division of Reproductive and Developmental Sciences, Oregon National Primate Research Center, Oregon Health and Science University, Beaverton, OR, USA

Jose Cibelli
Cellular Reprogramming Laboratory, Department of Animal Science, Michigan State University, East Lansing, MI, USA; LARCel, Programa Andaluz de Terapia Celular y Medicina Regenerativa, Andalucía, Spain

Paula E. Cohen
Department of Biomedical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY, USA

Marco Conti
Center for Reproductive Sciences and The Eli and Edythe Broad Center of Regeneration Medicine and Stem Cell Research, Department of Obstetrics and Gynecology and Reproductive Sciences, University of California, San Francisco, CA, USA

Scott A. Coonrod
Baker Institute for Animal Health, College of Veterinary Medicine, Cornell University, Ithaca, NY, USA

David S. Cram
Department of Anatomy and Developmental Biology, Monash University, Clayton, Australia

Madeleine Dólleman
Department of Reproductive Medicine and Gynecology, University Medical Center, Utrecht, the Netherlands

Ursula Eichenlaub-Ritter
Faculty of Biology, Institute of Gene Technology/ Microbiology, University of Bielefeld, Bielefeld, Germany

Anna P. Ferraretti
SISMeR, Reproductive Medicine Unit, Bologna, Italy

Tom P. Fleming
Centre for Biological Sciences, University of Southampton, Southampton General Hospital, Southampton, UK

Saiichi Furukawa
University of Tokyo, Tokyo, Japan

Fulvio Gandolfi
Laboratory of Biomedical Embryology, Centre for Stem Cell Research UNISTEM, Università degli Studi di Milano, Milan, Italy

Luca Gianaroli
SISMeR, Reproductive Medicine Unit, Bologna, Italy

Isabelle Gilbert
Université Laval, Quebec City, Quebec, Canada

Robert B. Gilchrist
Research Centre for Reproductive Health, Robinson Institute, School of Paediatrics and Reproductive Health, The University of Adelaide, Adelaide, Australia

Roger Gosden
Jamestowne Bookworks, Williamsburg, VA, USA

Alain Gougeon
CRCL, UMR INSERM and CNRS, Faculté de Médecine Laennec, Lyon, France

John B. Gurdon
Wellcome Trust/Cancer Research UK Gurdon Institute, University of Cambridge, Cambridge, UK

Karen E. Hemmings
Division of Reproduction and Early Development, Leeds Institute of Genetics, Health and Therapeutics, University of Leeds, Leeds, UK

Mary Herbert
Wellcome Trust Centre for Mitochondrial Research, Institute for Ageing and Health, Newcastle University, Bioscience Centre, International Centre for Life, Newcastle-upon-Tyne, UK

Hayden Homer
Mammalian Oocyte and Embryo Research Laboratory, Cell and Developmental Biology, Division of Biosciences and Reproductive Medicine Unit, Institute for Women's Health, UCL, London, UK

Aaron J. W. Hsueh
Program of Reproductive and Stem Cell Biology, Department of Obstetrics and Gynecology, Stanford University School of Medicine, Stanford, CA, USA

Karin Hübner
Department of Cell and Developmental Biology, Max Planck Institute for Molecular Biomedicine, Münster, Germany

Gayle M. Jones
GMJ A.R.T. Solutions, Melbourne, and Department of Anatomy and Developmental Biology, Monash University, Clayton, Australia

Kazuhiro Kawamura
Department of Obstetrics and Gynecology, St. Mariana University School of Medicine, Kawasaki, Japan

Boram Kim
Baker Institute for Animal Health, College of Veterinary Medicine, Cornell University, Ithaca, NY, USA

Keith E. Latham
Department of Animal Science, College of Agriculture, Michigan State University, East Lansing, MI, USA

Ruth Lathi
Department of Obstetrics and Gynecology, Reproductive Endocrinology and Infertility, Stanford School of Medicine, Palo Alto, CA, USA

Qinglei Li
Department of Veterinary Integrative Biosciences, Texas A&M University, College Station, TX, USA

M. Cristina Magli
SISMeR, Reproductive Medicine Unit, Bologna, Italy

Victoria L. Mascetti
Department of Surgery and Anne McLaren Laboratory for Regenerative Medicine, University of Cambridge, Cambridge, UK

Martin M. Matzuk
Departments of Molecular and Human Genetics, Pathology and Immunology, Molecular and Cellular Biology, and Pharmacology, Baylor College of Medicine, Houston, TX, USA

Serge McGraw
Departments of Pediatrics, Human Genetics, and Pharmacology and Therapeutics, McGill University and of the Research Institute of the McGill University Health Centre at the Montreal Children's Hospital, Montreal, Canada

Shoukhrat Mitalipov
Division of Reproductive and Developmental Sciences, Oregon National Primate Research Center and Departments of Obstetrics and Gynecology and Molecular and Medical Genetics, Oregon Stem Cell Center, Oregon Health and Science University, Beaverton, OR, USA

Kei Miyamoto
Wellcome Trust/Cancer Research UK Gurdon Institute, University of Cambridge, Cambridge, UK

Swapna Mohan
Department of Biomedical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY, USA

