Nucleic Acids and Molecular Biology 25

Hans Joachim Gross, Janusz M. Bujnicki (Eds.)

Y. Kikuchi
E.Y. Rykova
Editors

Extracellular Nucleic Acids

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Editors
Yo Kikuchi
Department of Environmental
and Life Sciences
Toyohashi University of Technology
441-8580 Toyohashi-city, Aichi
Japan
kikuchi@tut.jp

Elena Y. Rykova
Institute of Chemical Biology
and Fundamental Medicine,
Lavrentjeva Prospect, 8
630090 Novosibirsk
Russia
rykova@niboch.nsc.ru

Series Editors
Hans J. Gross
Institut für Biochemie
Biozentrum
Am Hubland
97074 Würzburg
Germany

Janusz M. Bujnicki
International Institute of Molecular
and Cell Biology
Laboratory of Bioinformatics and
Protein Engineering
Trojdena 4
02-109 Warsaw
Poland

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Preface

Nucleic acids have a wide variety of functions in the main systems of life, gene maintenance, and expression, and therefore nucleic acids as functional forms have been believed to exist only inside cells. Extracellular nucleic acids have long been known, but these nucleic acids were initially presumed to be derived from cells lysed by natural cell death or viruses and considered to be of limited importance. However, these extracellular nucleic acids have recently emerged as important players in the fields of medical and environmental microbiology. In recent years, extracellular nucleic acids have been shown to be involved in not only microbial evolution as genetic elements but to also have structural roles in bacterial communities, such as biofilms. Circulating nucleic acids have been found in human blood in normal and pathologic states. Moreover, they are expected to be the low invasive markers for diagnosis of several diseases. Extracellular nucleic acids have attracted attention as active modulators of the immune system in higher organisms.

This volume of the Nucleic Acids and Molecular Biology series is devoted to the extracellular nucleic acids, which are found in different biological fluids in the highest organisms and in the extracellular milieu of bacterial communities. Applications of the phenomena are also important topics for consideration.

Recently, characterization and the role of extracellular DNA of the bacterium *Pseudomonas aeruginosa* were clearly described by Tolker-Nielsen and colleagues. Chiang and Tolker-Nielsen begin this volume by describing new roles of extracellular DNA in bacterial biofilm formation.

Extracellular DNAs in the environment are thought to be used as genetic donors in natural transformation of several organisms. The following two chapters are related to evolution and microbial ecology. Lang and Beatty introduced extracellular DNAs packed in virus-like particles. Tani and Nasu discuss extracellular DNAs and microbial diversity from a global viewpoint.

Extracellular DNAs of high molecular weight seem to be more stable than previously imagined. Kaneko and Itaya describe a new technique for introduction of large DNAs from lysed cells into other species mimicking horizontal gene transfer in nature. Chapter 5 by Kikuchi also presents technology-oriented topics.

The physiology and biotechnology of a marine bacterium are described. In addition to the extracellular DNA, analysis of extracellular RNAs of this organism is described. A newly developed method for production of RNA drugs using this bacterium is also presented.

One of the current hot topics in biology, systemic RNA interference, in which some RNAs interfere with gene expression, is strongly related to the subject of extracellular RNAs. Timmons reviews these topics and leads to the following chapters for higher organisms.

Extracellular nucleic acids circulating in the human blood were first considered to be products of cell degradation. Later on, concentration and composition of the circulating nucleic acids were found to be significantly changed in the blood of patients with different pathologies, including cancer, compared with healthy subjects. Rykova et al. review the recent progress in the investigation of the circulating DNA and RNA as markers of cancer and other disorders, such as diabetes, trauma, stroke, myocardial infarction, complications in transplantation, etc. Development of the low invasive diagnostics of pregnancy-associated and fetal disorders based on the circulating DNA and RNA markers is described by Tsang and Lo. Tewari and Mitchell discuss the assessment of microRNAs as a promising approach for cancer diagnostics. Questions are brought up, which remain to be resolved to develop robust assays for the pathologies diagnosis and prognosis relevant for clinical use.

Gahan and Stroun present data highlighting appearance of extracellular nucleic acids in the human blood and indicating their important biological roles in normal and pathologic states of the organism. The chapter provides evidence that circulating nucleic acids can play signaling roles under normal conditions and become causative factors in the disease development. One of the important properties of nucleic acids and their metabolites is their potential to be active modulators of the immune system, which is detailed in two chapters. Koyama et al. describe the recognition and immune response of "nonself" nucleic acids during infection and "self" nucleic acids in autoimmune disorders. Some clinical applications of nucleic acids that utilize their immunogenic potential are illustrated. Klinman and Klaschik give the detailed description of the gene expression regulation by unmethylated CpG DNA triggered by Toll-like receptor 9 (TLR9). Synergistic modulation of gene expression by TLR9 and TLR3 ligands is also discussed.

This volume covers almost all current fields related to extracellular nucleic acids, including the fields of basic biology, ecology, and medical sciences. These are currently emerging fields of study. The reviews presented in this book will provide readers current knowledge into these exciting fields and also stimulate discussion to address new frontiers.

Toyohashi, Japan — Yo Kikuchi
Novosibirsk, Russia — Elena Y. Rykova

Contents

Contributors

Shizuo Akira Laboratory of Host Defense, WPI Immunology Frontier Research Center, Osaka University, 3-1 Yamada-oka, Suita, Osaka 565-0871, Japan

J. Thomas Beatty Department of Microbiology and Immunology, University of British Columbia, Vancouver, BC, V6T 1Z3, Canada

Wen-Chi Chiang Department of International Health, Immunology and Microbiology, Faculty of Health Sciences, University of Copenhagen, Blegdamsvej 3B, 2200 Copenhagen, Denmark

P.B. Gahan King's College London, Anatomy and Human Sciences, London Bridge, London SE1 1UL, UK

Ken J. Ishii Laboratory of Vaccine Science, WPI Immunology Frontier Research Center, Osaka University, 3-1 Yamada-oka, Suita, Osaka 565-0871, Japan, kenishii@biken.osaka-u.ac.jp; Laboratory of Adjuvant Innovation, National Institute of Biomedical Innovation, 7-6-8 Asagi Saito, Ibaraki, Osaka 567-0085, Japan

Mitsuhiro Itaya Institute for Advanced Biosciences, Keio University, Nipponkoku, Tsuruoka-shi, Yamagata 997-0017, Japan

Shinya Kaneko Graduate School of Bioscience and Biotechnology, Department of Life Science, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku, Yokohama-shi, Kanagawa 226-8501, Japan

Yo Kikuchi Department of Environmental and Life Sciences, Toyohashi University of Technology, 1-1 Hibarigaoka, Tempaku-cho 441-8580 Toyohashi, Japan, kikuchi@tut.jp

Sven Klaschik Cancer and Inflammation Program, National Cancer Institute, NCI in Frederick, Frederick, MD 21702, USA

Dennis Klinman Laboratory of Experimental Immunology, National Cancer Institute at Frederick, Frederick, MD 21702-1201, USA

Shohei Koyama Department of Respiratory Medicine, Tohoku University Graduate School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai-city, Miyagi 980-8575, Japan

P.P. Laktionov Institute of Chemical Biology and Fundamental Medicine, SB RAS, 8, Lavrentjeva Prosp., Novosibirsk 630090, Russia

Andrew S. Lang Department of Biology, Memorial University of Newfoundland, St. John's, NL A1B 3X9, Canada, aslang@mun.ca

Y.M. Dennish Lo Li Ka Shing Institute of Health Sciences and Department of Chemical Pathology, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, New Territories, Hong Kong SAR, China, loym@cuhk.edu.hk

Patrick S. Mitchell Human Biology Division, Fred Hutchinson Cancer Research Center, 1100 Fairview Avenue North, Mailstop D4-100, Seattle, WA 98109, USA; Molecular and Cellular Biology Program, University of Washington, Seattle, WA, USA

Masao Nasu Department of Environmental Science and Microbiology, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamada-oka, Suita, Osaka, 565-0871, Japan, nasu@phs.osaka-u.ac.jp

Elena Y. Rykova Institute of Chemical Biology and Fundamental Medicine, Siberian Division of the Russian Academy of Sciences, Lavrentiev Prosp., 8, Novosibirsk 630090, Russia, rykova@niboch.nsc.ru

M. Stroun OncoXL, Eclosion, 14m chemin des Aulx, 1228 Plan-les-Ouates, Geneva, Switzerland, Mauricestroun@bluewin.ch

Katsuji Tani Department of Environmental Science and Microbiology, Faculty of Pharmaceutical Sciences, Osaka-ohtani University, 3-11-1 Nishikiori-kita, Tondabayashi, Osaka 584-0854, Japan

Muneesh Tewari Human Biology Division, Fred Hutchinson Cancer Research Center, 1100 Fairview Avenue North, Mailstop D4-100, Seattle, WA 98109 USA, mtewari@fhcrc.org; Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, WA, USA; Public Health Sciences Division, Fred Hutchinson Cancer Research Center, Seattle, WA, USA

Lisa Timmons Department of Molecular Biosciences, University of Kansas Lawrence, KS, USA

Tim Tolker-Nielsen Department of International Health, Immunology and Microbiology, Faculty of Health Sciences, University of Copenhagen, Blegdamsvej 3B, 2200 Copenhagen, Denmark, ttn@sund.ku.dk

Jason C.H. Tsang Li Ka Shing Institute of Health Sciences and Department of Chemical Pathology, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, New Territories, Hong Kong SAR, China

V.V. Vlassov Institute of Chemical Biology and Fundamental Medicine, SB RAS, 8, Lavrentjeva Prosp., Novosibirsk 630090, Russia

Chapter 1
Extracellular DNA as Matrix Component in Microbial Biofilms

Wen-Chi Chiang and Tim Tolker-Nielsen

Contents

Abstract Bacteria in nature primarily live in surface-associated communities commonly known as biofilms. Because bacteria in biofilms, in many cases, display tolerance to host immune systems, antibiotics, and biocides, they are often difficult or impossible to eradicate. Biofilm formation, therefore, leads to various persistent infections in humans and animals, and to a variety of complications in industry, where solid–water interfaces occur. Knowledge about the molecular mechanisms involved in biofilm formation is necessary for creating strategies to control biofilms. Recent studies have shown that extracellular DNA is an important component of the extracellular matrix of microbial biofilms. The present chapter is focussed on extracellular DNA as matrix component in biofilms formed by *Pseudomonas aeruginosa* as an example from the Gram-negative bacteria, and *Streptococcus* and *Staphylococcus* as examples from the Gram-positive bacteria. Besides the role of extracellular DNA in biofilm formation, the mechanisms involved in DNA release from *P. aeruginosa*, *Streptococcus*, and *Staphylococcus* are addressed.

W-C. Chiang and T. Tolker-Nielsen (✉)
Department of International Health, Immunology and Microbiology, Faculty of Health Sciences, University of Copenhagen, Blegdamsvej 3B, 2200 Copenhagen, Denmark
e-mail: ttn@sund.ku.dk

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1.1 Introduction

Although the growth of bacteria in planktonic culture has been the mainstay of microbiological technique from the time of Pasteur to the present, and has provided an increasingly accurate understanding of prokaryotic physiology and genetics, it is now clear that bacteria in nature primarily live in surface-associated biofilm communities. Biofilm formation is believed to occur in a sequential process of (1) transport of microbes to a surface, (2) initial attachment, (3) formation of microcolonies, and (4) further proliferation and maturation; and the involvement of cellular motility, adhesins, matrix-forming exopolymers, and cell-to-cell signaling has been demonstrated (e.g., Davies et al. 1998; Espinosa-Urgel et al. 2000; Whitchurch et al. 2002; Klausen et al. 2003a, b; Friedman and Kolter 2004). During the last 10 years, there has been a rapidly increasing recognition of microbial biofilms as a highly significant topic in microbiology with relevance for many important areas in modern society such as drinking water supply systems, industrial settings, waste water treatment, bioremediation, chronic bacterial infections, nosocomial infections, and dental plaque. Figure 1.1 shows a confocal laser scanning microscope (CLSM) micrograph of a *Pseudomonas aeruginosa* biofilm with elaborate mushroom-shaped multicellular structures.

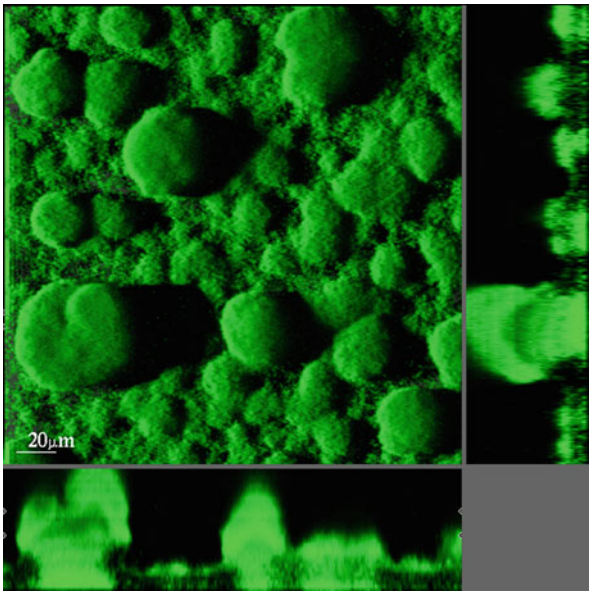

Fig. 1.1 CLSM micrograph acquired in a biofilm formed by Gfp-tagged *P. aeruginosa* PAO1. The central picture shows a top-down view, and the flanking pictures show side views. The size bar corresponds to 20 μm. Reproduced from Klausen et al. (2003b) with permission from Wiley-Blackwell publishing

One of the most distinctive features of biofilms is the extracellular polymeric substances that surround the bacteria and constitute the biofilm matrix. The composition of the biofilm matrix varies depending upon the bacterial species and the environmental conditions, but in general it consists of exopolymers such as polysaccharide, protein, and DNA (Sutherland 2001; Whitchurch et al. 2002). Apart from these exopolymers, outer membrane proteins and a variety of cell appendages such as fimbriae, pili, and flagella may also function as part of the biofilm matrix (Sutherland 2001). The components of the biofilm matrix are usually, but not always, produced by the bacteria themselves.

Because bacteria in biofilms, in many cases, display tolerance to host immune systems, antibiotics, and biocides, they are often difficult or impossible to eradicate (Costerton et al. 1999). Biofilm formation, therefore, leads to various persistent infections in humans and animals, and to a variety of complications in industry where solid–water interfaces occur. The extracellular matrix and the special physiology of bacteria in biofilms is believed to offer protection against various adverse factors including protozoan predation in environmental settings (Matz and Kjelleberg 2005), and host immune responses and antibiotic treatment in medical settings (Costerton et al. 1999). Knowledge about the molecular mechanisms that are involved in biofilm formation is necessary for creating strategies to control biofilms.

This chapter is focussed on extracellular DNA as matrix component in biofilms formed by *P. aeruginosa* as an example from the Gram-negative bacteria, and *Streptococcus* species and *Staphylococcus* species as examples from the Gram-positive bacteria. Besides the role of extracellular DNA in biofilm formation, the mechanisms involved in DNA release from *P. aeruginosa*, *Streptococcus*, and *Staphylococcus* are addressed.

1.2 Extracellular DNA as Matrix Component in *P. aeruginosa* Biofilm

Evidence for a role of extracellular DNA as matrix component in *P. aeruginosa* biofilms has been provided both for the *P. aeruginosa* PAO1 laboratory strain and for clinical *P. aeruginosa* isolates (Whitchurch et al. 2002; Nemoto et al. 2003). Biofilm formation by *P. aeruginosa* PAO1 in microtiter trays and in flow-chambers was inhibited in the presence of deoxyribonuclease I (DNase I) (Whitchurch et al. 2002). Moreover, young *P. aeruginosa* PAO1 biofilms, which had been grown in flow-chambers irrigated with DNase-free medium, were dispersed rapidly after addition of DNase I to the medium, whereas older *P. aeruginosa* PAO1 biofilms were not dispersed by DNase I treatment, suggesting that other components extracellular DNA stabilizes older *P. aeruginosa* PAO1 biofilms (Whitchurch et al. 2002). Matsukawa and Greenberg (2004) investigated the composition of the extracellular matrix of mature *P. aeruginosa* PAO1 biofilms, and found that extracellular DNA by far was the most abundant polymer, although exopolysaccharide

encoded by the *psl* genes appeared to be the most critical structural matrix component. Nemoto et al. (2003) found that mature biofilms formed by four different clinical *P. aeruginosa* isolates could be dispersed by DNase treatment, suggesting that extracellular DNA is the critical matrix component in mature biofilms formed by these *P. aeruginosa* strains. In a series of early studies, Murakawa (1973a, b) characterized extracellular "slime" produced by *P. aeruginosa*. The chemical composition of slimes from 20 clinical *P. aeruginosa* isolates was investigated, and it was found that slimes from 18 strains consisted primarily of DNA, while two strains with a mucoid phenotype produced slimes composed primarily of alginate.

The extracellular DNA in *P. aeruginosa* biofilms appears to be similar to *P. aeruginosa* chromosomal DNA. PCR and Southern analysis have suggested that the extracellular DNA released from *P. aeruginosa* in biofilms and planktonic cultures is similar to whole-genome DNA (Steinberger and Holden 2005; Allesen-Holm et al. 2006). In addition, it has been shown that different chromosomal genes could be transferred by transformation of $CaCl_2$-treated *P. aeruginosa* cells with extracellular DNA at the same frequencies as when transformation was done with an equivalent amount of purified intracellular DNA (Hara et al. 1981; Muto and Goto 1986).

A basal level of extracellular DNA present during the initial phase of *P. aeruginosa* biofilm formation is evidently generated via a pathway, which is not linked to quorum-sensing (a mechanism by which bacteria can monitor their cell population density through the extracellular accumulation of signaling molecules) (Allesen-Holm et al. 2006). However, the generation of large amounts of extracellular DNA during subsequent *P. aeruginosa* biofilm formation evidently depends on the interconnected Las, Rhl and Pqs quorum-sensing systems (Allesen-Holm et al. 2006; Nakamura et al. 2008). The generation of extracellular DNA in *P. aeruginosa* wild-type biofilms appears to be linked to quorum-sensing via a mechanism that results in lysis of a small subpopulation of the cells (Allesen-Holm et al. 2006; Barken et al. 2008). In support of a role of quorum-sensing in cell lysis, D'Argenio et al. (2002) reported that mutants that could not produce the PQS quorum-sensing signal molecule did not show autolysis, whereas mutants that overproduced PQS displayed high levels of autolysis. In addition, Heurlier et al. (2005) presented evidence that *P. aeruginosa* quorum-sensing mutants, unlike the wild-type, did not undergo cell lysis in stationary phase cultures. Quinolone compounds have previously been shown to induce prophages in bacteria (Phillips et al. 1987; Froshauer et al. 1996), and studies by Webb et al. (2003) and Hentzer et al. (2004) have suggested that quorum-sensing regulated DNA-release might be linked to bacteriophage induction in biofilms. However, membrane vesicles produced by *P. aeruginosa* might also have a role in DNA-release. *P. aeruginosa* releases membrane vesicles that have bacteriolytic effects and contain DNA (Kadurugamuwa and Beveridge 1996; Renelli et al. 2004). Extracellular DNA might be released either from vesicles that eventually lyse, or through the bacteriolytic activity of the vesicles which might lyse a small subpopulation of the *P. aeruginosa* cells. Recently, it was shown that PQS is necessary for vesicle formation in *P. aeruginosa* (Mashburn and Whiteley 2005; Nakamura et al. 2008). Experiments reported by

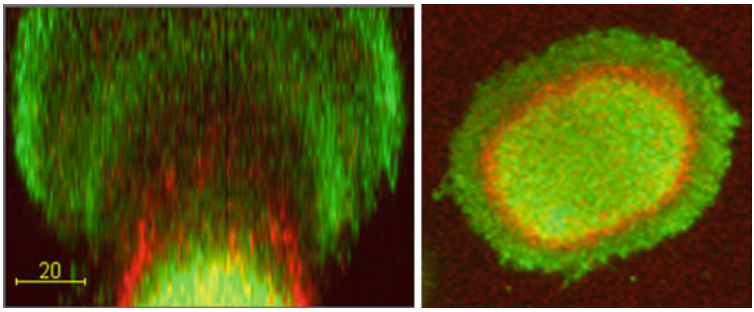

Fig. 1.2 CLSM micrographs acquired in a propidium iodide-stained biofilm formed by Gfp-tagged *P. aeruginosa* PAO1. The left image shows a vertical section through a mushroom-shaped multicellular structure, whereas the right image shows a horizontal section through a mushroom-shaped multicellular structure. The bacteria appear green fluorescent and the extracellular DNA appears red fluorescent. The size bar corresponds to 20 μm. Reproduced from Allesen-Holm et al. (2006) with permission from Wiley-Blackwell publishing

Yang et al. (2007) showed that high levels of iron suppressed *P. aeruginosa pqs* gene expression, DNA release, structural biofilm development, and the development of subpopulations with increased tolerance toward antimicrobial compounds.