Lawrence Nelson
Intramural Research Program on Reproductive and Adult Endocrinology, Integrative Reproductive Medicine Group, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD, USA

Robert J. Norman
The Robinson Institute Research Centre for Reproductive Health, School of Paediatrics and Reproductive Health, University of Adelaide, Adelaide, Australia

Roger A. Pedersen
Department of Surgery and Anne McLaren Laboratory for Regenerative Medicine, University of Cambridge, Cambridge, UK

Jia Peng
Departments of Molecular and Human Genetics, and Pathology and Immunology, Baylor College of Medicine, Houston, TX, USA

Melissa E. Pepling
Department of Biology, Syracuse University, Syracuse, NY, USA

Luca Persani
Department of Clinical Sciences and Community Health, University of Milan and Division of Endocrine and Metabolic Diseases, IRCCS Istituto Auxologico Italiano, Milan, Italy

Helen M. Picton
Division of Reproduction and Early Development, Leeds Institute of Genetics, Health and Therapeutics, University of Leeds, Leeds, UK

Carlos Plancha
Unidade de Biologia da Reprodução, Instituto de Histologia e Biologia da Deservolvimento, Faculdade de Medicina de Lisboa, Lisbon and Centro Médico de Assistência à Reprodução – CEMEARE, Lisbon, Portugal

Santhi Potireddy
Department of Biochemistry, Temple University School of Medicine, Philadelphia, PA, USA

Renee A. Reijo Pera
Center for Human Embryonic Stem Cell Research and Education, Institute for Stem Cell Biology and Regenerative Medicine, Department of Obstetrics and Gynecology, Stanford University School of Medicine, Palo Alto, CA, USA

Laura Rienzi
Centro GENERA, Clinica Valle Giulia, Rome, Italy

Rebecca L. Robker
The Robinson Institute Research Centre for Reproductive Health, School of Paediatrics and Reproductive Health, University of Adelaide, Adelaide, Australia

Heide Schatten
Department of Veterinary Pathobiology, University of Missouri, Columbia, MO, USA

Hans R. Schöler
Department of Cell and Developmental Biology, Max Planck Institute for Molecular Biomedicine and Medical Faculty, University of Münster, Münster, Germany

Stephanie Sherman
Department of Human Genetics, Emory University School of Medicine, Atlanta, GA, USA

David Silber
Infertility Center of St. Louis, St. Luke's Hospital, St. Louis, MO, USA

Sherman Silber
Infertility Center of St. Louis, St. Luke's Hospital, St. Louis, MO, USA and University of Amsterdam, Department of Obstetrics and Gynecology, Reproductive Endocrinology, Amsterdam, Netherlands

Marc-André Sirard
Université Laval, Quebec City, Quebec, Canada

Johan Smitz
Follicle Biology Laboratory, UZ Brussel, Brussels, Belgium

Dominic Stoop
Centre for Reproductive Medicine, UZ Brussel, Brussels, Belgium

Fumihiro Sugawa
Department of Cell and Developmental Biology, Max Planck Institute for Molecular Biomedicine, Münster, Germany

Koji Sugiura
University of Tokyo, Tokyo, Japan

Qing-Yuan Sun
State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing, China

Karl Swann
Institute of Molecular and Experimental Medicine, Cardiff University School of Medicine, Cardiff, UK

Jeremy G. Thompson
Research Centre for Reproductive Health, Robinson Institute, School of Paediatrics and Reproductive Health, The University of Adelaide, Adelaide, Australia

Jacquetta M. Trasler
Departments of Pediatrics, Human Genetics, and Pharmacology and Therapeutics, McGill University and of the Research Institute of the McGill University Health Centre at the Montreal Children's Hospital, Montreal, Canada

Alan Trounson
Californian Institute for Regenerative Medicine, San Francisco, CA, USA

Helen A. L. Tuppen
Wellcome Trust Centre for Mitochondrial Research, Institute for Ageing and Health, Newcastle University Medical School, Newcastle-upon-Tyne, UK

Doug M. Turnbull
Wellcome Trust Centre for Mitochondrial Research, Institute for Ageing and Health, Newcastle University Medical School, Newcastle-upon-Tyne, UK

Miguel A. Velazquez
Centre for Biological Sciences, University of Southampton, Southampton General Hospital, Southampton, UK

Dagan Wells
Nuffield Department of Obstetrics and Gynaecology, Institute of Reproductive Sciences, Oxford, UK

Claus Yding Andersen
Laboratory of Reproductive Biology, Juliane Marie Centre, University Hospital of Copenhagen, and Faculty of Health Science, University of Copenhagen, Copenhagen, Denmark

Hang Yin
Center for Reproductive Medicine and Infertility, Weill Medical College of Cornell University, New York, NY, USA

Preface

When approached by Nick Dunton of Cambridge University Press to edit the second edition of *The Biology and Pathology of the Oocyte*, my response was an emphatic yes, providing my co-editor Roger Gosden could be enticed from retirement. He agreed and we both wanted Ursula Eichenlaub-Ritter to be the third editor because we admired her expertise in the basic biology of the oocyte and her ability to get the job done. There has been incredible progress in the knowledge of the oocyte and applications for medicine that have regularly appeared since the first edition. We considered the areas of reproductive technology – IVF – and the areas of reprogramming somatic cell phenotype as spin-offs of the progress made in oocyte biology. Both these areas received Nobel Prizes in the last few years and are included in the contributions for the second edition. We were fortunate to have John Gurdon open the second edition the year (2013) after he won the Nobel Prize in Physiology or Medicine. He and his coauthor set the scene for a rather different perspective of the power and influence of the oocyte in modern biology. The contributors invited for the second edition are exceptional in their areas of oocyte biology, pathology, and applications to biotechnology and medicine. We think they have captured the excitement of the fast-moving frontier of the oocyte field.

The second edition will enthuse the reader interested in how the oocyte is formed, its function, and the underlying mechanisms of what is the most extraordinary cell in the body. It remains the germinal link from generation to generation and must undergo the most elaborate series of changes to be ready to accept the

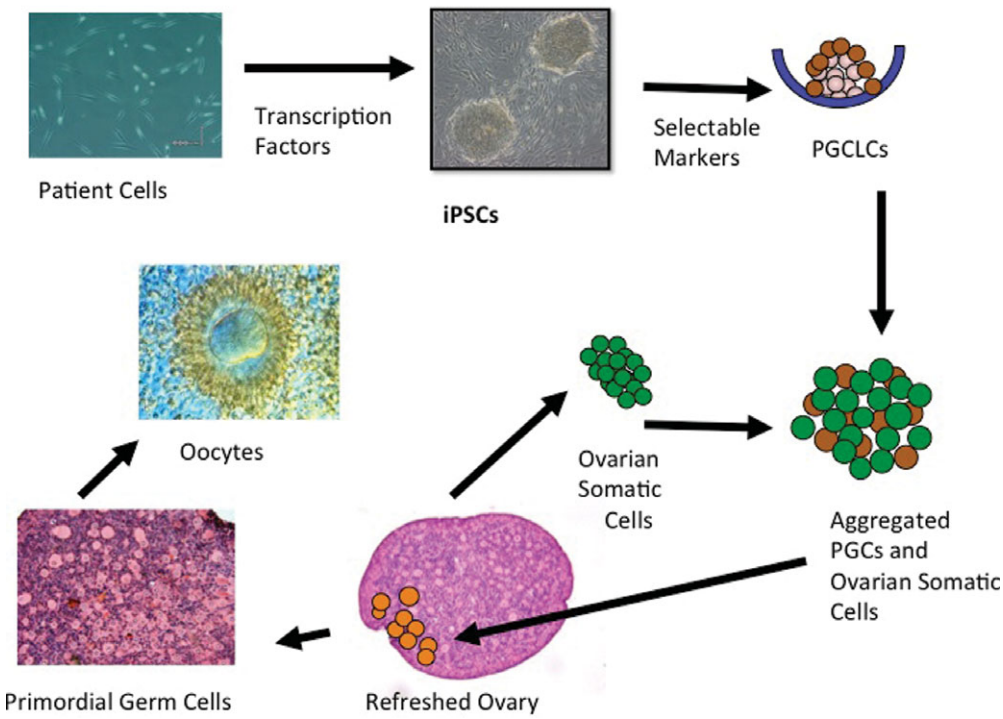

Figure P.1 A potential future strategy for generating viable new oocytes using technology to create iPSCs from adults, their germ cell differentiation and maturation in reconstructed ovaries. PGCLCs, primary germ cell-like cells; PGCs, primary germ cells. From Trounson [3].

genomic contribution of the most differentiated of all cells – the sperm. The oocyte must then enter the developmental program that enables an organism to arise with extremes in patterning and lineage differentiation consistent with the species of origin. In this exquisitely crafted program of development, it is possible to intervene to manipulate the oocyte for purposes of solving human infertility, to clone animals, develop pluripotent embryonic stem cells, and reprogram cell commitment in fully differentiated cells in animals including the human – so-called induced pluripotent stem cells (iPSCs). As a consequence we are able to address human infertility and avoid some of the worst inheritable genetic diseases, enable advances in selective animal breeding, and potentially address many human pathologies by using stem cell therapies.

While editing the second edition, we noted the astonishing reports of Hayashi *et al.* [1, 2] who were able to generate sperm and eggs in mice from embryonic stem cells (see Chapter 16). While it remains to be seen if other labs can replicate their observations, these could herald the ultimate method to derive new oocytes for research in the human and other species. Importantly, iPSCs derived from adult cells of female mice could be directed into primordial germ cells and selected for aggregation with fetal ovarian somatic cells to form viable follicles, oocytes, embryos, and live young – a possible future treatment for sterility (see Figure P.1). We would expect a considerable expansion of research in this area because of the implications for human sterility, animal reproduction, and conservation of threatened species. Perhaps other developments will arise around the germ cell and oocyte that will also accelerate the field in new directions. It is often difficult to predict what the next major advance will be. We hope funding bodies will continue to strongly support research on the oocyte as the NIH did with Dick Tasker's Egg Club.

We wish to thank all those contributors who selflessly gave their time to make the second edition a remarkable and very different book. We also thank Rob Sykes for all his assistance and enthusiasm in the publishing team and for Nick Dunton in getting us together. It has been a privilege to work with you all.