The extracellular DNA appears to be organized in distinct patterns in *P. aeruginosa* biofilms (Allesen-Holm et al. 2006). In flow-chamber-grown *P. aeruginosa* biofilms, which contain mushroom-shaped structures, the extracellular DNA is located primarily in the stalk-portion of the mushroom-shaped structures with the highest concentration in the outer parts of the stalks forming a border between the stalk-subpopulation and the cap-subpopulation (see Fig. 1.2). Evidence has been presented that formation of the mushroom-shaped structures in *P. aeruginosa* biofilms occurs in a sequential process involving a nonmotile bacterial subpopulation that forms the stalks by growth in certain foci of the biofilm and a migrating bacterial subpopulation that subsequently forms the mushroom caps via a process that requires type IV pili (Klausen et al. 2003a), flagellum-mediated surface motility, and quorum-sensing controlled DNA release (Barken et al. 2008). Extracellular DNA is primarily generated in the stalk microcolonies before they are colonized by the cap-forming subpopulation, and a high concentration of extracellular DNA is present in the outer layer of the stalk microcolonies (Allesen-Holm et al. 2006). It is currently not understood how the migration of the motile cells is coordinated so that they form mushroom caps. However, type IV pili bind to DNA with high affinity (Aas et al. 2002; van Schaik et al. 2005), and evidence has been provided that the high concentration of extracellular DNA on the outer parts of the mushroom stalks causes accumulation of the migrating bacteria, which in combination with bacterial growth, might result in the formation of the mushroom caps (Barken et al. 2008). In agreement with the spatial distribution of the extracellular DNA and a role of quorum-sensing in DNA release, experiments involving a *P. aeruginosa pqsA-gfp* reporter strain provided evidence that the *pqs* genes are expressed specifically in the outer layer of the stalks that subsequently become

capped by the migrating subpopulation (Yang et al. 2007). The extracellular DNA in *P. aeruginosa* biofilms appears to have a stabilizing effect, as mature *P. aeruginosa* PAO1 biofilms that were pretreated with DNase I were more susceptible to SDS treatment than biofilms that were not pretreated with DNase I (Allesen-Holm et al. 2006).

P. aeruginosa colonizes the lungs of cystic fibrosis (CF) patients and is a major cause of lung deterioration, health decline, and death of these patients (Høiby 2002; Moreau-Marquis et al. 2008). Several studies have provided evidence that *P. aeruginosa* forms biofilms in the CF lung (e.g., Lam et al. 1980; Baltimore et al. 1989; Worlitzsch et al. 2002; Høiby 2002), and the biofilm mode of growth is considered the major reason that these bacteria cannot be eradicated by host defenses or antibiotic treatment (Costerton et al. 1999). CF lungs contain large amounts of extracellular DNA from necrotized neutrophils (Lethem et al. 1990), and evidence has been presented that extracellular actin-DNA filaments can provide a matrix for biofilm formation by *P. aeruginosa* (Walker et al. 2005). The presence of extracellular DNA was shown to be important for *P. aeruginosa* biofilm formation in artificial CF sputum medium (Sriramulu et al. 2005). In addition to a role of extracellular DNA, it was reported that biofilm formation in artificial CF sputum medium depended on the presence of amino acids (Sriramulu et al. 2005). Evidence has been presented that *P. aeruginosa*, in part due to the presence of a high level of aromatic amino acids, produces large amounts of PQS when it is present in CF lungs (Collier et al. 2002; Palmer et al. 2005). Because PQS evidently plays a role in DNA release from *P. aeruginosa* (Allesen-Holm et al. 2006; Yang et al. 2007), PQS-mediated release of DNA from the bacteria might play a role in biofilm formation in the CF lung. In further support of this possibility, the amino acid content of CF sputum has been shown to correlate with the severity of the disease (Thomas et al. 2000). A recent study by Mulcahy et al. (2008) suggested that extracellular DNA in *P. aeruginosa* biofilm can induce antibiotic tolerance systems in the bacteria via chelation of cations.

1.3 Extracellular DNA as Matrix Component in Streptococcal Biofilm

Evidence for a role of extracellular DNA in biofilm formation by *Streptococcus* species is accruing. DNA uptake deficient mutants of *Streptococcus mutans* and *Streptococcus gordonii* were shown to be attenuated in biofilm formation (Loo et al. 2000; Li et al. 2002; Yoshida and Kuramitsu 2002), and the fact that these mutants are also deficient in generating extracellular DNA, suggests that the biofilm formation defect was caused by a lack of extracellular DNA. In accordance, the presence of DNase I was subsequently shown to attenuate biofilm formation by *S. mutans* and *Streptococcus intermedius* wild-type strains (Petersen et al. 2004, 2005). The ability to generate and take up extracellular DNA is quorum-sensing regulated in streptococci, and addition of exogenous quorum-sensing signal molecules to *S. mutans* and

S. intermedius cultures was shown to promote DNA release and biofilm formation, whereas simultaneous treatment with DNase I inhibited biofilm formation, suggesting that extracellular DNA was responsible for the increase in biofilm formation caused by addition of quorum-sensing signal molecules (Petersen et al. 2004, 2005). Evidence has been presented that proteins on streptococci, which are necessary for binding and uptake of extracellular DNA, play a role in biofilm formation. Petersen et al. (2005) reported that a *comGB* mutant of *S. mutans*, which is deficient in DNA binding, showed reduced biofilm formation. In the presence of DNase I, biofilm formation by the *S. mutans* wild type was reduced to a level similar to that displayed by the *comGB* mutant. The *comGB* mutant was not impaired in DNA-release as growth in the presence of quorum-sensing signaling molecules promoted DNA-release from both the wild-type and the *comGB* mutant.

Extracellular DNA in streptococcal populations is evidently generated via lysis of a subpopulation of the bacteria (e.g., Steinmoen et al. 2002, 2003; Moscoso and Claverys 2004; Shibata et al. 2005), and should therefore be similar to genomic DNA. The release of DNA in *Streptococcus pneumoniae* populations was shown to involve cell lysis through activity of cell wall hydrolases (LytA, LytC, and CbpD) (Steinmoen et al. 2003; Moscoso and Claverys 2004; Guiral et al. 2005). Evidence has been provided that competent *S. pneumoniae* bacteria (i.e., bacteria which are in a physiological state that allow them to take up extracellular DNA) trigger lysis of *S. pneumoniae* sibling bacteria that are noncompetent (Steinmoen et al. 2003; Moscoso and Claverys 2004; Guiral et al. 2005). The phenomenon evidently involves a system consisting of a bacteriocin (CibAB), its immunity factor (CibC), and the cell wall hydrolases (Guiral et al. 2005). Competent cells are immune to the bacteriocin, presumably because they also produce the immunity factor. A similar bacteriocin-based system appears to operate during DNA-release from *S. mutans* populations and *Streptococcus sanguinis* populations (Kreth et al. 2005; van der Ploeg 2005; Schlegel and Slade 1973). Kreth et al. (2008) recently provided evidence that hydrogen peroxide, produced by *S. sanguinis* and *S. gordonii* under aerobic growth conditions, increases the release of extracellular DNA.

The spatial organization of the extracellular DNA in streptococcal biofilms has to our knowledge not been investigated. However, because biofilms contain numerous microenvironments, the DNA-release mechanism described above will most likely lead to a stratified distribution of extracellular DNA in streptococcal biofilms.

Competence-triggered DNA release from streptococci has been proposed to ensure coordination between DNA release and uptake, thus favoring genetic exchange (e.g., Steinmoen et al. 2002, 2003). However, the finding that DNA release in *S. pneumoniae* cultures continued a long time after competence had disappeared suggested that genetic exchange is not the only purpose of competence-triggered cell lysis (Moscoso and Claverys 2004). Competence-triggered lysis of streptococcal cells is evidently important for biofilm formation, but also for the release of virulence factors such as pneumolysin and lipo-teichoic acid (Guiral et al. 2005). Because streptococci often are involved in biofilm-related infections such as those occurring in middle ears or lungs (e.g., *S. pneumoniae*) or on teeth (e.g., *S. mutans*), it is possible that the extracellular DNA plays a role in

stabilizing medically relevant streptococcal biofilms. In agreement with this possibility the *comB*, *comD*, *lytA*, and *cbpD* genes, which are necessary for DNA release, have all been implicated in the virulence of *S. pneumoniae* (Jedrzejas 2001; Bartilson et al. 2001; Lau et al. 2001; Hava et al. 2003).

1.4 Extracellular DNA as Matrix Component in Staphylococcal Biofilm

Evidence for a role of extracellular DNA in staphylococcal biofilm formation has been found for a number of species including *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Staphylococcus haemolyticus* (Qin et al. 2007; Eckhart et al. 2007; Rice et al. 2007; Seidl et al. 2008; Izano et al. 2008; Fredheim et al. 2009). Biofilm formation by *S. aureus*, *S. epidermidis*, and *S. haemolyticus* was inhibited when DNase was added at the initial stage of biofilm formation, indicating that extracellular DNA plays a fundamental role as biofilm matrix component. Moreover, DNase treatment was shown to disrupt mature biofilms formed by *S. aureus* and *S. haemolyticus*. On the contrary, Qin et al. (2007) found that DNase treatment could not disrupt mature *S. epidermidis* biofilms, indicating that other compounds than extracellular DNA stabilizes *S. epidermidis* biofilms at this stage, or alternatively that the presence of macromolecules or proteases in the mature biofilms may affect the efficiency of the DNase treatment. Fredheim et al. (2009) investigated the biofilm-forming capacities of a number of clinical *S. haemolyticus* isolates. Fifty-three isolates were able to form biofilms in microtiter trays with glucose tryptic soy broth, and all these biofilms were susceptible to DNase treatment, suggesting that extracellular DNA was the major matrix component in all the biofilms. Figure 1.3

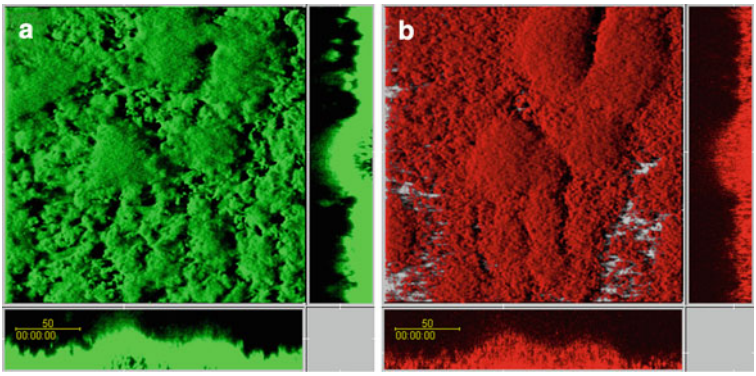

Fig. 1.3 CLSM micrographs acquired in a DDAO/SYTO 9-stained biofilm formed by *S. epidermidis*. The central pictures show top-down views, and the flanking pictures show side views. The green fluorescent bacteria and the red fluorescent extracellular DNA are shown in separate micrographs acquired at the same location. The size bars correspond to 50 μm. Reproduced from Qin et al. (2007) with permission from the Society for General Microbiology (SGM)

shows the extracellular DNA matrix in a flow-chamber-grown *S. epidermidis* biofilm. The extracellular DNA appears to be homogeneously distributed in *S. epidermidis* biofilm, in contrast to what is observed in *P. aeruginosa* biofilm.

Fournier and Hooper (2000) provided evidence that the ArlS–ArlR proteins in *S. aureus* control the rate of autolysis as well as biofilm formation. Attachment to polystyrene and subsequent biofilm formation in the wells of microtiter trays was much more pronounced for an *S. aureus arlS* mutant than for the wild-type strain. The *arlS* mutant exhibited increased autolysis and a pronounced decrease of extracellular proteolytic activity. The mutant did not overexpress the *S. aureus* autolysin AtlA, but the activity of the autolysin was increased due to the absence of secreted serine proteases. The available evidence suggests, therefore, that AtlA autolytic activity could provide extracellular DNA, which mediated cell-to-surface and cell-to-cell binding during *S. aureus* biofilm formation. In accordance, Biswas et al. (2006) subsequently provided evidence that an *S. aureus atlA* mutant, unlike the wild-type strain, is deficient in biofilm formation. Qin et al. (2007) provided evidence that extracellular DNA is generated in *S. epidermidis* biofilms by autolysin AtlE-mediated cell lysis of a subpopulation of the cells, and that the extracellular DNA promotes biofilm development of the remaining population. It has been shown that autolysin AtlE expression and biofilm formation are promoted in an *S. epidermidis agr* quorum-sensing mutant (Vuong et al. 2000), indicating that AtlE-mediated DNA release may be regulated by the *agr* quorum-sensing system.

In addition to *atlA*, the *cidA* and *lrgA* genes evidently encode a system that causes autolysis of *S. aureus* bacteria (Bayles 2003). It is hypothesized that these genes encode holins and antiholins, respectively, and may serve as molecular control elements of bacterial cell lysis. A recent study by Rice et al. (2007) indicated that CidA-mediated cell lysis plays a significant role in *S. aureus* biofilm formation, and that the released genomic DNA is an important structural component of *S. aureus* biofilm. The study by Rice et al. (2007) also suggested that AtlA contributes to cell lysis and DNA release in addition to CidA. Seidl et al. (2008) studied the role of catabolite control protein A (CcpA) in *S. aureus* biofilm formation, and found that CcpA up-regulated transcription of the *cidA* gene. In agreement with a role of CidA-mediated DNA release in *S. aureus* biofilm development, an *S. aureus ccpA* mutant, unlike the wild type, could not form biofilm under static or flow conditions. Trotonda et al. (2008) found that the MgrA regulator decreases *cidA* expression in *S. aureus*, and therefore decreases the amount of cell lysis and DNA release. In agreement with a role of extracellular DNA in *S. aureus* biofilm formation various *mgrA* mutants displayed enhanced biofilm formation compared to the wild type.

Staphylococcus species are major human pathogens that can cause community- and hospital-acquired infections (Lowy 1998; Mack et al. 2006; Rupp and Archer 1994). The infections significantly increase the financial loads on the healthcare system and cause severe patient suffering and high mortality. Besides an increase in antibiotic resistance of these species, as displayed, for example, by methicillin-resistant *S. aureus*, the ability of infecting staphylococci to tolerate antibiotic treatment is related to biofilm formation. Because extracellular DNA evidently is

an important matrix compound in *Staphylococcus* biofilms strategies aimed at disrupting this component may be useful for control of these biofilms.

1.5 Final Remarks

Because most bacterial populations are accompanied by extracellular DNA (Lorenz and Wackernagel 1994), and most bacterial species bind to DNA (Dubnau 1999), it appears that extracellular DNA may serve as a matrix compound in many different biofilms. On top of a basal level of DNA-release many bacterial species, especially those that are able to develop competence, possess a specific DNA-release program. A correlation between DNA-release and competence development has been established in many different bacteria including *Pseudomonas stutzeri* (Stewart et al. 1983), *Bacillus subtilis* (Lorenz et al. 1991), *Acinetobacter calcoaceticus* (Palmen and Hellingwerf 1995), *Neisseria gonorrhoeae* (Dillard and Seifert 2001), and *S. pneumoniae* (Steinmoen et al. 2002). Since many kinds of polymers can function as biofilm matrix material, it is difficult to conceive that bacteria should release large amounts of costly information material solely with the purpose of stabilizing biofilms. It is possible that bacteria release DNA both in order to exchange genetic material and form and stabilize biofilms. The relatively long-lasting physical proximity of bacteria in biofilms enable the constituent cells to establish long-term relationships with each other, and evidence has been presented that biofilms are optimal environments for transformation-based gene transfer (e.g., Li et al. 2001; Wang et al. 2002; Hendrickx et al. 2003). It thus seems that the biofilm populations may share a common gene pool, and that genomic plasticity may not only be an evolutionary factor with impacts on species developments, but also an inducible adaptive feature with short-term benefits.

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Chapter 2
Gene Transfer Agents and Defective Bacteriophages as Sources of Extracellular Prokaryotic DNA

Andrew S. Lang and J. Thomas Beatty

Contents

Abstract A large amount of extracellular DNA on the planet exists in the form of viruses and virus-like particles. Globally, these particles contain a large amount of DNA that appears to have originated from cellular organism genomes, as opposed to being truly viral. Although the exact frequencies with which packaging of cellular DNA happens and the full range of reasons for why this happens are not known, virus-like elements that package host DNA are known. These include defective phages and gene transfer agents. The potential that these elements have to contribute to the pool of extracellular prokaryotic nucleic acids is discussed in this chapter.

2.1 Viruses in the Environment and Packaging of Host DNA

Viruses contribute large amounts of extracellular nucleic acids to natural environments. The number of viral particles is estimated at a global total of $\sim 10^{31}$, on the basis of concentrations found in marine, aquatic, and terrestrial environments.

A.S. Lang (✉)
Department of Biology, Memorial University of Newfoundland, St. John's, NL, Canada A1B 3X9
e-mail: aslang@mun.ca

J.T. Beatty
Department of Microbiology and Immunology, University of British Columbia, Vancouver, BC, Canada V6T 1Z3

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There are as many as 10^6 to 10^8 viral particles per milliliter in the sea (Suttle 2005), 10^7 to 10^{10} per gram in marine and freshwater sediments (Danovaro et al. 2008), and 10^8 to 10^9 per gram of soil in terrestrial systems (Williamson et al. 2005). These numbers are likely underestimates because most of the enumeration techniques employed do not detect RNA viruses, and these are abundant in aquatic environments (Culley et al. 2006; Djikeng et al. 2009). In this chapter, we focus on particles that contain DNA.

The above numbers reflect the greater abundance of viruses relative to cellular organisms, with viruses generally being ~10-fold more abundant than prokaryotic cells. In the sea, viruses numerically represent >90% of all life forms; however, because of their small size, they actually represent only ~5% of the total biomass (Suttle 2007). With the exception of deep-sea sediments where there is a large amount of extracellular free DNA (Dell'Anno and Danovaro 2005), it appears that viruses and similar particles are the largest reservoirs of extracellular nucleic acids on Earth.

Although most virus particles contain exclusively viral DNA, some also contain DNA that originated from the host cell's genome. This can occur by virtue of the virus DNA packaging mechanism or through errors during the DNA replication and packaging stages of the virus replication cycle. Therefore, a large amount of prokaryotic genomic DNA is present within the global pool of viral extracellular DNA. If this host DNA is subsequently transmitted to another cell, the process is called transduction, and this process is known to be important in the movement of genes between organisms (Cheetham and Katz 1995; Jiang and Paul 1998; Ochman et al. 2000; Canchaya et al. 2003a; Brussow et al. 2004).

With the large numbers of viruses in the environment and the fact that some viruses package host cell DNA, the total amount of prokaryotic extracellular DNA in this reservoir is large. If only 0.1% of all viral particles contain some host DNA, this would be ~10^{28} particles globally. Furthermore, metagenomic studies have demonstrated the great abundance of host DNA in viruses. Although most of the sequences discovered in viral metagenomes are completely novel, a high proportion of the ones that can be identified are most similar to what are considered cellular sequences. In a study of marine viruses, host DNA averaged ~72% of the classifiable sequences in viral metagenomic libraries from four different oceanic regions (Angly et al. 2006), and this value averaged 95% for two hot springs (Schoenfeld et al. 2008). We found one published soil viral metagenomic study (Fierer et al. 2007) and, although the percentage of putative host cell genomic sequences was not available, it seems reasonable to assume that it would also be high in this environment. It is possible that the amount of host cell DNA in viral particles has been slightly overestimated, because a proportion of sequences classified as "host" may in fact have come from true phage DNA, and simply displayed homology to prophage sequences in a cellular genome that have not been recognized or annotated as such. However, these findings indicate that a significant fraction of viriome sequences is from host genomes.

2.2 Defective Phages and Extracellular DNA

Defective phages are phage-like elements that are incapable of self-transmission between host cells. There are several well-known examples of defective phages. These phage-like elements are produced from genes encoded within the genome of the producing organism, but they do not appear to be capable of transferring the packaged DNA to subsequent recipient cells. This distinguishes them from gene transfer agents (GTAs, discussed below).

One of the best-known defective phage is PBSX originally found in *Bacillus subtilis* 168 (Seaman et al. 1964; Okamoto et al. 1968), although related and functionally similar elements have subsequently been described in numerous *Bacillus* species and strains (Garro and Marmur 1970; Tsutsumi et al. 1990). PBSX is a phage-like structure (Fig. 2.1) that packages small (∼13 kb) apparently random pieces of the producing cell's genome. However, these particles do not appear to transfer this DNA to recipient cells, but rather act like bacteriocins and bind to and kill other strains of *Bacillus* that do not encode the particle. The genes responsible for producing PBSX are in a 28-kb cluster in the *B. subtilis* genome (Wood et al. 1990). The cluster consists of a head–tail structural module that appears to have undergone several deletion events, lysis genes, and a lysogeny module (Canchaya et al. 2003b); DNA replication functions are not present.

Defective phages that do not appear to carry out cell killing are also known, such as the BLP elements described in *Bartonella* (Anderson et al. 1994; Bowers et al.