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Alan Trounson, Roger Gosden, and
Ursula Eichenlaub-Ritter

Chapter 1

Insights into the amphibian egg to understand the mammalian oocyte

Kei Miyamoto and John B. Gurdon

Abstract

Amphibian eggs and oocytes have been widely used as a model system for understanding animal development. They have led to numerous major discoveries in cellular and developmental biology. These findings have greatly helped us to understand the physiology of mammalian oocytes. Amphibian eggs have also played an important role not only in revealing genomic conservation and plasticity historically, but also in gaining a mechanistic insight into nuclear reprogramming. This chapter summarizes major findings using amphibian eggs and oocytes, focusing on reprogramming aspects. We also discuss how *Xenopus* eggs can be used to study mammalian oocytes.

Introduction

For over 100 years, amphibian embryos have been the favored choice of material for research into mechanisms of early vertebrate animal development. This is because amphibian embryos are unusually large, being about 1 mm in diameter. The whole amphibian egg divides into an embryo whereas, in birds, for example, only a very small amount of material in the huge egg actually forms an embryo. All mammalian eggs are relatively inaccessible and are very small, usually 70–120 μm in diameter. European amphibia include the Urodeles (salamanders, newts, *Triturus*, etc.) as well as Anura (frogs, toads, *Rana*, *Bufo*). Members of these groups usually lay abundant eggs in natural pond water in the northern-hemisphere spring. The eggs are easy to culture. Their large size and consistency make them exceptionally favorable for microdissection and other manipulative experiments. This was the material used by Spemann, Hamburger, and Holtfreter and others.

The only disadvantage of most anuran species is that they produce eggs naturally only in the European spring, amounting to one or two months during the year. Soon after World War II, *Xenopus* became the favored choice for amphibian research. The interesting history of how this happened was largely coincidental [1]. The huge advantage of *Xenopus* is that it can be induced to lay eggs at any time of year, following an injection of mammalian pituitary hormone. The species is permanently aquatic, making its laboratory maintenance a great deal easier than for land-living amphibia. Since it naturally lives in highly infected pond water (in Africa) *Xenopus laevis* is exceptionally disease-free and easy to culture. Over the last 50 years, nearly all amphibian research has come to be conducted on *Xenopus* species.

The majority of experimental interventions now carried out on a range of vertebrate species, and especially in mammals, have their origin in work that started with amphibia. Moreover, many scientific discoveries and knowledge in amphibia have been extended to mammals. In this review, we trace back the origin of many experimental procedures and scientific findings that are now in widespread use in mammals, and find that these were first pioneered in amphibia.

Meiotic prophase germ line in *Xenopus laevis* and the mouse

In *Xenopus laevis*, the female germ cell, the so-called oocyte, is arrested in prophase of meiosis I (MI) in the ovary of the adult frog (Figure 1.1; stage I to VI). During this period, oocytes accumulate a stockpile of macromolecules and organelles that are required to support early embryonic development. Stage VI oocytes are fully grown and capable of reacting to progesterone from the surrounding follicle cells. They complete MI and are subsequently arrested in metaphase of meiosis II (MII). These matured

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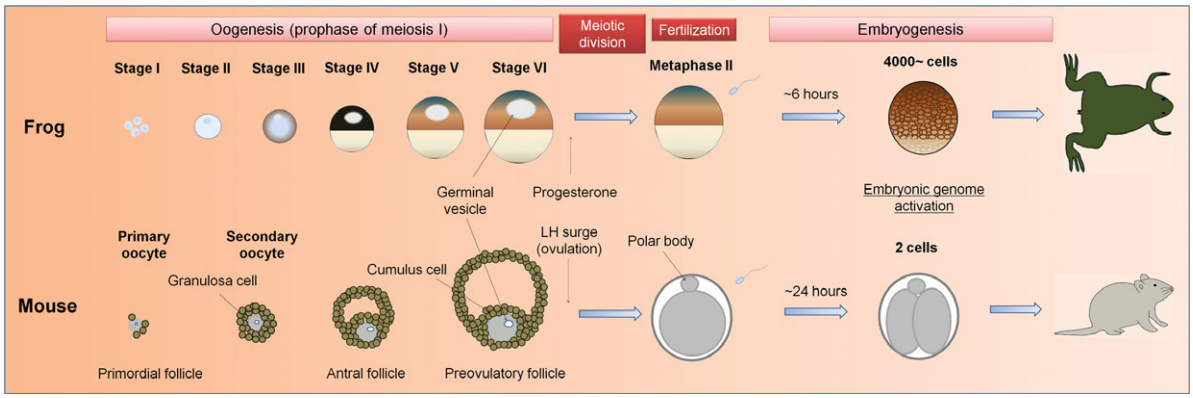

Figure 1.1 Oogenesis and embryogenesis in frog and mouse. Oocytes contain a giant nucleus referred to as the germinal vesicle. Upon resumption of meiosis, germinal vesicles are broken down and oocytes are matured to the metaphase II stage, followed by fertilization. Fertilized embryos undergo early cleavages directed by maternally stored factors without conspicuous transcription. Embryonic genome activation happens at the indicated cell stages, allowing embryos to develop further. LH, luteinizing hormone.

oocytes are then ovulated as unfertilized eggs (MII eggs). Upon fertilization, the egg is released from meiotic arrest and enters interphase. Early embryonic development is characterized by rapid progression through the cell cycle, consisting of repeated S- and M-phases. The stockpile of components present within the eggs supports this early development until the mid-blastula transition (MBT). Major embryonic gene activation starts at this MBT (Stage 8–8.5 embryos; 4000–8000 cells) and embryonic gene products then direct further embryonic development.