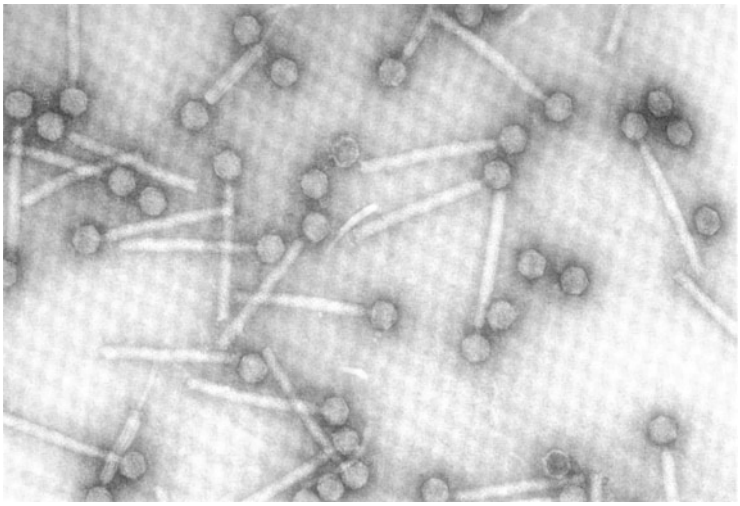

Fig. 2.1 The defective phage PBSX. Each particle contains a random 13-kb fragment of the producing cell's genome, but this DNA is not delivered to subsequent cells. The head structure has a diameter of 41 nm and the tails are ∼190 nm in length. Reproduced from the Okamoto et al. (1968), copyright 1968, with permission from Elsevier

1998; Barbian and Minnick 2000). Several species in the *Bartonella* genus produce phage-like particles that package 14 kb of apparently random pieces of the producing cell's genome, but there has been no report of lytic or gene transfer activity reported for these particles.

Since the advent of genome sequencing, it has become evident that the majority of bacterial genomes contain prophages (Casjens 2003). Not only are prophages widely distributed but they can also constitute a fairly significant portion of the genetic information in some organisms, accounting for as much as 20% of the total genome (Casjens et al. 2000). Therefore, it is possible that many species produce defective phages under certain circumstances, and these elements could contribute significantly to the global amount of extracellular nucleic acids.

2.3 Gene Transfer Agents and Extracellular DNA

Like defective phages, GTAs are phage-like elements that appear incapable of self-transmission between host cells. GTAs are distinguished from defective phages in a number of ways, one of which is the frequency that GTA particles transduce host cell DNA. The process of gene transfer by GTAs is analogous to transduction, but GTAs are different from transducing phages. GTAs constitutively package random pieces of the producing cell's genome; therefore, a collection of GTAs all contain host DNA that can be transferred to other cells. Generalized transducing phages, which also can transfer any region of host genome, have an overall low frequency of transducing particles in the population of phage particles released from cells. Therefore, while most particles in a transducing phage lysate are capable of infecting and eventually lysing another target cell, GTA particles only donate host DNA to a recipient cell without lysis. Also, in all cases where the information is available, GTA particles appear to package less DNA than is known or would be predicted to encode the GTA structure itself (Lang and Beatty 2001, 2007; Stanton 2007).

The first GTA that was discovered, RcGTA, is produced by the purple nonsulfur α-proteobacterium *Rhodobacter capsulatus* (Marrs 1974). RcGTA was discovered as a virus-sized, DNase-resistant, and protease-sensitive mediator of genetic exchange (Marrs 1974; Solioz et al. 1975). Purification of RcGTA particles and visualization by electron microscopy showed that RcGTA resembles a small tailed bacteriophage (Fig. 2.2), with a head diameter of approximately 30 nm (Yen et al. 1979). The mechanism of RcGTA release from cells is unknown; there is no detectable lysis in liquid cultures and no plaque-forming activity on plates (Marrs 1974).

The nucleic acid within the RcGTA particle is linear double-stranded DNA approximately 4-kb in length (Solioz and Marrs 1977; Yen et al. 1979). Several different lines of evidence indicate that RcGTA packages genomic DNA indiscriminately. All genetic markers tested can be transferred from donor to recipient cells via GTA, whether the trait is present in donor cells on the chromosome or on extrachromosomal elements (Solioz and Marrs 1977; Yen et al. 1979; Scolnik and Haselkorn 1984). Also, restriction digestion patterns and DNA renaturation kinetics

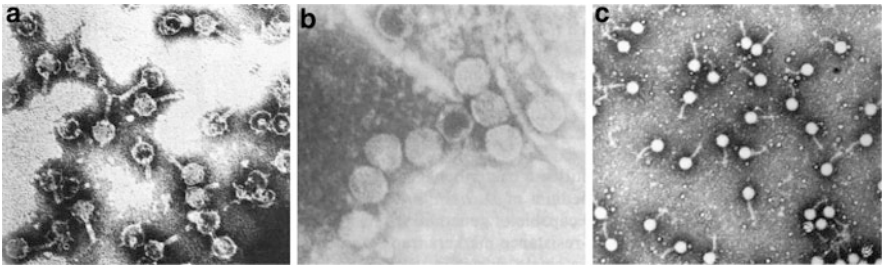

Fig. 2.2 Gene transfer agents. (**A**) RcGTA from *R. capsulatus*. RcGTA particles have a head diameter of ~30 nm, a tail length of ~50 nm, and package ~4-kb of DNA. Reproduced from Yen et al. (1979), copyright 1979, with permission from Elsevier and with kind permission of the authors. (**B**) Dd1 from *D. desulfuricans*. The particles, with a head diameter of 43 nm and a tail length of 7 nm, package 13.6-kb of DNA. Reproduced from (Rapp and Wall 1987) with kind permission of the authors. (**C**) VSH-1 from *B. hyodysenteriae*. These particles have a head diameter of 45 nm, a tail length of 64 nm, and package 7.5-kb of DNA. Reproduced from (Humphrey et al. 1997) with kind permission of the authors

(C_0t) analysis indicated that there are no detectable preferred DNA sequences in particles (Yen et al. 1979). These proposals have been verified by hybridizing RcGTA DNA to a *R. capsulatus* whole-genome microarray, which demonstrated that there is no gene packaging preference based on a location in the genome (Lang et al., unpublished). This property of RcGTA has made it a very useful tool for genetic manipulations of *R. capsulatus* (Marrs 1978; Scolnik and Marrs 1987), by mediating transfer of modified genes into the genomes of recipient cells (Scolnik and Haselkorn 1984; Lilburn et al. 1992; Lang and Beatty 2002), as well as facilitating gene mapping (Wall et al. 1975b; Yen and Marrs 1976; Wall and Braddock 1984). Allele replacement in recipients is dependent upon a cellular recombination system (Genthner and Wall 1984).

Based on its (then) novel properties, and because RcGTA functions solely to mediate gene exchange, it was suggested that it might represent a cell-evolved genetic exchange vector as opposed to a defective phage (Yen et al. 1979). However, it appears that an ancestor of RcGTA was present in the last common ancestor of all α-proteobacteria, and evolution of RcGTA from a prophage is the most parsimonious explanation of its origins (Lang and Beatty 2007). Considering the long history of GTA and GTA-like sequences in the α-proteobacteria, and that GTA genes have been maintained as a functional unit in *R. capsulatus* and many related species, it appears that RcGTA and homologous gene clusters provide a useful function for bacterial hosts. Perhaps the distribution of RcGTA genes reflects the benefits gained through genetic exchange and homologous recombination (Vos 2009).

Other GTA types have subsequently been discovered in a diverse array of prokaryotes (Fig. 2.2). These are Dd1 in *Desulfovibrio desulfuricans* (Rapp and Wall 1987), VSH-1 in *Brachyspira hyodysenteriae* (Humphrey et al. 1997), and VTA in *Methanococcus voltae* (Bertani 1999). The properties of these GTAs have been reviewed recently (Stanton 2007). A GTA that is genetically related to RcGTA was

also recently discovered in the marine α-proteobacterium *Ruegeria pomeroyi* (Biers et al. 2008).

The genetic basis for GTA production has been characterized for VSH-1 in the spirochete *B. hyodysenteriae*. VSH-1 is encoded by a set of genes that reside in a single location in the *B. hyodysenteriae* genome, but that are split into two units with an intervening set of non-VSH-1 genes (Matson et al. 2005; Stanton et al. 2009). One gene cluster consists of head, tail, and lysis genes, and the second cluster consists of three tail genes. The role of some of these genes in VSH-1 function has been verified by N-terminal sequencing of proteins from the particles. None of the VSH-1 structural genes have recognizable similarity to any genes of known function.

The RcGTA structure is encoded by a ~15-kb gene cluster (Lang and Beatty 2000) that resembles a "typical" tailed phage head to tail gene cluster (Casjens et al. 1992). The regulation of expression of these genes and production of RcGTA is controlled by the actions of several cellular regulatory modules. Three genes have been identified that are required for proper RcGTA production. One (*ctrA*) encodes a response regulator protein, another (*cckA*) a sensor kinase, and the third (*gtaI*) a homoserine lactone synthase required for quorum sensing (Lang and Beatty 2000; Schaefer et al. 2002). It was discovered in the 1970s that the frequency of RcGTA-mediated gene transduction is maximal in the stationary phase of cultures (Solioz et al. 1975). Cloning of the RcGTA structural genes led to experiments that showed this stationary phase regulation is due to induction of RcGTA structural gene transcription (Lang and Beatty 2000). These genes absolutely require *ctrA* for expression (Lang and Beatty 2000), and *gtaI* for the maximal stationary phase response (Schaefer et al. 2002). The specific regulation of RcGTA through these cellular systems, and particularly the requirement of quorum sensing for maximal RcGTA production, is compelling evidence that RcGTA is not simply a defective phage but a cellular machine, which functions to provide the advantage of increased genetic diversity through homologous recombination (Vos 2009) to *R. capsulatus*.

Interestingly, the *cckA* and *ctrA* genes also regulate flagellum-dependent motility in *R. capsulatus*, suggesting that these genes control two complementary responses to an unfavorable environment: (1) induction of motility, to allow movement to a more favorable environment; (2) induction of RcGTA to increase genetic exchange dependent on homologous recombination. Until these discoveries, it was thought that such regulatory genes function exclusively in cellular activities; so, RcGTA is novel, in part, because it resembles a phage morphologically, yet cellular regulatory proteins control GTA production.

It was subsequently discovered that homologs of the RcGTA genes are present in the genomes of most α-proteobacteria for which sequences are available (Lang and Beatty 2001, 2007; Lang et al. 2002; Biers et al. 2008). The RcGTA genes are especially well conserved in the taxonomic order *Rhodobacterales*, which contains the *Rhodobacter* and *Roseobacter* groups (each of these groups contains multiple genera). Indeed, there have been genome sequences completed for more than 20 *Roseobacter* group members (http://www.roseobase.org/), and it appears that all complete genome sequences contain complete RcGTA-like structural gene clusters

(Lang and Beatty 2007; Biers et al. 2008). Therefore, the demonstration of functional GTA production in *R. pomeroyi* is particularly important because this bacterium belongs to this *Roseobacter* group, indicating that the conserved RcGTA-like gene cluster is functional in genera other than *Rhodobacter*. The *Roseobacter* group comprises a collection of ecologically important and physiologically diverse species that are very abundant in some marine environments (Buchan et al. 2005; Wagner-Dobler and Biebl 2006; Brinkhoff et al. 2008). Therefore, these abundant marine bacteria all appear to have the genetic potential for GTA production, and it is possible that GTAs constitute a significant portion of the virus-like particles that are present in these environments. Because of the smaller amount of DNA packaged in these particles, it is unlikely they would be detected using the current methods of fluorescence microscopy (Wen et al. 2004) and flow cytometry (Brussaard et al. 2000) to enumerate viruses.

So far, GTA-mediated genetic exchange has been documented only with organisms in culture, and GTAs have not been observed in natural environments. The spirochete *B. hyodysenteriae* is a swine intestinal pathogen, and it was found that conditions similar to what this organism would encounter during colonization of the intestine trigger expression of the VSH-1 genes (Matson et al. 2007). One of the VSH-1 genes was used to probe different spirochete species, and this sequence was found in all six species tested from the *Brachyspira* genus but not within seven species from four other genera (Stanton et al. 2003). VSH-1-like gene clusters were subsequently also identified in the genome sequences of 3 *Brachyspira* species (Motro et al. 2009). Therefore, GTA production could be widespread in the *Brachyspira* genus and be occurring in its natural environments.

As a purple nonsulfur bacterium, *R. capsulatus*, displays tremendous physiological diversity and is capable of phototrophy, respiration, fermentation, nitrogen fixation and, chemolithotrophy (Madigan and Jung 2009), and therefore is found in a wide range of environments. Most strains of *R. capsulatus* can both produce RcGTA and take up RcGTA-borne DNA, although some strains are capable of only one or the other, and some strains do neither (Marrs 1974; Wall et al. 1975a). Considering the high level of GTA gene conservation in the *Rhodobacterales* order, and with documented production in two divergent species in this order, it is reasonable to suggest that a number of species in this group might produce GTAs. Therefore, RcGTA-like GTAs could be making a contribution to gene movements as well to the extracellular host DNA present in natural environments.

2.4 Concluding Remarks

There are large numbers of viruses and virus-like particles present in all of the major environments on Earth, and so constitute a huge amount of extracellular DNA. It is clear that this virus fraction of natural communities contains a large amount of genetic material that originated from within the genomes of cellular organisms. The exact mechanisms by which most of these cellular genes come to be

in the viral fraction remain to be determined. Phages that package host DNA are known, including defective prophage elements that exclusively package host DNA. There are also GTAs that package smaller pieces of host DNA and transfer this to other cells. The relative contributions of these different types of particles remain to be deciphered.

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Chapter 3
Roles of Extracellular DNA in Bacterial Ecosystem

Katsuji Tani and Masao Nasu

Contents

Abstract Extracellular DNA in the natural environment is a source of nutrients and gene pools for bacteria. In sediments and biofilms, gene transfer must occur among bacteria via extracellular DNA because the concentrations of both exogenous DNA and cells are high. Some bacteria actively release naked DNA or the membrane vesicle containing the DNA into the environment. Some bacteria also produce extracellular DNA by suicide and fratricide. Released DNA is used for DNA repair, transformation, and generation of genetic diversity. DNA is required for the initiation of biofilm formation and stabilization of biofilms. In biofilms, gene exchange and mutation must occur to generate diversity.

K. Tani
Department of Environmental Science and Microbiology, Faculty of Pharmaceutical Sciences, Osaka-ohtani University, 3-11-1 Nishikiori-kita, Tondabayashi, Osaka 584-0854, Japan
M. Nasu (✉)
Department of Environmental Science and Microbiology, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamada-oka, Suita, Osaka 565-0871, Japan
e-mail: nasu@phs.osaka-u.ac.jp

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3.1 Extracellular DNA in Natural Environments

Extracellular DNA, whose sources are cell constituents and viruses, is ubiquitous in the environment. Most of the naked extracellular DNA in the natural environment is released from dead cells. Bacterial extracellular DNA in the environment is produced by cell death, autolysis, phage infection, predation, and so on. Some bacteria actively produce extracellular DNA that is naked or encased in membrane vesicles. Bacteria also take up extracellular DNA and use it as a nutrient source, for DNA repair, and generation of genetic diversity. In the natural environment, dissolved extracellular DNA is an important source of nutrients for bacteria. It also provides a gene pool that can transform various competent cells. Extracellular DNA is also required for the initiation of bacterial biofilm formation. Here, we describe the main roles of naked extracellular DNA in bacterial ecosystems (Table 3.1).

Extracellular DNA is found in various natural environments. Its concentration in soil can be as high as 2 μg/g (Niemeye and Gessler 2002). In natural aquatic environments, concentrations range from 0.2 to 88 μg/L (DeFlaun et al. 1986; Karl and Bailiff 1989; Paul et al. 1987, 1991; Siuda and Güde 1996). Concentrations in aquatic sediments are up to three to four orders of magnitude greater than those in the water column. The DNA concentration in sea sediments ranges from 1 to 31 ng/g, an amount that is about 25-fold higher than that of the DNA contained in all the bacteria inhabiting this environment (Dell'Anno et al. 1999; Corinaldesi et al. 2005). In the world's deep-sea sediments, the DNA content in the uppermost 10 cm is about 0.5 Gt. Therefore, deep-sea sediments constitute the largest reservoir of extracellular DNA (0.30–0.45 Gt) in the world's oceans (Whitman et al. 1998; Dell'Anno and Danovaro 2005).

The size of the extracellular DNA molecules in natural environments ranges from a few hundred to 30,000 base pairs (bp) (DeFlaun et al. 1987). DNA fragments in this size range are sufficient to contain gene sequences. The turnover time of extracellular DNA that is not attached to a solid surface in aquatic environments is about 10 h (Paul et al. 1987, 1989). Degradation rates of extracellular DNA in marine sediments are much higher than of those in the water column because DNase activities are higher in the sediments. However, turnover times in marine sediments were longer than in the water column because of the high DNA content in the sediments (Dell'Anno and Corinaldesi 2004). DNA is adsorbed on solid surfaces such as sand and clay, thereby gaining protection against degradation by DNase in natural environments (Romanowski et al. 1991; Stewart and Sinigalliano 1990). Shorter DNA fragments tend to preferentially adsorb to soil particles compared with longer fragments (Ogram et al. 1994). It was also reported that more

Table 3.1 Roles of extracellular DNA in bacterial ecosystem

Source of nutrient	Carbon, nitrogen, phosphorus
Genetic material	Transformation
	DNA repair
Adhesively material	Biofilm formation
	Cell aggregation

DNA is adsorbed to sand at high salt concentration and low pH (Lorenz and Wackernagel 1987). Adsorption of DNA to surfaces may influence its availability to bacteria in natural environments.

3.2 Extracellular DNA as Nutrient

The contents of carbon, nitrogen, and phosphorus in DNA are approximately 40%, 20%, and 10%, respectively. Extracellular DNA is an important component of dissolved organic matter and is a source of energy and nutrients in aquatic ecosystems (Niels et al. 1994; Pinchuk et al. 2008; Palchevskiy and Finkel 2006; Finkel and Kolter 2001). Various marine bacteria can grow by utilizing dissolved DNA as their sole nutrient source. Marine bacteria were cultivated on low-molecular-weight (LMW; <250 bp) or high-molecular-weight (HMW; <10 kbp) DNA as the primary source of carbon, nitrogen, and phosphorus. Bacterial colonies were isolated and their 16S rDNA sequences and DNA availabilities were analyzed. Dominant bacterial species in LMW DNA culture (LMW-DC) were *Alteromonas*, *Vibrio*, and *Pseudoalteromonas*. In HMW DNA culture (HMW-DC), *Alteromonas* and *Pseudoalteromonas* were dominant. *Alteromonas* and *Pseudoalteromonas* in LMW-DC and HMW-DC belonged to different phylogenetic clades. Isolates from LMW-DC grew faster than isolates from HMW-DC in LMW-DC, while the extracellular DNase activity of the former was lower than that of the latter in HMW-DC. Studies of the effect of DNA size on bacterial community composition show that various bacteria degrade dissolved DNA in marine ecosystems (Lennon 2007). These results were obtained from analysis of colony-forming bacteria. Thus, culture-independent methods may provide a detailed clarification of which bacterial populations are involved in DNA turnover.

Deep-sea sediments constitute the largest reservoir of extracellular DNA (0.30–0.45 Gt) in the world's oceans (Dell'Anno and Danovaro 2005). This amount is about six- to eightfold higher than that of the DNA contained within all bacteria inhabiting this environment. Extracellular DNA is an important source of nutrients for deep-sea bacteria. It supplies 4%, 7%, and 47% of the daily carbon, nitrogen, and phosphorus demand of prokaryotes, respectively. Extracellular DNA in deep-sea sediments is a reservoir of available phosphorus and is involved in phosphorus cycling in bacterial ecosystems. Marine sediments facilitate the uptake and expression of exogenous DNA by transformable marine bacteria, and that sediments are a more likely niche for natural transformation than the water column in marine environments.

3.3 Extracellular DNA as Genetic Material

DNA brought into the prokaryotic cell is used to generate genetic diversity and repair genome DNA damage. Natural genetic transformation is a phenomenon in which cells take up extracellular DNA and maintain it stably as plasmids or integrate it into

the bacterial genome. Bacteria acquire competence through the regulated expression of genes for protein components of the uptake machinery. At least 40 genera are known to be competent under natural conditions (Lorenz and Wackernagel 1994; Dubnau 1999; Chen and Dubnau 2004). In *Bacillus subtilis*, extracellular DNA serves as a template for the repair of DNA damage (Wojciechowski et al. 1989).

Neisseria species, *Haemophilus influenzae*, and many other Gram-negative bacteria take up species-specific DNA for transformation (Graves et al. 1982; Scocca et al. 1974). Homologous recombination between the bacterial genome and extracellular DNA occurs efficiently in these bacterial cells because almost all sequences are similar. Some bacteria can recognize the specific sequences to acquire homospecific DNA. The specific sequences are the *Neisseria* DNA uptake sequence (DUS), 5′-GCCGTCTGAA-3′, and the *H. influenzae* uptake signal sequence (USS) 5′-AAGTGCGGT-3′ (Goodman and Scocca 1988; Danner et al. 1980). In *H. influenzae*, *H. somnus*, *Pasteurella multocida*, and *Actinobacillus actinomycetemcomitans*, which belong to the *Pasteurellaceae*, these signal sequences are present as 1,471, 1,205, 927, and 1,760 copies in the genome, respectively (Bakkali et al. 2004). *Neisseria* species have approximately 2,000 DUSs in the genome. Seventy-six percent of DUSs were found to have two semiconserved base pairs extending from the 5′ end of the DUS, constituting a 12-mer repeat. The 12-mer sequence outperforms the 10-mer DUS in efficiency of transformation in *Neisseria gonorrhoeae* and *N. meningitidis*. Ambur et al. propose that the 10-mer identity of DUS should be extended and recognized as a 12-mer DUS (Ambur et al. 2007). Half of the 12-mer DUSs exist as inverted repeats and may be involved in rho-independent transcriptional termination or attenuation. DUS plays an important role in DNA uptake and transcription in *Neisseria* species (Goodman and Scocca 1988).

Unlike *Neisseria* species, *Acinetobacter* species do not discriminate between homospecific and foreign DNA during transformation (Dubnau 1999). The frequency of transformation with foreign DNA is at least 10^9-fold lower than that with homologous DNA in *Acinetobacter* species. When foreign DNA was linked on one side to 1 kbp homologous sequence to the recipient genome, however, the integration frequency of foreign DNA increased at least 10^5-fold (de Vries and Wackernagel 2002). The tag of the homologous sequence facilitates illegitimate recombination because the sequence serves as an anchor. When the lengths of homologous DNA tags were 1 kbp, 300 bp, and 200 bp, the frequency of transformation with foreign DNA was 10^{-8}, 10^{-9}, and 10^{-10}, respectively. The 183 bp homologous sequence also facilitated recombination. Therefore, extracellular DNA containing homologous regions contributes to gene introgression among prokaryotes.

3.4 Extracellular DNA in Biofilm

The biofilm is a surface-adhered bacterial community enclosed in extracellular materials such as nucleic acids, proteins, and polysaccharides. In *Pseudomonas aeruginosa*, extracellular DNA was up to 50% more abundant than cellular DNA

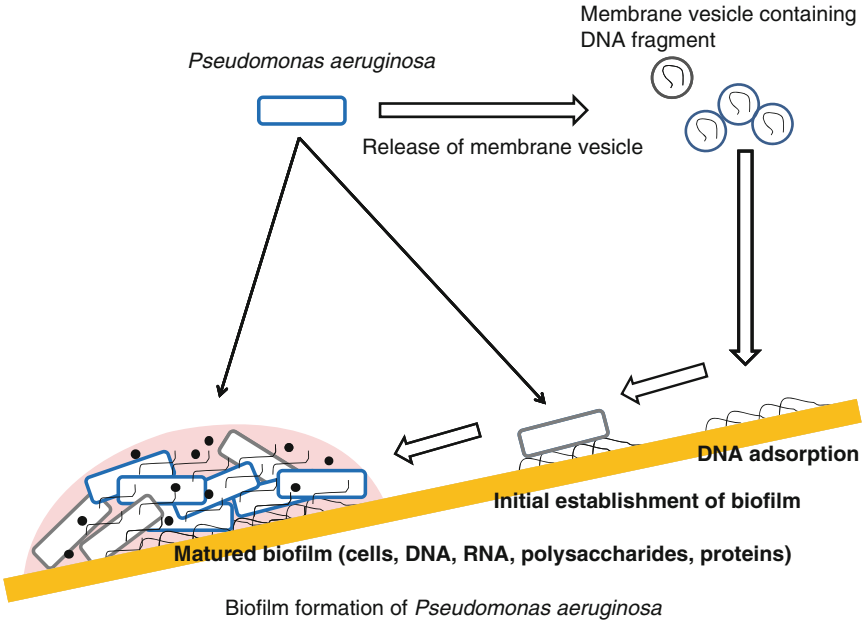

Fig. 3.1 Biofilm formation of *Pseudomonas aeruginosa*

in the unsaturated biofilm (Steinberger and Holden 2004). Extracellular DNA contributes to the initiation and stabilization of biofilms in which the concentrations of bacterial cells and exogenous DNA are higher than those in the water column (Whitchurch et al. 2002; Yang et al. 2007). Thus, bacteria produce extracellular DNA to create circumstances suitable for the generation of diversity by gene exchange and mutation (Driffield et al. 2008) (Fig. 3.1). Extracellular DNA is required not only for the initiation of biofilm formation but also for the stabilization of biofilms (Liu et al. 2008). In *P. aeruginosa* biofilms, extracellular DNA induces antibiotic resistance. DNA has antimicrobial activity by chelating cations that stabilize lipopolysaccharides and the outer membrane. At subinhibitory concentrations, DNA creates a cation-limited environment that results in induction of the cationic antimicrobial peptide resistance operon in *P. aeruginosa*. Resistance to cationic antimicrobial peptides and to aminoglycosides is increased up to 2,560-fold and 640-fold, respectively. Thus, extracellular DNA in the biofilm matrix contributes to cation gradients and inducible antibiotic resistance (Costerton et al. 1999; Mulcahy et al. 2008). Biofilms also increase the mutability of *P. aeruginosa* (Driffield et al. 2008). The mutation frequency for resistance to rifampicin and ciprofloxacin in biofilm cultures was up to 10^5-fold higher than in planktonic cultures. Downregulation of antioxidant enzymes in *P. aeruginosa* biofilms may increase the rate of mutagenic events due to the accumulation of DNA damage.

Extracellular DNA is required for biofilm formation in various bacteria. *Enterococcus faecalis* biofilms were grown on glass substrates and treated with DNase I

for 6, 12, and 24 h. After 6 or 12 h, the biofilms were sensitive to DNase I (Thomas et al. 2008). However, the effect of DNase I was less after 24 h of treatment. *E. faecalis* mutants deficient in DNA release were significantly reduced in the biofilm biomass. In *Staphylococcus epidermidis*, extracellular DNA is required in the initial phase of biofilm development (Qin et al. 2007; Izano et al. 2008). Sensitivities of staphylococcal biofilms to DNase I were compared. DNase I treatment inhibited the initiation of biofilm formation of both *S. aureus* and *S. epidermidis*, whereas DNase I detached a 24-h-old biofilm of *S. aureus* but not one of *S. epidermidis*. Extracellular DNA has different structural roles in *S. aureus* and *S. epidermidis* biofilms.

3.5 Uptake and Release of DNA

When bacteria use extracellular DNA as a source of nutrients or genetic material, they permit the passage of DNA across one membrane in gram-positive or two membranes in gram-negative bacteria. The mechanisms of DNA uptake are well known for natural transformation, which requires bacterial protein components for DNA uptake, but not for transformation induced by calcium treatment, heat shock, or electric pulses (Chen et al. 2005; Hamilton and Dillard 2006). Initially, exogenous DNA interacts with the cell surface. DNA bound to the cell surface is fragmented. In gram-negative bacteria, fragmented double-stranded DNA enters the periplasmic space across the outer membrane. Single-stranded DNA passes across the cytoplasmic membrane in both Gram-positive and -negative bacteria, and a complementary strand is degraded by nuclease. In *N. gonorrhoeae*, exogenous double-stranded DNA passes into the periplasmic space across the outer membrane through a channel formed by secretins. Secretins associated with the outer-membrane are components of the type IV pilus and form channels in which the central cavity has a diameter in the range of 6.5 nm. Double-stranded DNA is transported through a channel on the cytoplasmic membrane. ComE, a DNA receptor, is involved in the transport of double-stranded DNA. One strand of DNA in the periplasmic space enters the cytosol across the cytoplasmic membrane through a channel formed by ComA. The other strand is degraded and released into the periplasmic space. In Gram-positive bacteria that have no outer membrane, such as *B. subtilis* and *Streptococcus pneumonia*, double-stranded DNA taken up through the pseudopili binds the DNA receptor ComEA and is transported through a channel on the cytoplasmic membrane. One strand of DNA enters the cytosol across the cytoplasmic membrane through a channel formed by ComEC. A piece of single-stranded DNA is integrated into the genome of the recipient by homologous recombination that is dependent on RecA or other enzymes. A plasmid can be reconstituted and maintained if it is replicable in the recipient. In *Escherichia coli*, homologs of proteins involved in natural transformation in *H. influenza* are required in order to take up extracellular DNA as a nutrient source (Palchevskiy and Finkel 2006; Finkel and Kolter 2001). *E. coli* has homologs to eight *H. influenzae* genes

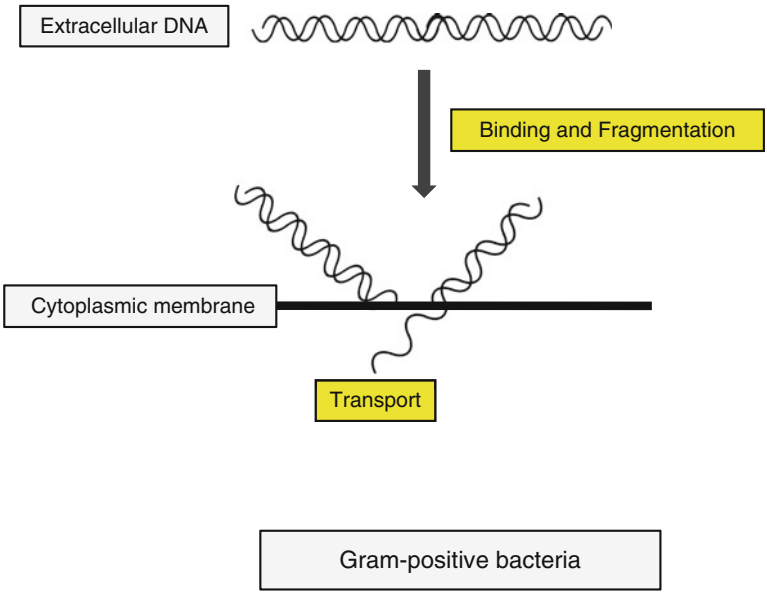

Fig. 3.2 DNA processing and transfer during transformation in Gram-positive bacteria

that are involved in competence and transformation. Their amino acid sequence homology to the com gene products of *H. influenzae* range from 12% to 74%. Homologs of these *E. coli* genes are present in many members of the gamma-proteobacteria, such as *Salmonella typhi*, *Klebsiella pneumoniae*, *Vibrio cholerae*, and *Pseudomonas aeruginosa*. Therefore, these bacteria also use homologs of the competence genes to consume extracellular DNA (Figs. 3.2 and 3.3).

Uptake of homospecific DNA enables efficient repair of the genome and generation of genetic diversity in bacteria, because the frequency of recombination with homospecific DNA is more than 10^9-fold higher than with heterospecific DNA. DNA may be released accidentally from bacterial cells by autolysis, phage infection, predation, or other forms of death. It is doubtful that enough homospecific DNA is provided to repair genome or generate genetic diversity by these accidental events only in natural environments. There are various strategies by which bacteria efficiently acquire DNA of their own and related species. *N. gonorrhoeae* recognizes DUS-specific DNA to take up homospecific DNA preferentially for efficient transformation. *N. gonorrhoeae* also secretes chromosomal DNA. Little is known about the mechanism by which secreted DNA is produced; however, it is clear that a type IV secretion system is involved in DNA donation (Hamilton et al. 2005). Type IV secretion systems are encoded in a 57 kbp region of the gonococcal genetic islands that are present in 80% of *N. gonorrhoeae* strains. This secretion system is noted for its ability to secrete both DNA and proteins. Donated DNA from own and

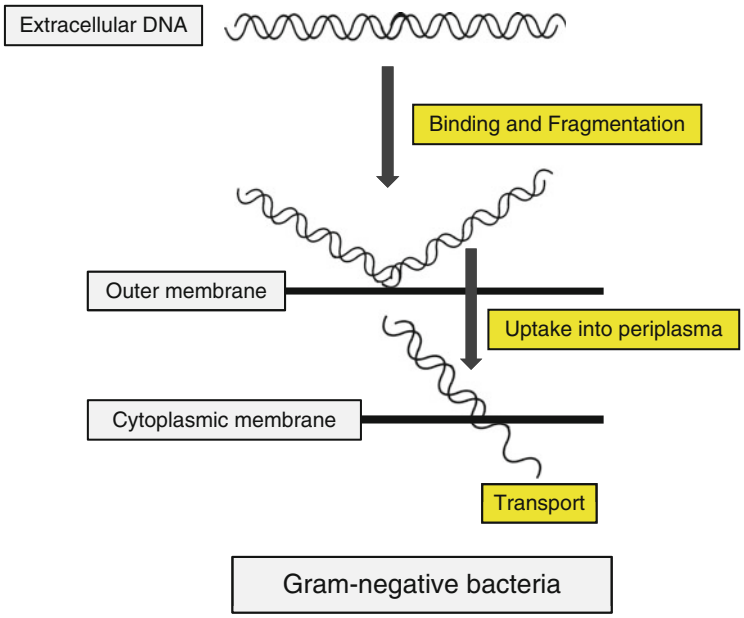

Fig. 3.3 DNA processing and transfer during transformation in Gram-negative bacteria

related species may contribute to the generation of genetic and functional diversity, such as antigenic variation and the spread of antibiotic resistance.

Extracellular DNA is produced by another mechanism of DNA exchange between streptococci (Fig. 3.4). The competence of streptococci is triggered by a secreted peptide pheromone called competence-stimulating peptide (CSP) through a quorum-sensing mechanism (Steinmoen et al. 2002, 2003; Petersen et al. 2005). Competent cells of streptococci produce lysins, which are cell lytic enzymes. The lysins are anchored to the cell surface of competent cells and cause the lysis of noncompetent cells through cell-to-cell contact. Bacterial DNA of noncompetent streptococcal cells is released for gene exchange. Streptococci behave as recipients and donors under a quorum-sensing controlled system. *Enterococcus faecalis* produces an autolysin to release its own DNA; the autolysin also lyses other *E. faecalis* cells (Thomas et al. 2008).

Gram-negative bacteria release membrane vesicles into the surrounding medium during normal growth. Vesicles contain lipopolysaccharide, periplasmic proteins, outer membrane proteins, phospholipids, and DNA. *E. coli* produces outer membrane vesicles that export virulence factors to host cells (Kolling and Matthews 1999; McBroom et al. 2006). *P. aeruginosa* releases membrane vesicles that supply the bacterial extracellular DNA that is required for early biofilm formation (Li et al. 1996). Autolysin is involved in the release of the vesicles that is regulated by the

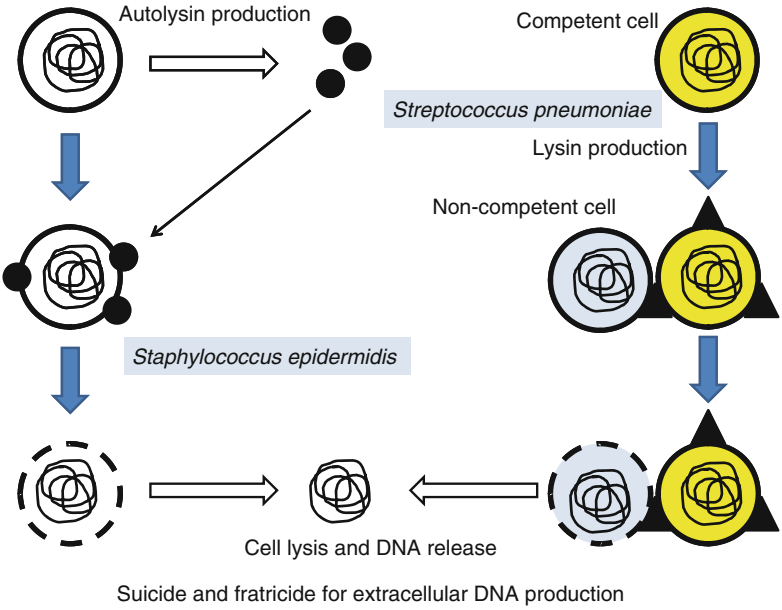

Fig. 3.4 Suicide and fratricide for extracellular DNA production

quorum-sensing systems in *P. aeruginosa* (Nakamura et al. 2008). *N. gonorrhoeae* also releases membrane vesicles. *Neisseria* species produce vesicles as a DNA delivery system for gene exchange because the DNA within the vesicles is protected against exogenous nucleases (Assalkhou et al. 2007). Many bacterial species produce lytic enzymes. Some bacterial species produce lytic enzymes that lyse the cells that produce them, releasing their own DNA into the environment. Some bacteria produce bacteriocins that kill related species but not themselves and unrelated species. The bacteriocin they produce kills nutrient competitors, whose cell lysates they use as nutrients. They may also produce bacteriocins to obtain DNA as genetic material.

Bacterial DNA is also released by interactions of bacteria and eukaryotes (Turk et al. 1992). Concentrations of dissolved DNA in filtered seawater cultures were monitored with or without addition of the marine nanoflagellate *Ochromonas* sp. The concentration of dissolved DNA was sixfold higher with the nanoflagellates than without them. The amount of DNA released suggested that most of the consumed bacterial DNA was ejected. Phagotrophic nanoflagellates thus represent an important source of extracellular DNA. The breakdown of released DNA produces inorganic phosphorus in the culture. The bacterial biomass grazed by protozoa plays an important role in the biogeochemical cycling of phosphorus in the marine environment. Matsui et al. studied the effects of cocultivation with either *Euglena gracilis* (Euglenophyta) or photosynthetic algae on the production of extracellular plasmid DNA by *E. coli* (Matsui et al. 2003). Transformable plasmids were released from *E. coli* by cocultivation with *E. gracilis*, *Microcystis aeruginosa*,

or *Carteria inversa*. Algae stimulate the release of transformable DNA from prokaryotes in the natural environment.

3.6 Uptake of Extracellular DNA by Indigenous Bacteria

The mechanism and frequency of DNA uptake have been well studied in some standard bacterial strains. However, it is difficult to estimate the frequency of DNA uptake by naturally occurring bacteria because they are not easily cultured by conventional methods. Recently, a culture-independent method based on the expression of the green fluorescent protein gene (*gfp*) has been used to monitor the transformation of *Acinetobacter* species (Hendrickx et al. 2003; Rizzi et al. 2008). Use of this technique revealed that transformation occurred with plasmid DNA at a 10^6-fold lower concentration than previously reported. However, the frequency of DNA uptake may be underestimated using this method because techniques based on transformation are limited by differences in the maintenance of DNA and the expression of specific genes.

In situ gene amplification methods can make monitoring of DNA in bacterial cells possible, independent of cultivation and gene expression (Tani et al. 1998; Maruyama et al. 2003; Kenzaka, et al. 2005). The broad-host plasmid pQE70 carrying the *gfp* gene was introduced into the river water samples; DNA uptake was monitored by the in situ rolling circle amplification, and expression of the *gfp* gene was monitored by epifluorescence microscopy (Maruyama et al. 2006). The frequency of DNA uptake by river bacteria ranged from 10^{-5} to 10^{-3}. Detection of DNA uptake as monitored by in situ DNA amplification was 20 times higher, at its maximum, than by direct counting of *gfp*-expressing cells (Table 3.2). This showed that the frequency of DNA uptake by naturally occurring bacteria is higher than previously estimated. Extracellular DNA may facilitate gene transfer among bacteria, especially in sea sediments and biofilms, in which free DNA and bacterial cells are present at high concentrations.

Table 3.2 Frequency of DNA uptake and *gfp* gene expression in river bacteria

Location	Sampling time (h)	GFP expression frequency	DNA uptake frequency
Kanzakigawa River	0	$<2.1 \times 10^{-6}$	$<1.4 \times 10^{-6}$
	7	$<2.1 \times 10^{-6}$	$<1.3 \times 10^{-5}$
	13	$<1.9 \times 10^{-6}$	$<3.4 \times 10^{-5}$
	25	$<1.3 \times 10^{-6}$	$<1.4 \times 10^{-4}$
	170	1.0×10^{-6}	$<5.3 \times 10^{-4}$
Minohgawa River	0	$<3.1 \times 10^{-5}$	$<2.1 \times 10^{-5}$
	7	$<3.1 \times 10^{-5}$	4.6×10^{-4}
	13	$<2.9 \times 10^{-5}$	1.6×10^{-3}
	25	$<2.9 \times 10^{-5}$	2.3×10^{-3}
	170	9.1×10^{-5}	1.6×10^{-3}

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Chapter 4
Stable Extracellular DNA: A Novel Substrate for Genetic Engineering that Mimics Horizontal Gene Transfer in Nature

Shinya Kaneko and Mitsuhiro Itaya

Contents

Abstract Molecular biology technology generally requires naked DNA purified in a test tube. However, extracellular DNA in the natural environment can contribute to breeding and growth as nutrition or genetic information. Transfer and maintenance of DNA is called horizontal gene transfer (HGT), a mechanism that has certainly played a role during evolution. The naked DNA received by particular microbes through natural transformation processes would almost certainly be extracellular. However, this form of transformation has been rarely studied compared with the other two major mechanisms of HGT: transduction and conjugation.