Mouse oocytes, as well as *Xenopus* oocytes, are arrested at prophase of MI in the ovary. During oogenesis, mouse oocytes increase their size from ~10 μm to 80 μm while actively transcribing the maternal genome for subsequent embryonic development (Figure 1.1). When the luteinizing hormone (LH) surge stimulates the resumption of meiosis, oocytes surrounded by cumulus cells are released from fully grown follicles. Oocytes are re-arrested at the MII stage until fertilization takes place. Major embryonic genome activation is first observed at the 2-cell stage.

As summarized above, maternal factors required for early embryonic development are accumulated in both *Xenopus* and mouse oocytes. During this oogenesis period, oocytes at the first meiotic prophase contain a giant nucleus referred to as the germinal vesicle (GV). The *Xenopus* GV reaches a diameter of 400 μm, which is more than 100 times larger than that of a mature mouse oocyte itself. It also stores huge amounts of macromolecules and nuclear organelles for intensive transcription; these include extrachromosomal nucleoli (~1500), Cajal bodies (50–100), and RNA polymerase II whose activity is sufficient for 100 000 somatic nuclei [2]. Notably, *Xenopus* GV oocyte genomes form so-called lampbrush chromosomes with actively transcribing chromatin loops and are found throughout chromosomes. Chromatin loops are maximally extended during early oogenesis and retracted towards the fully grown stage (stage VI). In the mouse, although lampbrush-like chromosomes have not been identified, oocyte genomes are also actively transcribed and produce a large stockpile of maternal RNA and protein. The chromatin structure of a mouse oocyte has been extensively studied. Follicular activation, at the beginning of oogenesis, is characterized by the loading of an oocyte-specific linker histone H1foo (closely related to the *Xenopus* histone B4) into the oocyte nucleus. Chromatin in growing mouse oocytes is initially decondensed and supports active transcription. As oogenesis proceeds, chromatin becomes progressively condensed and transcriptionally silenced, forming a heterochromatin rim around the oocyte nucleolus. Recent research suggests that histone-modifying enzymes play roles in the mouse oocyte chromatin remodeling associated with changes in transcriptional abilities [3]. Revealing mechanisms of oocyte transcription and its associated chromatin structure helps our understanding not only of germ cell development but also of the maternal contribution to early embryonic development.

Signaling in early embryogenesis

The first pivotal experiment which demonstrated signaling in development was that of Spemann and Mangold [4]. By transplanting tissue from one embryo

into another (distinguished by pigment markers), it was proved that one set of cells can alter the fate of other cells placed near them. The Spemann signaling center exists in early amphibian embryos at the early gastrula stage. Subsequently, Nieuwkoop [5] demonstrated that signaling also occurs much earlier in development from the vegetal cells to the overlying animal cells. This Nieuwkoop center is the first known source of signaling in animal development and is responsible for the formation of the mesoderm layer.

In more recent years the mechanism of the Spemann signaling process has become greatly clarified. This is particularly due to the work of De Robertis [6] who has identified a number of signaling and other molecules that regulate the signaling process and in particular the distance in an embryo over which a signal factor acts. A network of such signaling centers and of the counteracting molecules that restrict the strength or distance of signaling has been identified [7].

Many such signaling processes work as morphogen gradients. This means that the concentration of signaling factor decreases with distance from its source. Most importantly, cells are able to sense the strength of the signal, at the position in which they lie, and differentiate in directions related to the strength of the signal that they receive. The mechanisms of morphogen gradient interpretation continue to attract wide interest [8]. The phenomenon is of great importance because the single source of signal can generate several different cell types according to the strength and duration of signal that a cell receives. The regulation of morphogen gradient interpretation is complex because it depends on the rate of movement of the morphogen, its stability, and particularly on the abundance of counteracting factors which can inactivate the morphogen [9].

Signaling in embryos is now well established in mammalian development. Gene ablation technologies and the availability of cultured pluripotent stem cells, such as embryonic and epiblast stem cells, in mice accelerated our understanding of how signaling pathways function in mammals [10, 11]. Signaling pathways that play a key role in early post-implantation development, such as Wnt and transforming growth factor beta (TGFβ), have been extensively studied [12].

Cell-free system

Components of amphibian eggs can be efficiently extracted by crushing them in an appropriate buffer. These cell-free extracts retain many egg proteins intact and as a result numerous cellular events, such as transcription, translation, cell-cycle progression, chromatin remodeling, and even reprogramming, are reproduced in the extracts to some extent. *Xenopus laevis* eggs have been widely used as a source of extracts due to their large size and abundance. Egg extracts are valuable for identifying molecules and molecular mechanisms involved in cellular events since many biochemical approaches can be applied to extracts. For example, specific proteins can be depleted from extracts by immunodepletion using antibodies in order to assess the roles of these proteins. To carry out such knockout experiments is very challenging in living embryos. In addition, the complexity of a live cell or egg can be somewhat simplified in extracts. We summarize below major discoveries and recent applications of egg extracts to understand reprogramming.