S. Kaneko
Graduate School of Bioscience and Biotechnology, Department of Life Science, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku, Yokohama-shi, Kanagawa 226-8501, Japan
M. Itaya (✉)
Institute for Advanced Biosciences, Keio University, Nipponkoku, Tsuruoka-shi, Yamagata 997-0017, Japan
e-mail: mita2001@sfc.keio.ac.jp

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In this chapter, we present our recent discovery that plasmid DNA released from lysed *Escherichia coli* remains surprisingly stable in the environment. This extracellular DNA, of up to 100 kb and possibly larger, can undergo an HGT-like process into a recipient bacterium capable of natural transformation.

This form of DNA transfer, mimicking HGT, could facilitate the delivery of engineered genes without the need for biochemical purification, and permit experimental research into the mechanisms of HGT.

Abbreviations

amp	Ampicillin
BAC	Bacterial artificial chromosome
bla	β-Lactamase gene
bp	Base pair(s)
cat	Chloramphenicol acetyl transferase gene
ccc	Covalently closed circular
*c*I	*c*I Repressor gene
CMM	Culture mix method
GFP	Green fluorescent protein
GpBR	Genomic pBR322 sequence
HGT	Horizontal gene transfer
kb	Kilobases
kbp	Kilobase pairs
km	Kanamycin resistance gene
tet	Tetracycline resistance determinant gene

4.1 Introduction

Horizontal gene transfer (HGT) is a major force in producing diversity in gene content and genome structure across species and higher taxonomic boundaries (Fig. 4.1). Recent advances in comparative genome sequence analysis have identified many examples of HGT among microorganisms (de la Cruz and Davies 2000; Ochman et al. 2000; Chivian et al. 2008). HGT generally proceeds through three major molecular mechanisms: natural transformation (Lorenz and Wackernagel 1994), conjugation (Ippen-Ihler 1990), and transduction (Kokjohn 1989). While traversing between two bacteria by conjugation or transduction, DNA molecules are protected by specific gene products encoded by host genomes, phage genomes, or plasmids. Consequently, the range of species that can use conjugation or transduction is small. In contrast, natural transformation uses naked DNA, referred to here as “extracellular DNA”. As there is little requirement for specific DNA sequences,

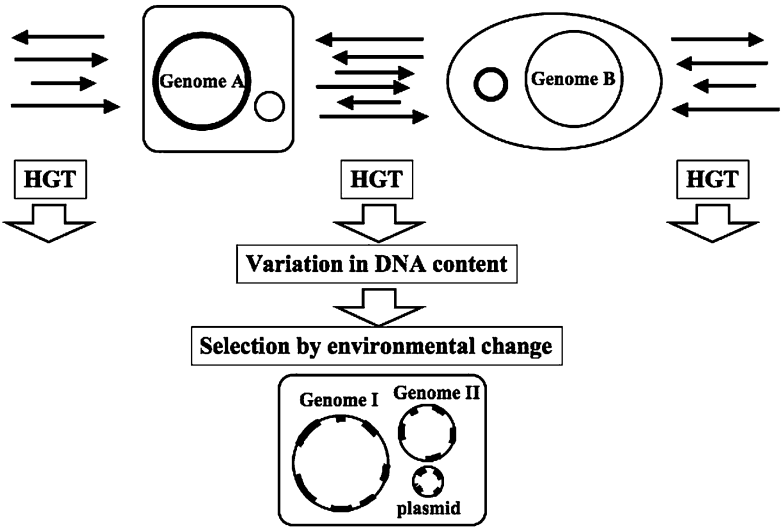

Fig. 4.1 Horizontal gene transfer (HGT) generates bacterial genome mosaics at all levels. Three major molecular mechanisms contribute to the HGT process: transduction, conjugation, and transformation

any DNA stably present in the environment may serve as a substrate for HGT. One of the important factors in transformation-mediated HGT is the stability and dissemination of DNA released from living or dead cells, which we shall hereafter term "DNA donors". Another important factor is the presence of cells able to recognize and incorporate this extracellular DNA. We designate these cells "recipient cells capable of developing competency". As shown below, our recent findings on the movement of giant plasmid DNAs from a donor *Escherichia coli* to a recipient *Bacillus subtilis* via extracellular DNA may lead to the establishment of novel DNA delivery systems applicable to a broad range of recipient cells.

4.1.1 Transformable Recipient Microorganisms

The extracellular DNA employed in this chapter was high molecular weight DNA polymer released into the natural environment. It does not decompose and is a suitable substrate for a transformable recipient microorganism. Some bacteria can enter a physiological state called "competence" through the expression of a number of competence-related genes. Those genes encoding DNA uptake and processing systems are often functionally similar to subunits of the type IV pili and type II secretion systems (Chen et al. 2005). Interestingly, it was reported that the bacterial "SOS" response to stress-induced DNA damage activates the cells' potential for genetic transformation and promotes HGT within a microcosm ecosystem

(Beaber et al. 2004; Prudhomme et al. 2006). These naturally transformable bacterial species, some of which are archaebacteria, have been widely found among the taxonomic and tropic groups; e.g., *Acinetobacter calcoaceticus*, *Deinococcus radiodurans*, *Lactobacillus lactis*, *Pseudomonas stutzeri*, *Streptomyces* spp., *Staphylococcus aureus,* and *Synechocystis* sp. strain 6803 (Lorenz and Wackernagel 1994; Peget and Simonet 1994; Tønjum et al. 1995; de Vries et al. 2001; de Vries and Wackernagel 2004). Recent reports have described that *Agrobacterium*, *Legionella*, and *E. coli* are also transformable in special growth environments (Baur et al. 1996; Demanéche et al. 2001a; Stone and Kwaik 1999). In addition, Demanéche et al. (2001b) and Cérémonie et al. (2006) observed DNA uptake from soil under certain conditions such as in electrical fields caused by thunderstorms and lightning discharges. Frischer et al. (1994) found that 10% of isolated marine bacteria were competent to take up plasmid DNAs and that 14% were able to take up chromosomal DNA. These observations suggest that extracellular DNA that is stable in the natural environment is an important source of genetic information. If these DNA molecules were incorporated randomly through natural transformation, the resulting increase in genetic variation would make the cells more adaptable to various environmental alterations, as shown in Fig. 4.1.

4.1.2 Natural Transformation Demonstrated in the Laboratory Test-tube

According to the HGT database (http://genomes.urv.cat/HGT-DB/), in which approximately 500 prokaryotes have been analyzed to date, the putative proportion of horizontally transferred genes is extremely variable: for example, *Mycoplasma mobile* 163K (0.6%), *Synechocystis* PCC6803 (6.9%), *E. coli* K12 (8.3%), *Bacillus subtilis* (12.8%), and *Pyrobaculum islandicum* (25.3%) (Garcia-Vallve et al. 2000, 2003). However, it is generally unknown which DNA segments were introduced by natural transformation. DNA incorporated by transformation leaves little or no trace, in contrast to that incorporated by conjugation or transduction, which generally comprises functional or cryptic genes encoding proteins such as integrases or factors related to plasmid conjugation systems or phages (Juhas et al. 2009).

Experimental research into natural transformation has been attempted in several transformable bacteria. Paul et al. (1992) showed transfer between Gram-negative bacteria. The authors observed the transfer of nonconjugative broad-host-range plasmids from heat-killed *E. coli* to naturally transformable *Vibrio* species processed in liquid and filter matings. Delivery among Gram-positive bacteria was reported by Murayama et al. (2004), who demonstrated that extracellular DNA released from a lysed *B. subtilis* cell population was taken up and incorporated by a competent *B. subtilis* strain present in the same medium. Similar, natural, intraspecies transformation was also described in *Streptococcal* strains as a fratricidal mechanism for generating genetic diversity in a restricted environment (Johnsborg and Hávarstein 2009).

4.1.3 Why Is Natural Transformation Important for Molecular Biology Research and Application?

Gene cloning, the first step in many molecular biology studies, is based entirely on the principles of HGT. In typical molecular cloning technology, as shown in Fig. 4.2, fragmented DNAs are combined with appropriate vectors in a test tube. Then, delivery to *E. coli* K-12, the Gram-negative bacterium most studied and most widely used as a molecular cloning host, is conducted using three biological processes. Cloned DNAs, the sequences of which have been modified or altered using *E. coli* molecular engineering, are normally transferred to another host of interest. Binary plasmid vectors are used for this purpose. Binary plasmids that possess another origin of DNA replication are able to propagate in the designated host and reenter into *E. coli*. The shuttling nature of these binary plasmids is secured by the method of transfer from *E. coli* to the new host. The transfer process is conducted by electroporation in most cases, or in some cases using the competence of the new host. These methods require purified plasmid DNA in a liquid form to be mixed with the designated hosts. Well-established laboratory protocols allow purification of plasmid DNA in solution (Sambrook et al. 1989), and HGT can be effectively performed in the laboratory. The plasmid DNA purified from *E. coli* is usually restricted to cloned DNA fragments of up to several kilobase-pairs (kbp), mainly because of the intrinsic stability of the vector plasmid. As DNA

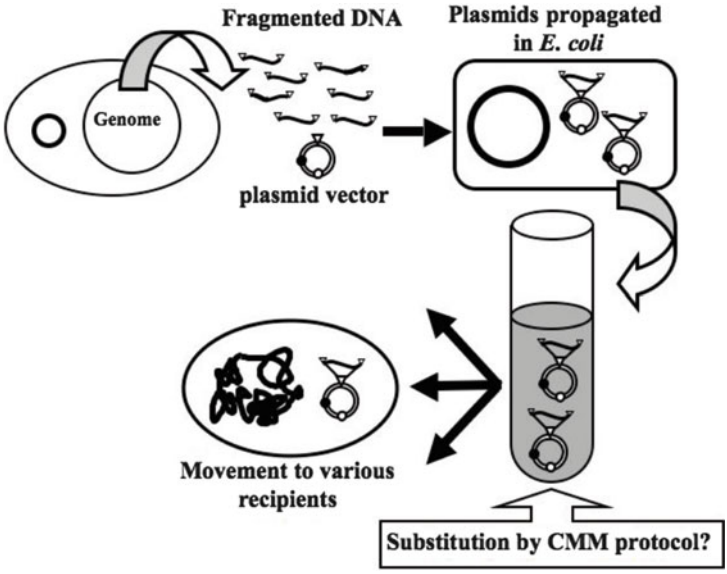

Fig. 4.2 Molecular cloning in the common host *E. coli* and transfer to another bacterium is regarded as one-way HGT, and is represented by *bold arrows*. Isolation of intact plasmid DNA in the test tube is shown being substituted by our discovery of stable extracellular DNA, as described in Fig. 4.3a. Culture mix method (CMM) protocol is shown in Fig. 4.3b

size increases, the handling of plasmids in solution becomes more difficult. In particular, preparation of higher molecular weight DNA (>100 kbp) from *E. coli*, such as from Bacterial Artificial Chromosome (BAC) vectors, is difficult because of strand breaks caused by physical shearing and contamination by nucleases during biochemical isolation (Kaneko et al. 2005). *In vitro* preparation of DNA as large as hundreds of kilobases needs to be conducted under special conditions in which pipeting must be minimized (Gibson et al. 2008). It was our initial motivation to use laboratory *E. coli*-based HGT systems to avoid DNA purification steps and increase the maximum length of DNA that could be transferred. In addition, such protocols, if applied more generally, could lead to well-designed systems to permit the investigation of HGT in nature.

4.2 Production of Stable Extracellular DNA Released from Donor Cells

Extracellular DNAs are generally brought into the environment spontaneously from lysed cells undergoing cell death, except when they are secreted via the type IV system (Hamilton et al. 2005; Suzuki et al. 2009). Therefore, to mimic natural processes, the production of extracellular DNAs should not involve biochemical procedures. In the laboratory, movement into various species is currently assisted by electroporation or natural transformation.

4.2.1 Extracellular DNAs Released from Cells Lysed in the Laboratory

We have surveyed the stability of DNA emerging from *E. coli* K-12 under various medium conditions. Fortuitously, we found that plasmid DNA released into Luria–Bertani (LB) medium during lambda induction of *E. coli* was extremely and surprisingly stable for some period. A mutant lambda lysogenic *E. coli* was used to prove this. The *E. coli* lambda lysogen grows normally in LB medium at 30°C. Due to a mutation in the *c*I repressor gene of lambda, *c*I857, the CI product loses its repressor ability at the elevated temperature of 37°C, causing induction of lambda phage. Subsequent phage maturation leads to cell lysis (Arber 1983). Nearly complete lysis of *E. coli* was obtained after 2 h at 37°C (Fig. 4.3a). After lysis, cellular materials, including genomic DNA and existing plasmid DNA, were released into the LB medium. Our attention was focused on the released plasmid DNA in terms of its stability both in chemical structure and in biological activity. *E. coli* whole lysate was subjected to a normal plasmid preparation protocol (Kaneko and Itaya 2010). As shown in Fig. 4.3a, the plasmid DNA prepared from LB medium containing lysing *E. coli* at 37°C was indistinguishable from that

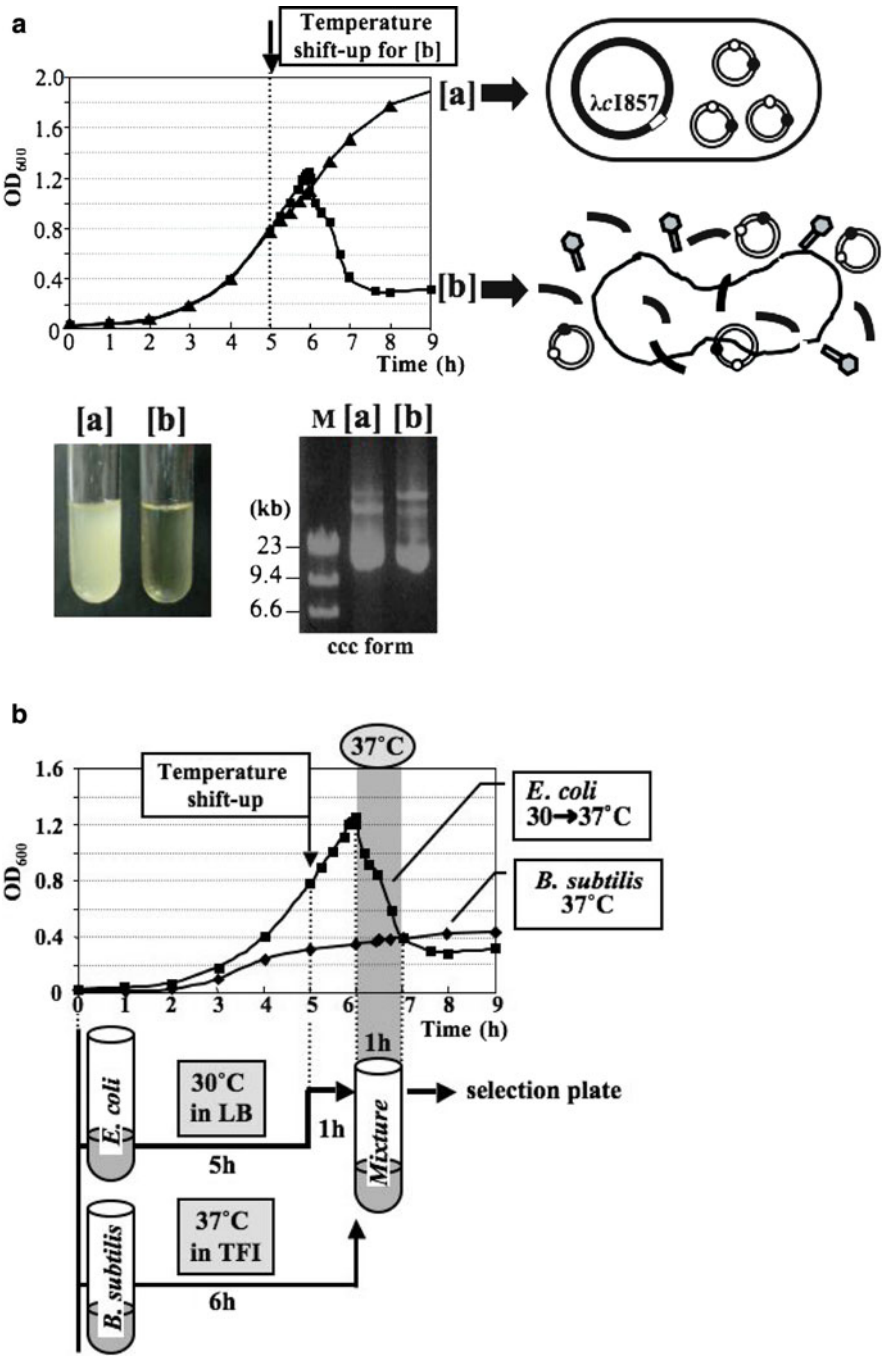

Fig. 4.3 (**a**) Analysis of extracellular plasmid DNA released from *E. coli*. The graph shows the growth of *E. coli* over time as measured by Optical Density (OD) 600 nm in LB medium.

containing intact cells at 30°C, within the resolution of agarose gel electrophoresis. Furthermore, the yield of recovered plasmid DNA from the LB medium was surprisingly high (Kaneko and Itaya 2010).

4.2.2 *Extracellular DNA: An Active Substrate in the Natural Transformation of a Gram-Positive Bacterium*

The plasmid DNA investigated here was from a binary plasmid that replicates both in *E. coli* and *B. subtilis,* a Gram-positive bacterium. *B. subtilis* Marburg 168, registered as 1A1 in the *Bacillus* Genetic Stock Center (BGSC), Ohio, is known to form molecular machinery to actively take up naked DNA from the environment. Cells able to incorporate DNA in this way are called "competent" cells. Competence develops during the transition from log phase to stationary phase induced by an excreted small polypeptide called the competence factor (Smith et al. 1981; Dubnau 1991a, b; Chen et al. 2005). Natural transformation by competent *B. subtilis* shows sequence nonspecificity (Chen et al. 2005) and normally proceeds by mixing competent cells and naked DNA prepared separately in the laboratory.

In our preliminary investigations, competent *B. subtilis* was used to validate the quality of plasmid DNA that had just been released from lysing *E. coli*. To ensure that the plasmid remained a high-quality substrate, regardless of the presence of *E. coli* cell debris, we developed the protocol for the Culture Mix Method (CMM) described in Fig. 4.3b (Kaneko and Itaya 2010). We believe that the CMM constitutes HGT in the laboratory, because the *B. subtilis* transformants resulting from HGT could be selected by a plasmid-associated marker. The binary plasmid shuttling between *E. coli* and *B. subtilis*, pGETSGFP (17 kb; Fig. 4.4a) (Ohashi et al. 2003), carries a tetracycline resistance determinant gene (*tet*) and a green fluorescent protein gene (*GFPuv*), which is expressed only in *B. subtilis*. Accordingly, transformants selected by tetracycline at 37°C can be monitored with green luminescence under UV light (365 nm). All *B. subtilis* transformants examined possessed intact pGETSGFP, as shown by agarose gel electrophoresis after *Eco*RV digestion (Fig. 4.4a). It is most likely that the plasmid released from lysed *E. coli* remained stably in the same structure as in the live cell; i.e., in the covalently closed circular (ccc) form. Complete suppression of transformation in the presence of

Fig. 4.3 *(continued)* The lambda *c*I857^{ts} lysogen starts lysing the bacteria after transfer to the elevated temperature of 37°C (*squares*). Without the temperature shift, the *E. coli* continue to grow (*triangles*). Although the medium from lysed cells [b] was clear, plasmid DNA was present and apparently undamaged (i.e., in the covalently closed circular (ccc) form) compared with that from nonlysed cells [a]. The "M" lane contains size markers as indicated on the *left*. (**b**) A standard protocol for the culture mix method (CMM). The method is based on the lysis kinetics of *E. coli*, mixing these cells with competent *B. subtilis* cells separately prepared in competent developing medium, designated TFI, and adjusted to give the best results. There is no requirement for biochemical treatment of the DNA in this protocol (Kaneko and Itaya 2010)

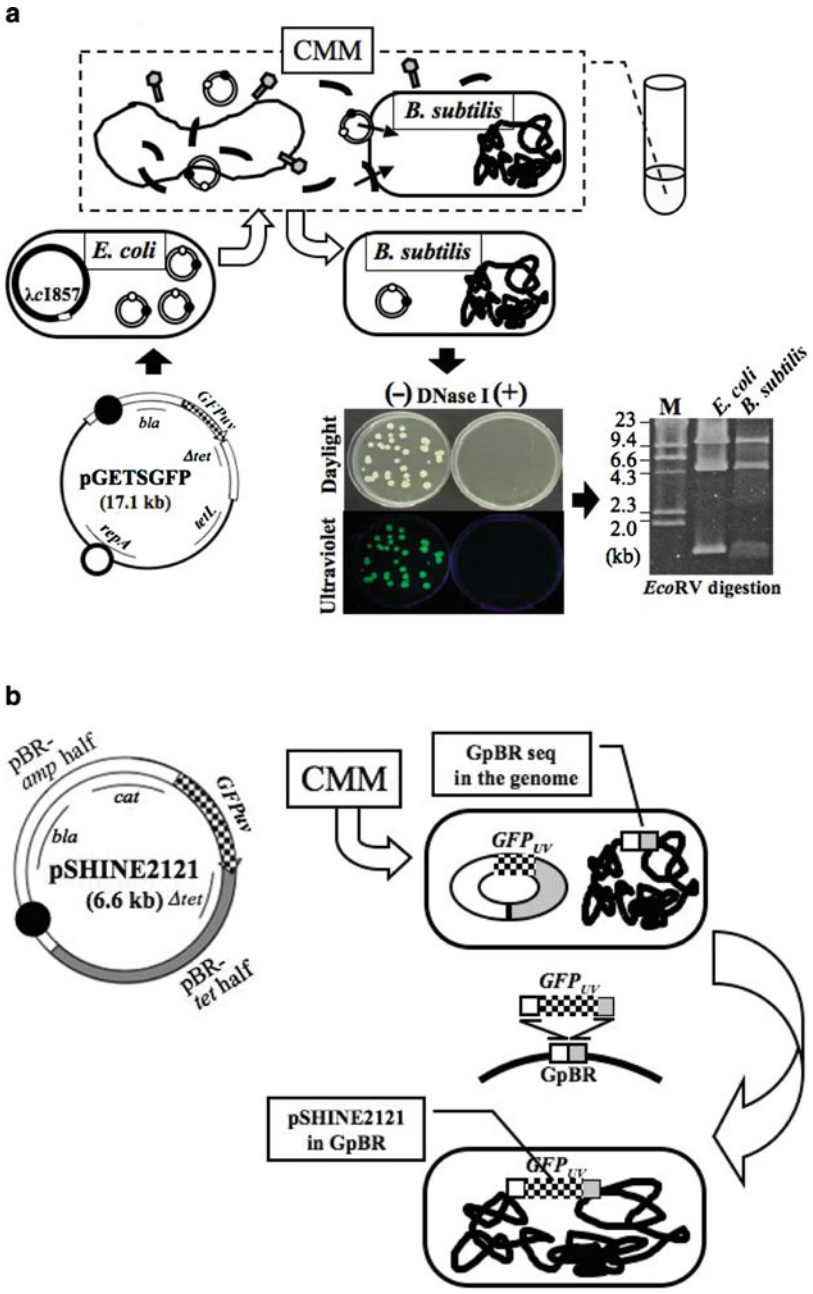

Fig. 4.4 (**a**) Horizontal plasmid transfer from *E. coli* to *B. subtilis*. The plasmid pGETSGFP possesses two replication origins: the *closed circle* for *E. coli* and the *open circle* for *B. subtilis*. Effective transfer by the CMM protocol was completely suppressed by the addition of DNase I during mixing of the cultures (Fig. 4.3b). Identical pGETSGFP structures in the donor (*E. coli*

commercial DNase I (3.4 μg/ml), as shown in Fig. 4.4a, strongly indicated that the extracellular DNA was sensitive to DNase I. In addition to investigating the importance of plasmid quality as a substrate for *B. subtilis* competent cells, we also studied transformation kinetics to assess the quantity of plasmid required (unpublished data).

The size limit, if any, of the CMM for competent *B. subtilis*, is interesting and important for its further application. As *B. subtilis* efficiently takes up purified DNA >100 kb long (Itaya 1999; Itaya et al. 2005, 2008; Kaneko et al. 2003, 2005, 2009), we examined the efficiency of large plasmid transfer by the CMM. Other larger binary plasmids that shuttle between *E. coli* and *B. subtilis* were used, being *E. coli* BAC-based plasmids of approximately 100 kb, carrying a large fragment originating from the mitochondrial genome of *Arabidopsis thaliana* (Kaneko et al. 2005). Unexpectedly, although these DNAs were huge compared with those in the standard CMM protocol shown in Fig. 4.3b, they showed the expected transfer to *B. subtilis,* albeit with a substantial reduction in efficiency. Although the factors that affect the efficiency remain to be investigated, these results indicated that plasmids as large as 100 kb released into LB medium remained an effective substrate for competent *B. subtilis*.

4.3 Engineered Natural Transformation in the Laboratory

4.3.1 Integration of Extracellular DNA into the Engineered Recipient Genome

Plasmid DNA normally replicates in several hosts. The host range seems slightly broader than that of phage-mediated transduction or conjugation; however, for optimal genome engineering (Itaya 2009), it is preferable to integrate DNA directly into the host genome beyond species or genera barriers. The *B. subtilis* genome has been employed to investigate this goal too.

E. coli plasmids lacking a replication origin (*ori*) for *B. subtilis* should be also taken up normally by competent *B. subtilis* cells. However, because the plasmid cannot replicate, the original plasmid would eventually be lost by segregation.

Fig. 4.4 *(continued)* lane) and recipient (*B. subtilis* lane) indicated that the extracellular plasmid is stable. Using the CMM, plasmids 100 kb in size are able to traverse between bacteria, as described in the text (kaneko and Itaya 2010). (**b**) Horizontal DNA transfer to integration into the *B. subtilis* genome. The fate of transferred plasmids possessing replication origins only for *E. coli* (the *closed circle*) is shown. In the recipient *B. subtilis* that carries the same plasmid sequence in its genome, indicated as GpBR, the plasmid integrates at the GpBR locus through homologous recombination. CMM-mediated integration of DNA >100 kb was observed, as described in the text (Itaya and Kaneko 2010). *bla, tet,* and *cat* indicate β-lactamase gene, tetracycline resistance determinant gene, and chloramphenicol acetyl transferase gene, respectively. *repA* gene functions for replication of *B. subtilis*. pBR-*tet* half and pBR-*amp* half indicate two pBR322 half regions carrying *tet* and ampicillin resistance gene (β-lactamase gene)

On the other hand, if the plasmid carries sequence homologous to that of the *B. subtilis* genome, it can integrate into the genome via homologous recombination, as shown in Fig. 4.4b. A pBR322-based *E. coli* plasmid, pSHINE2121 (6.6 kb), which carries *GFPuv* genes but lacks a replication origin for *B. subtilis* (Ohashi et al. 2003; Fig. 4.4b), was examined in the CMM (Itaya and Kaneko 2010). The pSHINE2121 plasmid also carries the same 4.3 kb of pBR322 sequence as the recipient *B. subtilis* genome, so that when this plasmid is transferred into competent *B. subtilis* by the CMM, it should become integrated into the genomic pBR322 sequence via homologous recombination. Indeed, all *B. subtilis* colonies selected by plasmid-associated antibiotic marker exhibited green fluorescence clearly visible under UV light (365 nm). Although direct purification of the extracellular stable pSHINE2121 in the *E. coli* lysate was not attempted, complete suppression by adding DNase I (3.4 μg/ml) suggested that the CMM was applicable to the delivery of large DNA molecules into the genome of *B. subtilis*.

4.3.2 *Integration of 100 kb DNA into* B. subtilis *by the CMM*

As transfer of plasmids >100 kb from *E. coli* to *B. subtilis* had been demonstrated, we inferred that similar plasmids also lacking a replication origin for *B. subtilis* should integrate if homologous sequence were provided in the recipient genome.

Several >100 kb BAC-based plasmids were tested in the CMM using recipient *B. subtilis* that possessed a BAC sequence in its genome (Kaneko et al. 2005). Several colonies possessed the entire BAC plasmid in the genome (Itaya and Kaneko 2010). These results suggest that extracellular DNA, even 100 kb long (the equivalent of 100 genes), was accurately transferred and integrated into the recipient genome by our current CMM protocol.

4.4 Conclusions and Future Perspectives

It has been reported that the extracellular DNA in natural habitats forms complex structures associated with various substances. These other substances are not only cellular proteins or slime layers but also sand and soil particles, including those in both terrestrial and aquatic environments (Stotzky 1989; Ippen-Ihler 1990). These complexes would result in the stable presence of even higher molecular weight DNA (Lorenz and Wackernagel 1988, 1992, 1993, 1994; Romanowski et al. 1991, 1993; Khanna and Stotzky 1992). It remains to be examined whether these accompanying substances facilitate uptake by the recipient cells, compared with the uptake of highly purified naked DNA in solution. The extracellular DNA released from *E. coli* was clearly stable enough to act as a substrate for *B. subtilis* competent cells, although it is unknown whether the stable plasmids were still bound with certain proteins, as they would have been in the cytoplasm (Kaneko and Itaya 2010;

Itaya and Kaneko 2010). The experimental observations described in this chapter illuminate the role of extracellular DNA produced in the natural environment (Baur et al. 1996; Stone and Kwaik 1999; Demanéche et al. 2001a). Transformation-mediated HGT by our simple protocol (the CMM) can facilitate rapid and convenient transfer of large DNA molecules and is not limited to the combination of *E. coli* and *B. subtilis*. Possible donors and recipients for the CMM should be investigated, together with the development of appropriate binary plasmids.

It should be emphasized that *E. coli* is an exceptional workhorse as a host cell for construction of nearly all plasmids in DNA engineering. On the other hand, we have developed an alternative cloning and manipulation system using *B. subtilis*, called the *Bacillus* GenoMe (BGM) vector system (Itaya et al. 2005, 2008; Tsuge et al. 2007; Itaya 2009). In the BGM vector system, the 4,215 kb genome of *B. subtilis* (Itaya 1993) plays the central role as a cloning vehicle. Once DNA is cloned into the BGM vector, flexible manipulation is guaranteed regardless of molecule size, as shown in Fig. 4.5. We succeeded in cloning the entire 3,500 kb *Synechocystis* PCC6803 genome (Itaya et al. 2005), the 135 kb rice chloroplast genome (Itaya et al. 2008), the 17 kb mouse mitochondrial genome (Yonemura et al. 2007), and a 355 kb mouse genomic segment inserted into a BAC vector (Kaneko et al. 2009). In the BGM cloning technology, however, the preparation of undamaged, naked DNA is one of the bottlenecks, particularly when DNA size exceeds 100 kb (Kaneko et al. 2005).

The present CMM can remove the need for the painstaking biochemical isolation steps from *E. coli*, as indicated in Fig. 4.5 (Kaneko and Itaya 2010). Furthermore, with its few and simple steps, this protocol is suited to multiplex analysis and automation (Itaya and Kaneko 2010). Our discovery of stable extracellular DNA

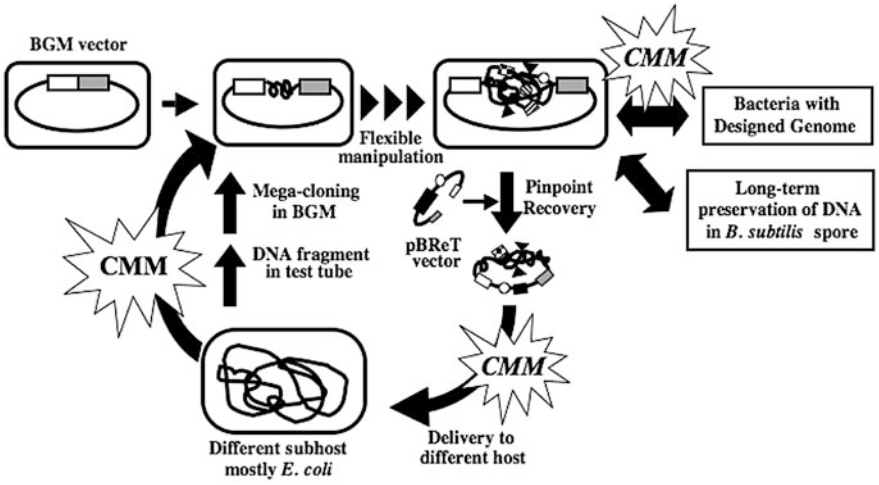

Fig. 4.5 The present CMM is featured in a production cycle using the *B. subtilis* genome (BGM) vector drawn based on Kaneko et al. (2005). Future possible applications for the CMM are shown in *italics*

from the most widely used host in molecular cloning, *E. coli*, was not accidental: we first observed it when an urgent need existed for the preparation of stable substrate for *B. subtilis* transformation. We believe that mimicking transformation-mediated DNA transfer, although to date it has only been established for one-way transfer from *E. coli* to *B. subtilis*, will contribute deeply to our understanding of HGT in general and during evolution. It also permits new approaches to the manipulation of bacteria that could be beneficial to humans, even though they are novel recombinant genomes produced through *B. subtilis* or yeast (Itaya et al. 2008; Gibson et al. 2009; Lartigue et al. 2009; Itaya and Kaneko 2010).

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Chapter 5
Extracellular Nucleic Acids of the Marine Phototrophic Bacterium *Rhodovulum sulfidophilum* and Related Bacteria: Physiology and Biotechnology

Yo Kikuchi

Contents

Abstract Extracellular nucleic acids of high molecular weight are present ubiquitously throughout the environment, such as seawater and soil. These nucleic acids were formerly thought to be derived from cells by cell death, but recent studies have shown that they are at least partly derived from the active release of nucleic acids from some bacterial cells. Marine phototrophic bacteria, *Rhodovulum sulfidophilum* and *Rhodovulum* sp. strain PS88, produce extracellular nucleic acids and form flocs, i.e., structured communities of cells. This chapter describes highly efficient extracellular nucleic acid production of *Rhodovulum* sp. strain PS88 and the physiological relationships between extracellular nucleic acids and flocculation in *Rdv. sulfidophilum*. The structures of extracellular soluble DNA and RNA of *Rdv.*

Y. Kikuchi
Department of Environmental and Life Sciences, Toyohashi University of Technology, 1-1 Hibarigaoka, Tempaku-cho 441-8580, Toyohashi, Japan
e-mail: kikuchi@tut.jp

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sulfidophilum are also described. Moreover, proposals for application of these properties to industrial productions of nucleotides and RNA drugs are discussed.

5.1 Introduction

Rhodovulum sulfidophilum is a purple phototrophic marine alphaproteobacterium that can grow by either anoxygenic photosynthesis or respiration with a wide variety of organic compounds as electron donors and carbon sources. This bacterium was first isolated from marine mud flats and named *Rhodopseudomonas sulfidophila* (Hansen and Veldkamp 1973). The name was subsequently changed to *Rhodobacter sulfidophilus* with the creation of the new genus *Rhodobacter* (Imhoff et al. 1984). In 1994, Hiraishi and Ueda proposed that all marine *Rhodobacter* species, but not the freshwater and terrestrial species, should be transferred to the new genus *Rhodovulum* based on their extensive investigation of DNA–DNA hybridisation and 16S ribosomal DNA sequence comparison of these strains (Hiraishi and Ueda 1994). *Rdv. sulfidophilum* is the type species of this genus. This is a gram-negative bacterium and cells divide by binary fission. The bacterium can grow under anaerobic conditions in the light or under aerobic conditions in the dark. The G+C content of the DNA ranges from 66.3 to 66.6 mol% (Hiraishi and Ueda 1994). Although unique features in the photosynthetic apparatus of the genus *Rhodovulum* have been reported (Masuda et al. 1999, 2000), only extracellular nucleic acids and related topics regarding these bacteria will be described here.

In this review, production, physiological roles, and structural analyses of extracellular nucleic acids of the genus *Rhodovulum* will be discussed. Furthermore, application of these bacteria to the production of mononucleotides and RNA drugs will be described.

5.2 Flocculation and Extracellular Nucleic Acid Production

Several strains of the genus *Rhodovulum* form structured communities of cells called flocs. Bacterial flocs have long been studied because of their importance for efficient wastewater treatment (Butterfield 1935). Flocculation usually causes sedimentation of cells. An example of cell sedimentation is shown in Fig. 5.1a (see also Sect. 5.2.2). Self-flocculating bacteria are very useful to achieve continuous wastewater treatment, as those are expected to be retained in activated sludge.

5.2.1 Rhodovulum *sp. Strain PS88*

Watanabe et al. (1998) isolated a photosynthetic bacterium, *Rhodovulum* sp. strain PS88, with flocculating ability from the sea sediment mud from shrimp cultivation

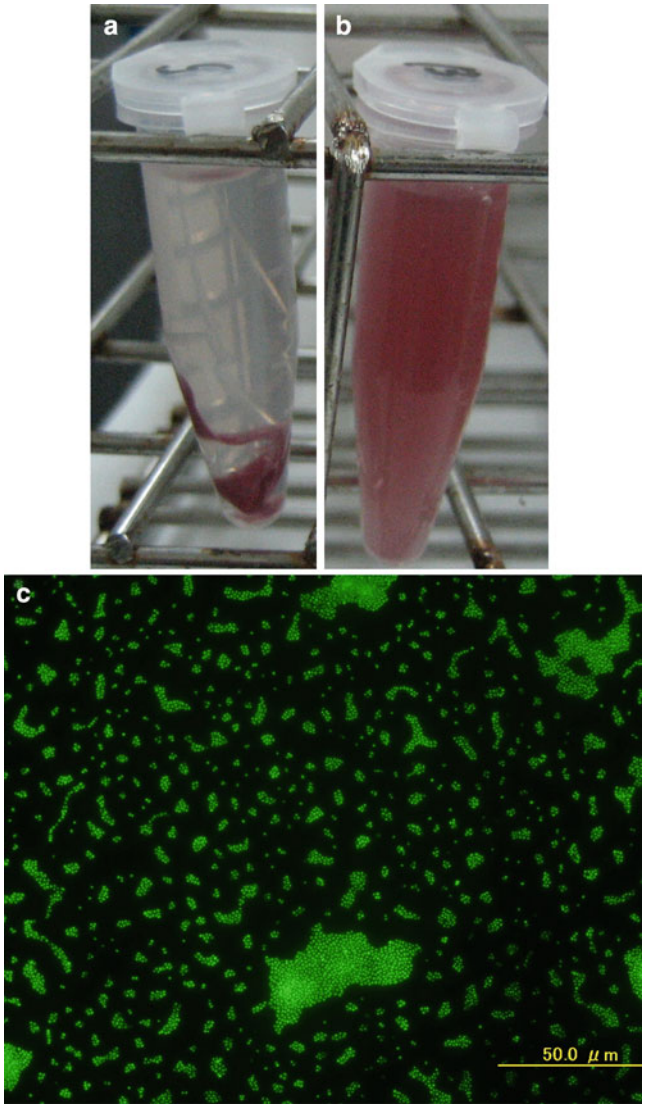

Fig. 5.1 Photographs of *Rhodovulum sulfidophilum*. (**a** and **b**) Cultivation tubes. *Rdv. sulfidophilum* DSM 2351 was cultivated in poor medium M5-M (**a**) and rich medium MB-M (**b**) under anaerobic conditions in the light at 25°C. After 90 h of incubation, each tube was gently shaken to remove cells from the inner wall of the tube. After standing for 5 min, photographs were taken. Sedimentation of cells (floc) was seen only in the tubes with poor medium (**a**). (**c**) Microscopic observation of cells stained with SYBR Green I. The *bar* indicates 50 μm. See text for full description

farms in Thailand. This bacterium forms floc and produces extracellular nucleic acids under aerobic conditions in the dark or anaerobic conditions in the light. This is the first report describing the flocculation and the extracellular nucleic acid

production of the genus *Rhodovulum* since its establishment. This bacterium flocculates in glutamate/malate medium (Lascelles 1956) and the flocculating ability is enhanced with increasing NaCl concentration.

Flocculation is usually quantified by comparison of cell mass in the top and bottom half fractions in the cultivation tube (Suzuki et al. 2009a; Watanabe et al. 1998). To obtain these two fractions, the cultivation tube is first gently shaken. The culture is left to stand for 5 min and then divided into the upper fraction (top half) and the lower fraction (bottom half). The protein concentrations of the fractions are used as the cell mass. Flocculation (F) is usually calculated as follows:

$$F(\%) = [(C_{LP} - C_{UP})/C_{TP}] \times 100$$

where C_{LP} and C_{UP} are the amounts of protein in the lower and upper fractions, respectively, and C_{TP} is the amount of total protein ($C_{LP} + C_{UP}$).

When cultivated in medium containing 5% NaCl, flocculation of *Rhodovulum* sp. strain PS88 reaches more than 70%. This strain produces an extracellular polymeric substance (EPS) consisting of polysaccharides, proteins, uronic acids, DNA and RNA. The yields of DNA, RNA and protein were reported to be 8.3, 62.5 and 48.5 mg/g dry cells, respectively. Although it has been reported that the EPS is extracted from the cell surface in flocs and that the nucleic acids are active in flocculation, almost 20% cell disruption during extraction of EPS has been reported (Watanabe et al. 1998). Therefore, it is possible that part of the yield includes the amounts of intracellular DNA and RNA. Although the nucleic acids have not yet been identified physiologically or biochemically, this high yield of nucleic acids is attractive for application of this strain to industrial nucleic acid production (described in Sect. 5.4). However, it has not been elucidated how the nucleic acids are produced extracellularly.

5.2.2 *Extracellular DNA and Flocculation of* Rhodovulum sulfidophilum

The relationship between extracellular nucleic acids and flocculation of *Rdv. sulfidophilum* has been described. Kikuchi and colleagues characterised the structures of extracellular nucleic acids themselves and the DNA release in *Rdv. sulfidophilum* (Ando et al. 2006; Suzuki et al. 2009a, b). For this study, *Rdv. sulfidophilum* DSM 2351 was used as this strain showed relatively rapid growth and the highest level of production of the extracellular nucleic acids among the strains of *Rhodovulum* species tested.