DNA replication

Xenopus egg extracts that are widely used at present were first reported by Lohka and Masui [13]. Unfertilized frog eggs are collected in a test tube and crushed by centrifugation. After centrifugation, the cytoplasmic fraction is used as an extract (Figure 1.2A). When demembranated sperm nuclei are incubated in this extract, these nuclei are immediately decondensed (Figure 1.2B) and start to form nuclear envelopes and pronuclei, accompanied by DNA replication. This is followed by breakdown of nuclear envelopes and chromosome condensation. This egg extract was further developed and could also replicate purified DNA [14]. Egg factors required for the DNA replication, such as Orc (origin recognition complex, subunit 2) [15], cdc6 (cell division cycle 6) [16], and MCMs (minichromosome maintenance proteins) [17], have been found and characterized in this system.

Cell-cycle analysis

Many important findings using *Xenopus* egg extracts have been achieved in the field of cell-cycle analysis. After the first egg extract, in which a single cell cycle is reproduced [13], Hutchison *et al.* [18] and Murray and Kirschner [19] developed and established the cycling extracts in which multiple cell cycles are reproduced. This led to the identification of cyclin-dependent protein kinase, CDK1, and cyclin B as necessary regulators for mitotic entry. CDK1 and cyclin B are also known as maturation-promoting factor (MPF). Molecules that modulate MPF activity have been extensively studied. Extracts have been also prepared from eggs arrested in

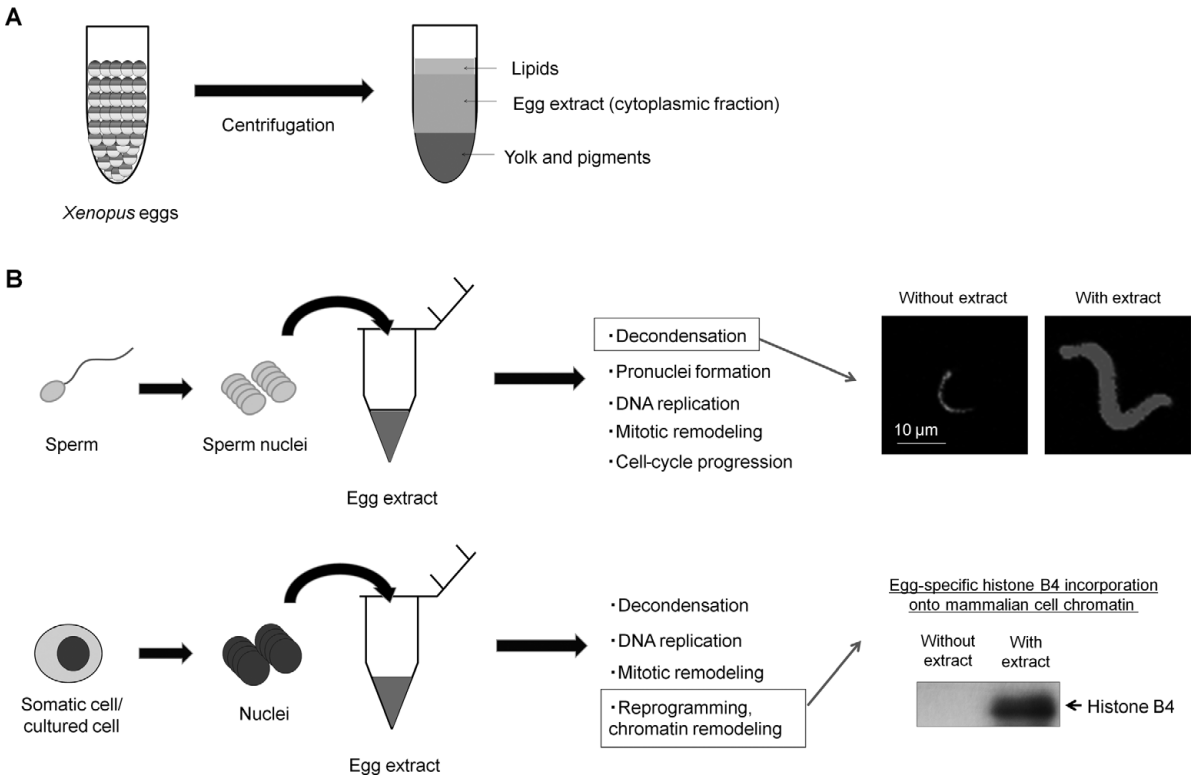

Figure 1.2 *Xenopus* egg extracts and their utility. (A) *Xenopus* eggs collected in a test tube are crushed by centrifugation and separated into three fractions. The middle fraction containing cytoplasm and membranes is used as an egg extract. (B) Various kinds of cellular and molecular events are reproduced in *Xenopus* egg extracts. When sperm nuclei are incubated in egg extracts, rapid decondensation of sperm nuclei is observed as shown. Egg extracts also induce several changes in somatic nuclei. Notably, a part of reprogramming, which includes oocyte linker histone B4 incorporation onto chromatin as revealed by the western blotting, is induced in somatic nuclei.

metaphase of the second meiotic division by an activity called cytostatic factor (CSF) [20]. CSF in connection with MPF activity plays an essential role in MII arrest to prevent parthenogenesis. Although some differences in molecular behavior between *Xenopus* and the mouse have been reported [21], the *Xenopus* egg cell-free system is a powerful tool to analyze biochemical interactions and signaling pathways involved in this meiotic arrest, egg activation, and early embryonic cell cycles.

Chromatin remodeling (sperm decondensation, mitotic remodeling and chromatin assembly)

As previously mentioned, sperm decondensation and male pronucleus formation were induced in frog egg extracts. By utilizing this property, nucleoplasmin in egg extracts was identified as a factor to decondense sperm nuclei and remove protamines from sperm nuclei [22, 23]. Oocyte linker histone B4 is also involved in sperm chromatin remodeling [24].

In addition to the above-mentioned replicating cell-cycle extracts, CSF extracts maintain the metaphase state so that they can induce nuclear envelope breakdown, chromosome condensation, and spindle assembly [20]. The roles of chromosomal proteins, such as topoisomerase IIα and histone H1, in mitotic chromosome assembly have been examined [25, 26]. Condensin necessary for mitotic chromosome condensation was identified using mitotic extracts [27].

Chromatin is formed when double-stranded or single-stranded plasmid DNA molecules are incubated in egg extracts [28, 29], providing unique opportunities to study chromatin assembly. Histones stored in eggs carry distinct patterns of histone modification [30]. Changes in histone modification are related to those of transcriptional activities in oocytes.

Therefore, it would be interesting to study chromatin structures in eggs/oocytes using this system.

Recapitulation of reprogramming in egg/oocyte extracts

Since *Xenopus* egg extracts mimic sperm nuclear remodeling after fertilization, it is reasonable to speculate that egg extracts, at least to some extent, can recapitulate somatic nuclear reprogramming that is induced after nuclear transplantation to eggs. Kikyo *et al.* [31] first reported that somatic nuclei incubated in *Xenopus* egg extracts are remodeled towards an embryonic state in which somatic proteins are lost, while egg proteins are incorporated into somatic chromatin. They have shown that the ATP-dependent chromatin remodeling factor ISWI plays a key role in this process. This system has also led to the identification of FRGY2a/b as a critical factor for nucleolar disassembly [32]. These are the first reports to identify actual egg factors involved in somatic cell reprogramming in vitro, proving that the egg cell-free system is a good route to manifest reprogramming mechanisms.

Subsequently, several reports have shown that reprogramming activities of egg extracts are conserved in mammalian somatic nuclei. The incorporation of *Xenopus* egg factors into mammalian chromatin was observed, including oocyte type lamin LIII [33] and histone B4 (Figure 1.2B) [34]. Moreover, the ability of egg extracts to trigger induction of mammalian embryonic gene expression has been shown [34, 35]. Egg and oocyte extracts from another amphibian species, the axolotl, also exhibit strong epigenetic reprogramming activities in mammalian nuclei [36]. These findings emphasize the utility of amphibian egg extracts as a tool to study reprogramming of mammalian nuclei, especially for the purpose of identifying egg factors with reprogramming activities.

Application of cell-free systems for understanding mammalian oocytes

The *Xenopus* egg cell-free system has greatly advanced molecular understanding of many cellular events, as mentioned above. Factors and mechanisms originally found in this system have been extensively tested and validated in mammalian in vivo systems. Therefore, the *Xenopus* cell-free system has served as a foundation for revealing molecular mechanisms. If a similar kind of cell-free system can be developed in mammals, our molecular understanding of mammalian oocytes may advance rapidly. This idea has been hampered by the fact that we cannot collect enough mammalian oocytes for making functional extracts. Nevertheless, some attempts to produce these have been made [37, 38], although further sophistication is needed. It might be a good idea to start first with one specialized oocyte extract that can reproduce only one aspect of cellular events.

Special manipulations

Nuclear transfer in eggs and oocytes

Spemann did an ingenious delayed nucleation experiment in which the nucleus of one of the first eight cells of an amphibian embryo was shown to lead to the formation of a normal embryo [39]. This demonstrated the totipotency of one of the first eight cells of an embryo, but did not test later stages. The first major success in nuclear transplantation was that of Briggs and King [40] when they were able to transplant the nuclei of *Rana pipiens* blastula cells into enucleated eggs of that species and obtain normal embryos. When they tried the same experiment using nuclei from slightly later stages, they were no longer able to obtain normal development [41]. They reached the entirely reasonable conclusion that, as development proceeds, the nuclei of somatic cells lose their totipotency. In 1958 nuclear transplantation had succeeded in *Xenopus*. A series of experiments culminated in the finding that totally normal, sexually mature adult animals could be obtained by transplanting the nuclei of embryo endoderm cells into enucleated eggs (Figure 1.3A) [42]. Subsequently it was found that the nuclei of differentiated intestinal epithelium cells could also yield normal, sexually mature animals. This was the proof that cell differentiation does not necessarily involve any loss of genetic totipotency. It is now generally accepted that, with very special exceptions like antibody-producing cells, all cells of the body have the same complete genome. In recent time, notably following the work of Takahashi and Yamanaka [43], the principle of totipotency of somatic cell nuclei has led to extensive work aiming to derive embryonic stem cells from adult tissue cells, with a view to drug testing and possibly cell replacement therapy.