It has been reported that flocculation and release of extracellular DNA into the culture medium by *Rdv. sulfidophilum* DSM 2351 are strongly affected by the medium composition (Suzuki et al. 2009a). Two different media, M5-M and MB-M, were tested for cultivation of this strain. M5-M and MB-M are poor and rich media, respectively, because only the latter contains yeast extract. MB-M contains

0.5% sodium malate, 0.05% yeast extract, 0.1% ammonium sulphate, 2% sodium chloride, 1% basal salt solution, 0.1% vitamin solution and 20 mM sodium phosphate (pH 7.0) (Maki et al. 2003; Suzuki et al. 2009a). The composition of M5-M was the same as that of MB-M with removal of the 0.05% yeast extract. Cultivation of this strain was performed under anaerobic conditions in the light at 25°C (Suzuki et al. 2009a). These two media were very powerful tools for elucidation of the relationship between flocculation and extracellular nucleic acids of this organism.

First, Suzuki et al. (2009a) reported that flocculation (%) increased rapidly to 80% after 40 h of cultivation in poor medium (M5-M), and the floc formed was retained until 120 h of cultivation. In the rich medium (MB-M), however, almost no flocculation was observed during the whole 120-h cultivation period (Suzuki et al. 2009a). Photographs of the cultivation tubes of poor and rich media are shown in Figs. 5.1a, b, respectively. Flocculated cells were observed only at the bottom of the M5-M tubes (Fig. 5.1a), while no sedimentation was observed in the MB-M tubes (Fig. 5.1b). An example of microscopic observation of *Rdv. sulfidophilum* DSM 2351 is also shown in Fig. 5.1c. This is from 42-h culture of MB-M under anaerobic conditions in the light. Small flocs can be seen, although no sedimentation occurred under these conditions.

Suzuki et al. (2009a) also measured the amounts of extracellular DNA that were solubilised in the culture medium. The amount of this extracellular soluble DNA released from the cells at 120 h in culture reached 1.4 μg/mL in the rich medium, but only 0.2 μg/mL in the poor medium. This extracellular soluble DNA was not removed from the floc by artificial manipulation as described in the previous section for the PS88 strain, but it was easily prepared from the culture supernatant after removal of cells by centrifugation. Therefore, they referred to this preparation as extracellular "soluble" DNA.

They also found that the floc was degraded by deoxyribonuclease or ribonuclease treatment. Based on these experiments, they reported that the DNA is of primary importance for maintenance of the floc and that RNAs are also necessary for floc maintenance. The floc is maintained by DNA in the poor medium, but no floc is observed in the rich medium and extracellular soluble DNA is present in the culture medium. Table 5.1 shows a comparison of the values of flocculation and the amounts of extracellular soluble DNA at 90 h cultures from the poor and rich media. In the poor medium, 85% flocculation was observed, whereas the amount of extracellular soluble DNA was only 0.2 μg/mL. In contrast, in the rich medium, cultures with 4% flocculation contained 1.2 μg/mL of extracellular soluble DNA. These observations suggested that the DNA, which plays a role in floc maintenance in the poor medium,

Table 5.1 Flocculation (%) and the amount of extracellular soluble DNA under different cultivation conditions. The values are from 90-h cultures under anaerobic conditions in the light (Suzuki et al. 2009a; see text)

	Poor medium (M5-M)	Rich medium (MB-M)	Poor medium (M5-M) + α-cyclodextrin
Flocculation (%)	85	4	0
Extracellular soluble DNA (μg/mL)	0.2	1.2	0.8

may be released into the culture in the rich medium. The relationship between flocculation and extracellular soluble DNA formation was also observed in the experiment using a quorum sensing inhibitor (described in Sect. 5.2.3).

5.2.3 *DNA as the Cell-to-Cell Interconnecting Compound in Floc of* Rdv. sulfidophilum

Quorum sensing is a mechanism by which bacteria can monitor their population density through extracellularly produced signal molecules (DeKievit and Iglewski 2000). For a long time, bacteria were thought to be independent single-celled organisms with no communication among cells. However, cell-to-cell communication was found to exist in many bacteria (reviewed by DeKievit and Iglewski 2000). A single cell produces signal molecules called autoinducers, and all cells can detect their population density based on the concentration of these autoinducers. Through expression of the genes responsive to these autoinducers, bacteria behave collectively to survive. For example, migration to a more suitable environment, sporulation and biofilm formation are observed. In many gram-negative bacteria, the signal molecule involved in quorum sensing is acylated homoserine lactone (AHL) (DeKievit and Iglewski 2000). Cyclodextrins are known to form complexes with AHL in the bacterial culture medium and to show an inhibitory effect on quorum sensing (Ikeda et al. 2002). As a quorum sensing system has been reported in *Rhodobacter* (Puskas et al. 1997), a genus closely related to *Rdv. sulfidophilum*, a quorum sensing system was also expected to operate in *Rdv. sulfidophilum*. Suzuki et al. (2009a) used this inhibitor (α-cyclodextrin) to investigate whether quorum sensing is involved in floc formation of *Rdv. sulfidophilum*. This experiment provided supportive data for elucidation of the relationship between floc formation and extracellular soluble DNA production. They found that the quorum sensing inhibitor α-cyclodextrin did not affect floc formation but inhibited floc maintenance. In the presence of α-cyclodextrin in the culture medium, normal floc formation was observed during the initial 50 h of cultivation. After 60 h of cultivation (late log phase), however, the floc was degraded rapidly, whereas the floc is usually retained at least until 120 h of cultivation in poor medium in the absence of α-cyclodextrin. At 90 h of cultivation, no floc was observed. In contrast, the amount of extracellular soluble DNA increased in the presence of α-cyclodextrin. The increase in extracellular soluble DNA production occurred concomitant with the decrease in floc. The values of flocculation and soluble DNA level from 90-h cultures containing α-cyclodextrin are shown in Table 5.1. The amount of extracellular soluble DNA reached 0.8 μg/mL at 90 h in culture (Table 5.1) and 1 μg/mL at 140 h of cultivation (Suzuki et al. 2009a). This value is comparable to that (1.4 μg/mL) seen in rich medium (described in Sect. 5.2.2). These observations indicated that the DNA would be released from the floc by inhibition of floc maintenance. As described above, the floc was degraded by deoxyribonuclease treatment. Taken together, these experiments indicated that extracellular DNA is the primary cell-to-cell

interconnecting compound of floc formed by *Rdv. sulfidophilum*, although the DNA releasing mechanism from the floc during quorum sensing inhibition has not yet been determined.

5.3 Analysis of Extracellular Nucleic Acids

Extracellular nucleic acids of bacteria have long been known to exist and have some functions in natural genetic transformation or formation of biofilm and floc (Lorenz and Wackernagel 1994; Murakawa 1973; Nemoto et al. 2003; Okubo et al. 2006; Okubo and Hiraishi 2007; Sakka and Takahashi 1981; Steimoen et al. 2002; Vlassov et al. 2007; Whitchurch et al. 2002). However, the extracellular nucleic acids themselves had not been well characterised prior to 2005.

5.3.1 Extracellular RNAs of Rdv. sulfidophilum

Ando et al. (2006) first reported the detailed structures of extracellular RNAs of *Rdv. sulfidophilum*. They cloned and sequenced cDNAs of the extracellular RNAs, and showed that the extracellular soluble RNAs of *Rdv. sulfidophilum* DSM 2351 are mainly fully mature-sized transfer RNAs (tRNAs) and fragments of 16S and 23S ribosomal RNAs (rRNAs). These sequence data are available in the DDBJ with the accession numbers D16423 and AB201391 to AB201407 (http://www.ddbj.nig.ac.jp/searches-e.html). Ando et al. (2006) also reported that the RNA sequences were encoded on the genome of *Rdv. sulfidophilum* as expected, and at least eight modified nucleotides that are usually seen in tRNAs, e.g., 2′-O- methyladenosine, 2-methyladenosine, 6-methyladenosine, 7-methylguanosine, 2′-O-methylcytidine, 5-methyluridine, dihydrouridine and pseudouridine, were identified in both extracellular and intracellular RNAs. Based on these results, they concluded that extracellular and intracellular RNAs were the same and that extracellular RNAs may be released from the cells after being fully processed, including precursor RNA processing and base modification (Ando et al. 2006).

Their analysis also indicated that these extracellular soluble tRNAs were non-aminoacylated, although almost all intracellular tRNAs were aminoacylated (Suzuki et al. 2009b). It has not yet been determined where deaminoacylation of aminoacylated tRNAs occurs, or whether only non-aminoacylated free tRNAs are released from the cells. It is likely that non-enzymatic deaminoacylation of tRNAs occurs after release from the cells into the culture medium, because the pH value of the culture medium reached 7.6 and the half-life of the aminoacyl bond in aminoacylated tRNA at pH 7.5 and 25°C is almost 60 min (Pingout et al. 1977). Interestingly, however, many of extracellular tRNAs have a mature 3′-terminal CCA sequence without any modifications, such as aminoacylation (Ando et al. 2006; Suzuki et al. 2009b), although these sequences are usually very susceptible to

ubiquitous exonucleases. Indeed, Suzuki et al. reported that *Rdv. sulfidophilum* does not produce any ribonucleases in the culture medium, at least within a cultivation period of 90 h (Suzuki et al. 2009b, 2010). This property is very important in application of this organism to industrial nucleic acid production (see Sect. 5.4.2).

5.3.2 *Extracellular DNA of* Rdv. sulfidophilum

In the case of biofilm formation, *Pseudomonas aeruginosa* also produces extracellular DNA. The DNA functions as a cell-to-cell interconnecting matrix component in biofilm (Allesen-Holm et al. 2006; Whitchurch et al. 2002). By Southern blotting analysis and other methods, Tolker-Nielsen and colleagues reported that the extracellular DNA of *P. aeruginosa* is similar to whole-genome DNA (Allesen-Holm et al. 2006; see also Chap. 1 of this volume). Suzuki et al. adopted a similar strategy to characterise the extracellular DNA of *Rdv. sulfidophilum* (Suzuki et al. 2009a). They prepared DIG-labelled probes of five genes, *nifH*, *pufQ*, *regA*, *soxA* and the formyl methionine tRNA gene. These genes were thought to show the widespread locations on the genome from the data of the known genome of *Rhodobacter sphaeroides* (Mackenzie et al. 2001), which is closely related to *Rdv. sulfidophilum*. Using these probes, Southern blotting analyses were performed for extracellular soluble DNA and intracellular genomic DNA. The restriction fragments separated on gels by electrophoresis were probed with labelled sequences from the five genes. All genes tested were present in extracellular soluble DNA and the patterns of extracellular soluble DNA restriction fragments containing these genes were the same as those of the genomic DNA. If the genomic organisation of *Rdv. sulfidophilum* is similar to that of *Rba. sphaeroides*, these observations suggested that almost all regions of the genome are released from the cells into the extracellular environment (Suzuki et al. 2009a). This was also confirmed by Southern blotting analysis using whole extracellular soluble DNA as a probe. This probe hybridised to all of restriction fragments of the genomic DNA separated by pulsed field gel electrophoresis (Suzuki et al. 2009a). These results indicated that the extracellular soluble DNA is almost the same as whole genomic DNA.

5.3.3 *Mechanism for Release of Extracellular DNA and RNA of* Rdv. sulfidophilum

Kikuchi and colleagues concluded that both the DNA and RNA produced in culture medium by *Rdv. sulfidophilum* originated from intracellular nucleic acids of this organism. As the extracellular soluble DNA production increased with quorum sensing inhibitor (Suzuki et al. 2009a; see Sect. 5.2.3), the release of nucleic acids into culture medium seems to be controlled by quorum sensing either directly or indirectly. It has been reported that bacterial DNA release controlled

by quorum sensing is caused by partial cell lysis, because cytoplasmic enzymes, such as β-galactosidase, are released from the cells concomitantly with the DNA (Steimoen et al. 2002). Suzuki et al. also observed that intracellularly expressed β-galactosidase and artificial RNAs were released with natural DNA and RNA from the cells (Suzuki et al. 2009a, 2010; Suzuki and Kikuchi unpublished results). From these results, it seems likely that the extracellular nucleic acids of *Rdv. sulfidophilum* are produced by controlled lysis of a subpopulation of the bacteria.

5.4 Biotechnology

5.4.1 Utility as a Nucleotide Source

Pure crystals of 5′-ribonucleotide salts, such as sodium salt of 5′-guanylic acid or 5′-inosinic acid, have umami taste (palatability) and are used as flavour enhancers in food production and cooking (Kuninaka 1960). Other ribonucleotides are also important sources of chemotherapeutic agents (Perigaud et al. 1992). At present, these nucleotides are industrially produced mainly by enzymatic hydrolysis of yeast RNA (Kuninaka et al. 1959; Olmedo et al. 1994). RNAs from *Saccharomyces*, *Candida* and *Torula* species are used.

Noparatnaraporn et al. (2000) proposed that *Rhodovulum* sp. strain PS88 can be used for practical RNA production. They reported that the maximum RNA production was 460 mg RNA/L of broth when this strain was cultivated continuously in a 1-L jar-fermenter under aerobic conditions in the dark with acetate medium containing 3% NaCl (Noparatnaraporn et al. 2000; Watanabe et al. 1998). This value includes intracellular RNA and extracellular insoluble RNA (in the EPS, see Sect. 5.2.1). Under these conditions, they also found 66.5 mg of extracellular soluble RNA/L of broth, which is about 2–3 times higher than the level of yeast cells. *Rhodovulum* sp. strain PS88 is now expected to be useful as an RNA source.

5.4.2 RNA Drug Production by Engineered Plasmids

As *Rdv. sulfidophilum* produces extracellular RNA, the extracellular production of RNAs of defined sequence using this bacterium has been proposed (Kikuchi et al. 2007; Suzuki et al. 2010). In Sect. 5.4.1, a strain of *Rhodovulum* was suggested to be useful for production of mononucleotides, but *Rdv. sulfidophilum* may be useful as a source of RNAs of artificially designed sequence if this bacterium can be genetically manipulated.

Recent studies have shown that many small RNAs are key players in the regulation of gene expression and that higher order structures in RNA sequences, such as riboswitches or ribozymes, act as regulators of expression (Breaker 2004; Chu and Rana 2007; Nellen and Hammann 2005). In addition to these natural RNA

functions, artificial siRNAs, ribozymes and RNA aptamers are also expected to be potential candidates for RNA therapeutics (Khan 2006; Que-Gewirth and Sullenger 2007). An RNA aptamer has already been developed as an RNA drug for inhibition of macular degradation by specifically targeting the vascular endothelial growth factor (Ng et al. 2006). In both basic studies of RNA and RNA drug production, efficient methods for preparation of homogeneous RNA molecules are very important. Especially, in the case of RNA drug production, highly economically efficient production in large quantities may be required. Such RNAs have been prepared by in vitro transcription (Milligan et al. 1987) or chemical synthesis (Marshall and Kaiser 2004). However, as these methods require use of expensive enzymes, synthetic DNA templates and chemicals, they are costly and labour intensive and are not suitable for preparation of RNAs in large quantities. The production of artificial RNAs in vivo using microorganisms has great potential. The in vivo expression and preparation of artificial RNAs have been reported previously (Ponchon and Dardel 2007; Umekage and Kikuchi 2009). Ponchon and Dardel (2007) reported in vivo production of recombinant RNAs using *Escherichia coli*. They proposed a system called a "tRNA scaffold" in which the RNA products were designed to be included in a tRNA structure to avoid degradation by endogenous ribonucleases. This was successful, but the products contained the flanking sequences of tRNA on both sides. Umekage and Kikuchi (2009) developed a method for in vivo production of circular RNAs using *E. coli*. The circular products are stably maintained in the cells, because the molecules are resistant to cellular exonucleases. With the use of *E. coli* as the host, it is always necessary to devise a method, such as tRNA scaffold or circularisation, to avoid degradation by cellular nucleases.

Kikuchi and colleagues proposed a new method for in vivo production of artificial RNAs using *Rdv. sulfidophilum* (Kikuchi et al. 2007; Suzuki et al. 2010). As described in Sect. 5.3.1, *Rdv. sulfidophilum* does not produce any ribonucleases in the culture medium (Suzuki et al. 2009b, 2010). Therefore, most of the tRNAs released into the culture medium retain the mature non-aminoacylated 3′-terminal CCA sequence (Suzuki et al. 2009b), although these sequences are very susceptible to ubiquitous exonucleases, as described in Sect. 5.3.1. These observations suggested that *Rdv. sulfidophilum* may be useful for fermentative extracellular production of artificial RNAs. For this purpose, they used *Rdv. sulfidophilum* DSM 1374^{T}, because gene manipulation methods in this strain have been well established (Masuda et al. 1999, 2000). The concept of the proposed artificial and functional RNA production method is shown schematically in Fig. 5.2.

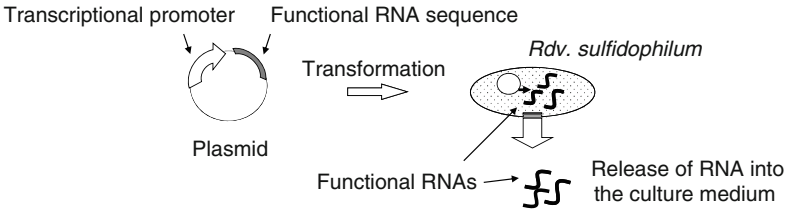

Fig. 5.2 Extracellular production of artificial and functional RNAs by *Rdv. sulfidophilum*

They developed a method for extracellular production of an RNA aptamer (Kikuchi et al. 2007; Suzuki et al. 2010). A streptavidin RNA aptamer (Srisawat and Engelke 2001) was chosen as an RNA drug product model. They designed a plasmid to produce a streptavidin RNA aptamer directly in the culture medium using *Rdv. sulfidophilum* DSM 1374^T. The designed aptamer sequence was flanked on both sides by self-cleaving hammerhead ribozyme sequences (Forster and Symons 1987). The hammerhead ribozyme sequence is a self-cleaving sequence originating from plant virusoids. This self-cleavage is an indispensable step for virusoid maturation and occurs at a specific site on the RNA sequence without the help of any protein enzyme (Forster and Symons 1987). This artificial gene was designed to be transcribed by the rRNA promoter (*rrn*) (Suzuki et al. 2010) and *puf* terminator (Masuda et al. 1999, 2000) of *Rdv. sulfidophilum*. Using *Rdv. sulfidophilum* harbouring this plasmid, 100 ng of streptavidin RNA aptamer/L of culture was produced extracellularly under aerobic conditions in the dark (Suzuki et al. 2010). The product RNA was tested for function, and it was demonstrated that the RNA produced retained its specific binding ability to streptavidin. No differences were detected in this binding function between this in vivo product and the product of in vitro transcription (Suzuki et al. 2010). This was the first demonstration of extracellular production of a functional artificial RNA in vivo. The product was released into the extracellular environment. This property makes it possible to develop a continuous production system in one vessel because collection and disruption of the cells are not necessary to obtain the product. The yield was not yet practical even for laboratory use, but several approaches are expected to yield improvements. High copy number of the plasmid vector and strong promoters may be effective. As described in Sect. 5.2.1, *Rhodovulum* sp. strain PS88 produces extracellular natural RNAs in a yield of 62.5 mg/g dry cells (Watanabe et al. 1998). This genus has great potential for RNA production in large quantities. For industrial production of RNA drugs, extracellular production may be useful for the future development of an efficient process engineering system using continuous cultivation. As *Rdv. sulfidophilum* produces almost no ribonucleases, further physiological investigations and optimisation of the conditions will lead to the future development of RNA drug production systems.

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Chapter 6
Systemic RNAi in *C. elegans* from the Viewpoint of RNA as Extracellular Signals

Lisa Timmons

Contents

Abstract The elegantly simple organism *Caenorhabditis elegans* has played an important role in the RNAi field. In 1997, the first RNAi experiment was performed in this nematode by engineering a specific sequence of double-stranded RNA (dsRNA) and injecting the molecules into the organism. Subsequently, an RNAi response was noted not only in the treated animal but also in its progeny. In later experiments that utilized Green Fluorescent Protein to assess RNAi in individual cells, systemic RNAi phenocopies were observed in most nonneuronal tissues. These early observations of systemic RNAi revealed a hitherto unknown feature of RNA molecules: an ability of locally delivered dsRNAs to traffic to distant somatic and germline cells and to gain entry into those cells – an ability not generally observed for structurally similar DNA molecules. Understanding the precise nature of the RNA molecules that facilitate RNAi as well as the cellular mechanisms that respond to them will provide insights into the unusually robust nature of RNAi in *C. elegans* as well as highlight potential trafficking routes for endogenous RNAs.

L. Timmons
Department of Molecular Biosciences, University of Kansas, Lawrence KS, USA
e-mail: timmons@ku.edu

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6.1 Introduction

The cuticle of *Caenorhabditis elegans* serves as a tough exoskeleton that maintains morphology, facilitates movement, protects against osmotic shock or dehydration, and is important for interaction and protection from other environmental factors (Johnstone 1994). In the laboratory, the cuticle is a formidable barrier that prevents access of molecules such as drugs, DNA, or RNA to the cells of the animal. When cells are exposed to dsRNA, a potent gene silencing, or RNAi, response is mounted against nucleotide sequences identical to the dsRNA. The cellular mechanisms that facilitate an RNAi response against dsRNA also function in cellular strategies that protect against viruses and other pathogens (Fritz et al. 2006; Lu et al. 2005). Thus, RNAi is considered an antiforeign genome response, and, by implication, dsRNA is an abnormal molecule against which cells actively survey and respond. Thus, the *C. elegans* cuticle serves as a passive defense against the entry of foreign genomes, including dsRNA.