For technical reasons, it was nearly 40 years after the first successful nuclear transplantation in amphibia

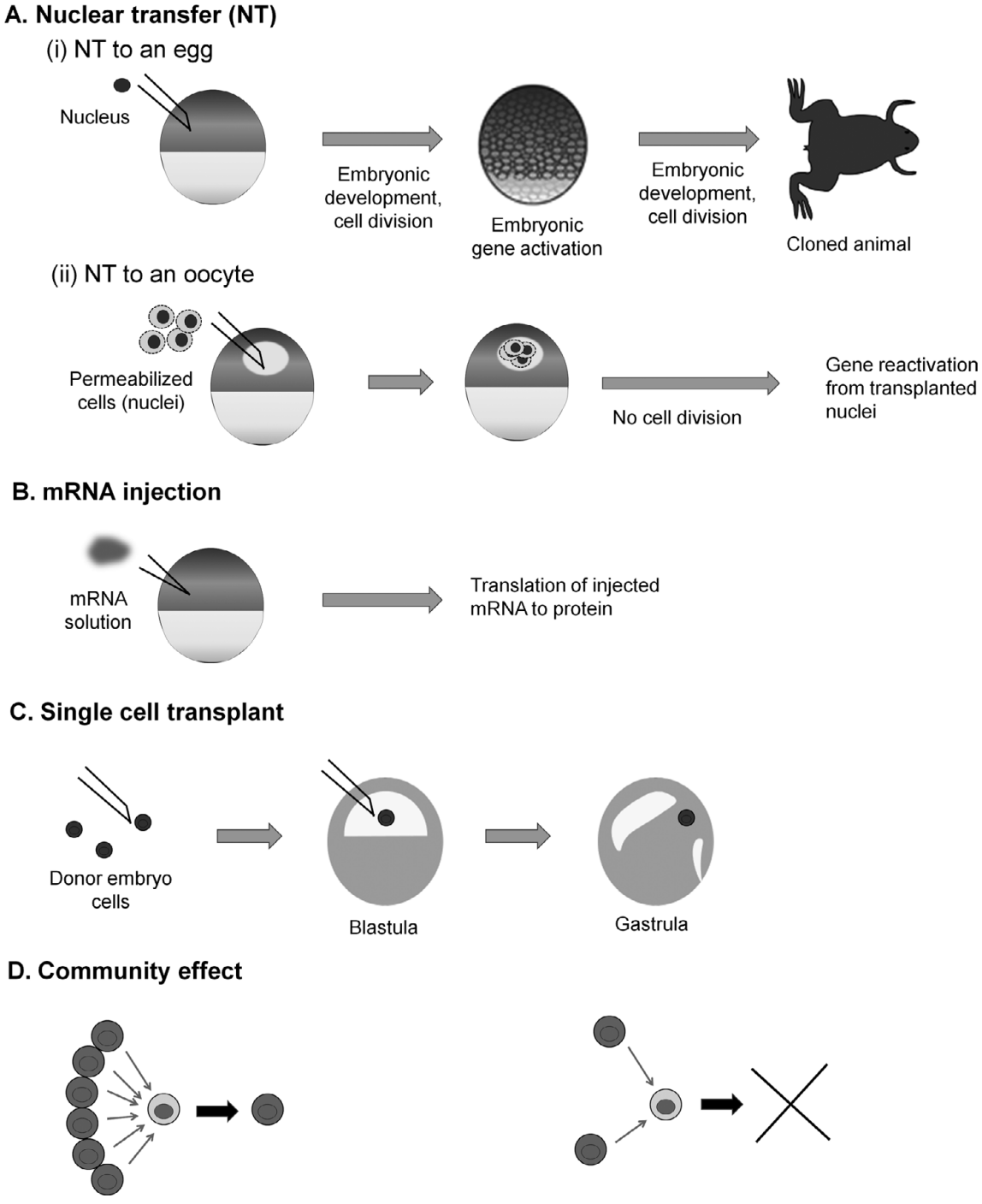

Figure 1.3 Special manipulations using *Xenopus* eggs and oocytes. (A) Two types of nuclear transfer (NT) are available in *Xenopus*. NT to MII eggs generates NT embryos, which finally give rise to cloned animals. Hundreds of nuclei can be injected into a nucleus of the *Xenopus* oocyte. Injected nuclei do not change to another cell type, but, instead, previously silenced genes are reactivated. Direct transcriptional reprogramming of somatic nuclei without the need for cell division is induced in this oocyte NT unlike NT to an egg, in which cell divisions are required before initiation of embryonic gene transcription. (B) In vitro synthesized mRNAs are injected into the cytoplasm of eggs/oocytes and are readily translated. (C) Embryo cells are separated from each other. A single cell is injected into the cavity of a blastula embryo and the fate of the injected cell can be traced. (D) Cell transplantation experiments led to the finding of the community effect, the phenomenon in which cells in close proximity to each other contribute some signal factor and receive more signals from their neighbors, thereby allowing efficient differentiation.