Microinjection is a common methodology that is used to deliver molecules past the cuticle and into the tissues of the animal, and microinjection was the first delivery method utilized to get dsRNA into the organism (Fire et al. 1998). Since then, three additional dsRNA delivery methods have gained prominence in the *C. elegans* field (Fig. 6.1). Two methods rely on the worm to ingest dsRNA and likely allow the organism to acquire dsRNA in the same manner as nutrients are acquired. The ingestion-based delivery protocols are vernacularly referred to as *soaking* and (bacterial) *feeding* methods. In *soaking* experiments, animals are placed in an aqueous solution of dsRNA, and ingestion of the solution results in an RNAi response (Tabara et al. 1998). The *feeding* method takes advantage of the fact that *C. elegans* normally feeds upon *E. coli* in the laboratory. When bacteria are engineered to express dsRNA corresponding in sequence to a worm gene, animals reared on such food can display an RNAi phenocopy (Timmons et al. 2001; Timmons and Fire 1998). DNA-based transgenes can also be used to deliver dsRNA to the animal. Transgenic *C. elegans* animals can transcribe dsRNAs from *C. elegans* promoters, allowing for in vivo transcription of dsRNAs (Hull and Timmons 2004; Tavernarakis et al. 2000; Timmons et al. 2003). In all the different methods of dsRNA delivery, *injection, soaking, feeding,* and *transgene* delivery, systemic RNAi responses can be observed in the treated animal and in its progeny. While the systemic nature of RNAi is remarkable in this organism, a few cells, notably some neuronal cells, have an intrinsic resistance to RNA (See Sect. 6.3.2).

6.2 Systemic RNAi and Trafficking Pathways Associated with Method of Delivery

The numerous and convenient dsRNA delivery methods utilized in various *C. elegans* laboratories raise a pertinent question: Do the different delivery methods depend upon identical sets of cellular dissemination and response mechanisms in order for

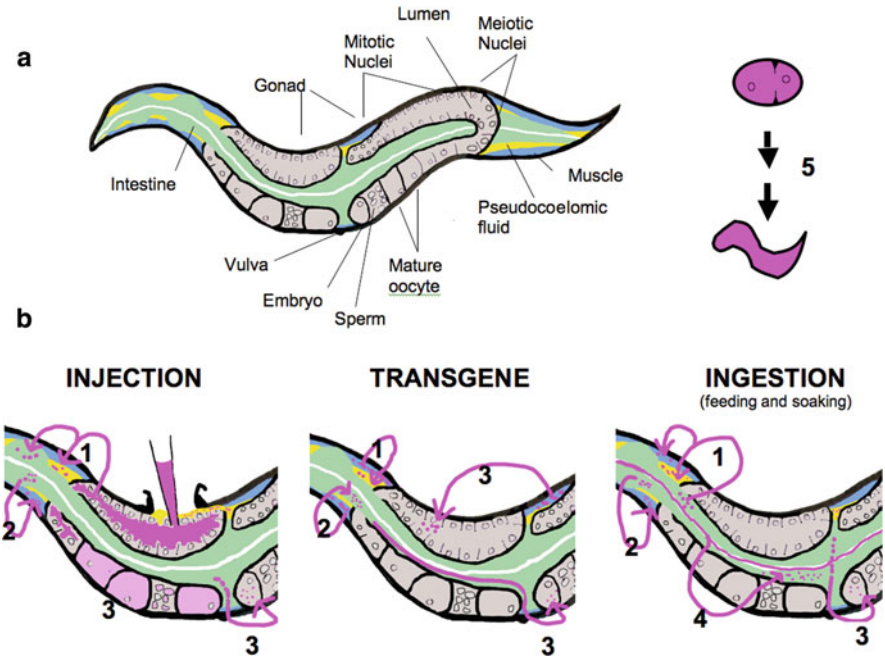

Fig. 6.1 (**a**) The free-living nematode *C. elegans* is a multicellular hermaphrodite with some cells organized into organs that are surrounded by basement membrane. Tissues depicted here include the intestine (green cells surrounding a white lumen), somatic muscle (*blue*), and gonad (*gray*). Cells in the postembryonic animal derive nutrients and oxygen from the pseudocoelomic fluid (*yellow*). The gonad is a multicellular structure that leads to the production of approximately 300 oocytes and fewer numbers of sperm (Hubbard and Greenstein 2005). Meiosis is an ongoing process in adults, and all stages of meiosis can be observed in a single gonad. Germline precursor cells first divide mitotically, proceed into meiosis, and later develop into mature oocytes. The immature germline nuclei are incompletely enclosed by plasma membrane; thus, the gonad is a syncytium of germline nuclei. (**b**) Four different methods allow for delivery of dsRNA into the organism: *injection, transgene-based transcription,* and the ingestion-based methods, *feeding* and *soaking*. The pink color depicts dsRNAs that are delivered experimentally, as well as their metabolic products (RNA silencing molecules). The numbers and associated pink arrows highlight possible trafficking routes for RNA silencing molecules. In the representative transgene-delivery experiment, dsRNA is transcribed from a muscle-specific promoter (*blue tissue*). (1) indicates potential pathways that move RNAs through the pseudocoelom. (2) indicates a potential pathway that involves movement from one cell directly into another. The pathway that directs RNA silencing molecules to the germ line (3) is predicted by the fact that the progeny of treated animals can display RNAi defects. The ingestion-based *feeding* and *soaking* methods reveal a trafficway that requires uptake of dsRNAs into intestinal cells, indicated in (4). A potentially distinct systemic mechanism that allows a fertilized, dsRNA-laden embryo to develop into an adult with a systemic RNAi phenocopys indicated in (5).

a systemic RNAi phenocopy to be elicited? A first step in addressing this question is to look at possible trafficking routes that may be taken by dsRNA molecules and to compare how those routes are affected in the different delivery methods.

6.2.1 *Systemic and Heritable Nature of RNAi in Response to Injected dsRNA*

C. elegans does not have a closed circulatory system. Fluid that fills the pseudocoelomic cavity provides a means for nutrient and oxygen transport and intercellular communication. The pseudocoelomic cavity arises during embryogenesis, lies beneath the body wall of the organism, and is bounded by the hypodermis, neuronal cells, the gonad, and the intestine (Fig. 6.1b). The pseudocoelomic fluid bathes the basal surface of most tissues in the animal; therefore, molecules in the fluid can easily gain access to cells. Tissues such as the intestine and gonad also have a fluid-filled lumen. The contents of the intestinal lumen consist of digested bacteria and other environmental matter, and the entry of molecules from the lumen into intestinal cells is regulated. Barrier junctions prevent mixing of the pseudocoelomic fluid in the body cavity with the luminal contents of the intestine. These barrier junctions also likely prevent the direct movement of ingested dsRNAs from the intestinal lumen into the pseudocoelomic fluid. Molecules required during oocyte and early embryonic development are commonly trafficked from somatic cells into the gonad in a regulated fashion, and these molecules are separated from the pseudocoelomic fluid by barrier junctions (described in Hall and Altun (2008) and Fig. 6.1).

When dsRNA is *injected* into *C. elegans*, the microinjection needle is typically inserted into the lumen of the gonad or the intestine (Fig. 6.1b). Irrespective of where the needle is placed in the animal, systemic RNAi phenocopies are observed in both the treated animal and in the progeny (Fire et al. 1998). Observations of systemic RNAi in the treated animal indicate an ability of dsRNA (or RNA silencing signals derived from the input dsRNA) to travel to distant cells, gain entry into those cells, and trigger RNAi responses in most cells. Because tissues are torn when the microinjection needle is inserted, it may be the case that dsRNA molecules simply enter the body cavity through the broken tissue barriers. The RNA molecules might then travel through the pseudocoelomic fluid, thereby gaining access to cells in the same manner as nutrients (Fig. 6.1b(1)). In another model for trafficking of RNA silencing molecules, subsequent to the cellular entry of dsRNAs, a relay transfer mechanism might allow for trafficking of RNAs from one cell directly into another (Fig. 6.1b(2)). While such models are not mutually exclusive, in both cases, a mechanism that allows for the entry of dsRNA into somatic cells is required for a fully systemic RNAi phenocopy.

Observations of systemic RNAi in progeny highlight potential trafficking routes taken by dsRNA that lead from the site of *injection* to the germline cells of the injected animal (Fig. 6.1b(3)). Due to the architecture of the gonad, dsRNA that is injected directly into the lumen has direct access to germline cells. The immature germline nuclei are not completely surrounded by plasma membrane in the distal part of the gonad, and luminal dsRNA may contact and intermix with germline cytoplasm. Thus, injection of dsRNA into the gonad allows for delivery of dsRNA into cells, albeit haploid oocytes. In these kinds of microinjection experiments,

systemic RNAi responses are typically observed in many progeny (over 100), and these responses can persist into the adult stage (Fig. 6.1(5)).

It is not altogether surprising that RNAi phenocopies are observed in the progeny of dsRNA-injected animals: *C. elegans* can be injected with a large number of dsRNA molecules and only about 10 rounds of cell division are required to produce an adult animal (Sulston and Horvitz 1977; Sulston et al. 1983). Thus, the large dose of dsRNA in a fertilized embryo may not be diluted to ineffectiveness by embryonic cell divisions. Furthermore, even when more dilute solutions of dsRNA (~10 dsRNA molecules per cell) were injected into the gonad, RNAi phenocopies were still observed in progeny animals (Fire et al. 1998). This latter result highlighted the potency of the RNAi mechanism and predicted the presence of mechanisms that amplify the RNAi response (See Sect. 6.3.1.2). Even more remarkable is the observation that, for some genes, RNAi phenocopies can persist into the seventh postinjection generation (Alcazar et al. 2008; Vastenhouw et al. 2006).

Distinct mechanisms may be required to allow for the distribution and maintenance of RNA silencing molecules during development (Fig. 6.1(5)). In theory, developmental RNAi mechanisms may involve a continuous trafficking of RNA silencing molecules from cell to cell; alternatively, mitosis-related mechanisms that distribute contents to daughter cells may allow the RNAi response to be maintained from the time of exposure in the germ line to adulthood.

6.2.2 *Systemic Nature of RNAi in Response to Transgene Delivery of dsRNA*

Transgenes can be configured to express dsRNAs in *C. elegans*. There are two basic cloning strategies used in order to generate plasmids that are capable of expressing dsRNA in *C. elegans* (Fig. 6.2a). In a two-stranded strategy, a *C. elegans* promoter sequence is placed upstream of the coding region of the gene to be silenced. A second construct drives expression of the antisense strand (Fig. 6.2a). In this strategy, sense and antisense RNA strands are expressed in the same cell and anneal in vivo. A second strategy produces an RNA that can fold back upon itself into a double-stranded hairpin (Fig. 6.2b). Hairpins are transcribed from a single *C. elegans* promoter that drives expression of two copies of the target gene oriented in inverted-repeat configuration. Transgene delivery of dsRNAs can elicit RNAi in the cells in which the dsRNA is transcribed, provided that the cell is RNAi-competent (Fig. 6.2c).

When ubiquitously expressing promoters are used, a systemic RNAi response can be elicited in those cells that have the capacity to perform RNAi (Tavernarakis et al. 2000). While this is a particularly convenient means of generating large numbers of RNAi-affected animals, the observation that RNAi is systemic in this experimental system is not particularly surprising, as dsRNA is transcribed directly in each cell. However, when *C. elegans* promoters that normally drive tissue-specific expression are used, systemic RNAi responses can also be observed. The use of the *green fluorescent protein* gene effectively demonstrates these results:

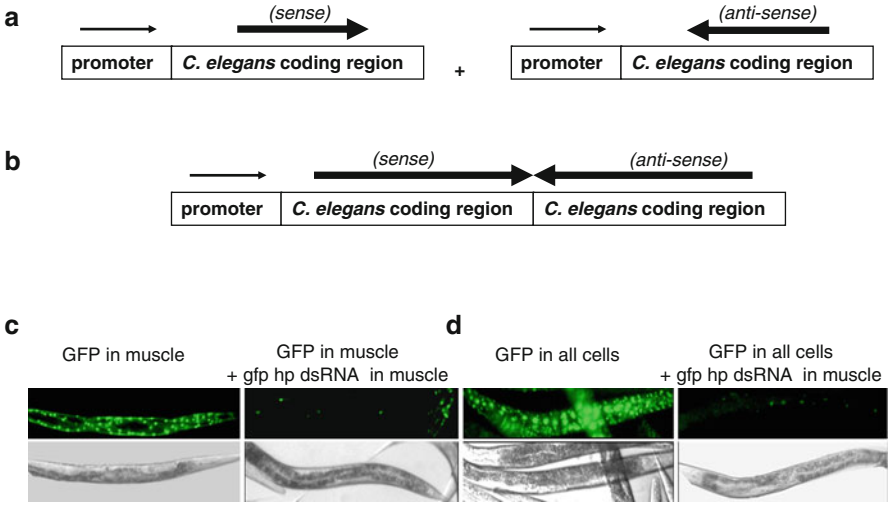

Fig. 6.2 Common configurations that allow for transcription of dsRNAs in transgenic animals. (**a**) A two-plasmid system produces sense and antisense RNA strands that anneal in vivo. Both plasmids are injected, and both are subsequently incorporated into the same multicopy transgene array by the animal. (**b**) A single-plasmid system produces a hairpin dsRNA. (**c**) An example of RNAi induced in muscle cells by a hairpin dsRNA expressed in muscle cells. (**d**) An example of systemic RNAi observed in response to dsRNA expressed in muscle cells. In both (**c**) and (**d**), *green fluorescent protein* sequences are used as both trigger and target

doubly transgenic animals can be generated that express both GFP reporter mRNA as well as dsRNA corresponding in sequence to *gfp* (Fig. 6.2c) (Timmons et al. 2003; Winston et al. 2002). In such experiments, GFP fluorescence serves as an indirect readout of RNAi activity that is observable in all cell types of this transparent organism.

Empirically, the fact that systemic RNAi phenocopies are observed when dsRNA is transcribed in specific tissues leads to two hypotheses: *one*, that RNA silencing molecules might be able to traffic directly from one cell to another (Fig. 6.1b(2)); or *two*, that dsRNA or related RNA silencing molecules can exit the cells in which they are transcribed and enter distant cells by traveling through the pseudocoelomic fluid (Fig. 6.1b(1)). These models are not mutually exclusive. Furthermore, transgene delivery of dsRNA provides evidence that cells have an ability to take up RNA silencing molecules as cells that are not transcribing dsRNA display RNAi phenocopies. In wild-type animals, an RNAi response requires the presence of the dsRNA-expressing transgene; multigenerational responses are not typically observed in wild-type progeny that fail to inherit the transgene (Fig. 6.1b (3)). Interestingly, in vivo transcription of dsRNA in neuronal cells does not always improve the ability of these cells to mount an RNAi response, an indication that the RNAi resistance in neurons is not due to a dsRNA delivery problem (See Sect. 6.3.2).

6.2.3 *Systemic RNAi in Response to Ingestion of dsRNA*

Soaking and *feeding* methods allow for the ingestion of dsRNAs by *C. elegans,* and such treatment can result in systemic RNAi phenocopies (Tabara et al. 1998; Timmons and Fire 1998). RNAi can be observed in treated animals as well as in progeny, and this especially the case in soaking experiments where concentrated solutions of dsRNA can be utilized. Again, it is possible to make specific predictions regarding trafficking routes for dsRNA in this organism (Fig. 6.1b). Intestinal cells must first take up dsRNA from digested material in the lumen of the gut (Fig. 6.1b(4)). Intestinal cells can subsequently display an RNAi response, yet they are not the only somatic cells that are affected when soaking and feeding methods are used; therefore, intestinal cells must have the capacity to release RNA silencing molecules. The RNA molecules may traverse from cell-to-cell directly (Fig. 6.1b (2)), or silencing RNAs may first be extruded into the pseudocoelomic fluid by the intestinal cells (Fig. 6.1b(1)). A specific and unidentified trafficking route may result in extrusion of RNA molecules from intestinal cells; alternatively, RNA molecules might enter the intestinal cell by fluid-phase endocytosis and exit from the basal surface using transcytosis mechanisms, thereby gaining access to the pseudocoelomic fluid and adjacent cells. Because the animals remain intact when ingestion methods are utilized and because progeny can also display an RNAi phenocopy, ingestion methods highlight the remarkable capacity for germline cells to take up silencing molecules (Fig. 6.1b(3)).

6.3 RNA Silencing Mechanisms in *C. elegans*

The use of GFP in RNAi experiments has allowed investigators to indirectly follow a trail of RNA silencing molecules from untreated tissue to affected cells. However, trafficking pathways are not easily elucidated even in this relatively simple creature. One complicating factor is that the dsRNA introduced into the organism is metabolized into other forms of RNA. Each metabolic form of RNA may traffic in a different manner and may be recognized by distinct RNA-binding proteins within the cell. The ability of each of the products of dsRNA metabolism to move systemically has not been fully investigated. Furthermore, we do not yet know the capacity of different cell types to respond to each of these metabolic forms. Thus, a complete elucidation of systemic RNAi mechanisms and the RNA silencing molecules that trigger their function will require a better understanding of RNA metabolism as well as tissue-specific aspects of RNA silencing mechanisms.

After dsRNA gains entry into the cell, the first few steps taken by the cell involve the action of Dicer and RISC (Filipowicz 2005; Jaskiewicz and Filipowicz 2008).

In higher organisms, a long (>30 bp) sequence of dsRNA can trigger the interferon pathway, which normally provides an antiviral protection that can lead to global cellular mRNA degradation and cell death. *C. elegans* lacks components

of the interferon pathway, and this gives researchers more flexibility, in that longer dsRNA molecules can be utilized to trigger RNAi. By design, the dsRNA molecule is engineered to have the same sequence as the target mRNA. Within the cell, long dsRNAs first trigger the activity of Dicer, a dsRNA-specific endonuclease, which converts the dsRNA molecules to small interfering RNAs (siRNAs) that are roughly 22 bp in length (Fig. 6.3) (Hamilton and Baulcombe 1999; Knight and Bass 2001). One strand of the siRNA, the guide strand, is preferentially stabilized in the cell. The guide strand is transferred to a multiprotein enzyme complex with mRNA cleavage capacity known as RISC (for RNA-induced Silencing Complex). The sequence information in the guide strand is utilized by RISC to cleave specific mRNAs with sequence complementarity, and this occurs in the cytoplasm (Montgomery and Fire 1998; Montgomery et al. 1998; Tijsterman and Plasterk 2004). The products of Dicer and RISC activity are used as substrates in other RNA silencing mechanisms: For example, the mRNA that is targeted by RISC is used as a template by RNA-dependent RNA polymerases (RdRPs) for the production of secondary siRNAs. This amplified pool of secondary siRNAs then participates in additional RISC-based mechanisms and in gene silencing mechanisms that act at the transcriptional level (See Sect. 6.3.1.1; Fig. 6.3).

When dsRNA is introduced into organisms, the cells are poised to respond. Indeed, some RNAi-related enzymatic reactions, such as RISC-mediated mRNA decay, can be recapitulated in cellular extracts (Aoki et al. 2007; Hammond et al. 2000; Wang et al. 2009; Zamore et al. 2000). The extent to which RNAi responses are conserved and the immediate nature of the RNAi response to exogenous dsRNA led to the speculation that RNAi and related mechanisms might be used for essential, ongoing, endogenous activities in cells. This hypothesis was quickly borne out from phenotypic observations of RNAi-defective mutants and the functional identification of endogenous RNAs that are not translated into protein (noncoding RNAs, or ncRNAs) (reviewed in Boisvert and Simard (2008); Grishok (2005); Grishok and Mello (2002)).

The dsRNA response mechanisms referred to as RNAi are part of a larger collection of cellular silencing activities known as "RNA silencing" that normally respond to endogenous ncRNAs. A large number of ncRNAs that exist in the *C. elegans* genome have been identified from deep sequencing and from computational approaches (Ambros et al. 2003; Claycomb et al. 2009; Grad et al. 2003; Gu et al. 2009; Lagos-Quintana et al. 2001; Lau et al. 2001; Lee and Ambros 2001; Lim et al. 2003; Ohler et al. 2004; Ruby et al. 2006). Based on their structure or function, these ncRNAs can be divided into different subclasses, such as microRNAs, endogenous siRNAs, 21U-RNAs, 21G-RNAs, and 22G-RNAs. The different classes of ncRNAs are utilized in different RNA silencing pathways, and their activities have important roles in development, metabolism, tumorigenesis, chromosome organization, and cell death.

Each RNA silencing pathway is composed of distinct protein complexes that typically include an Argonaute family member. Argonaute proteins can bind to a specific silencing RNA (a "trigger" or guide RNA)(Joshua-Tor 2006; Siomi and

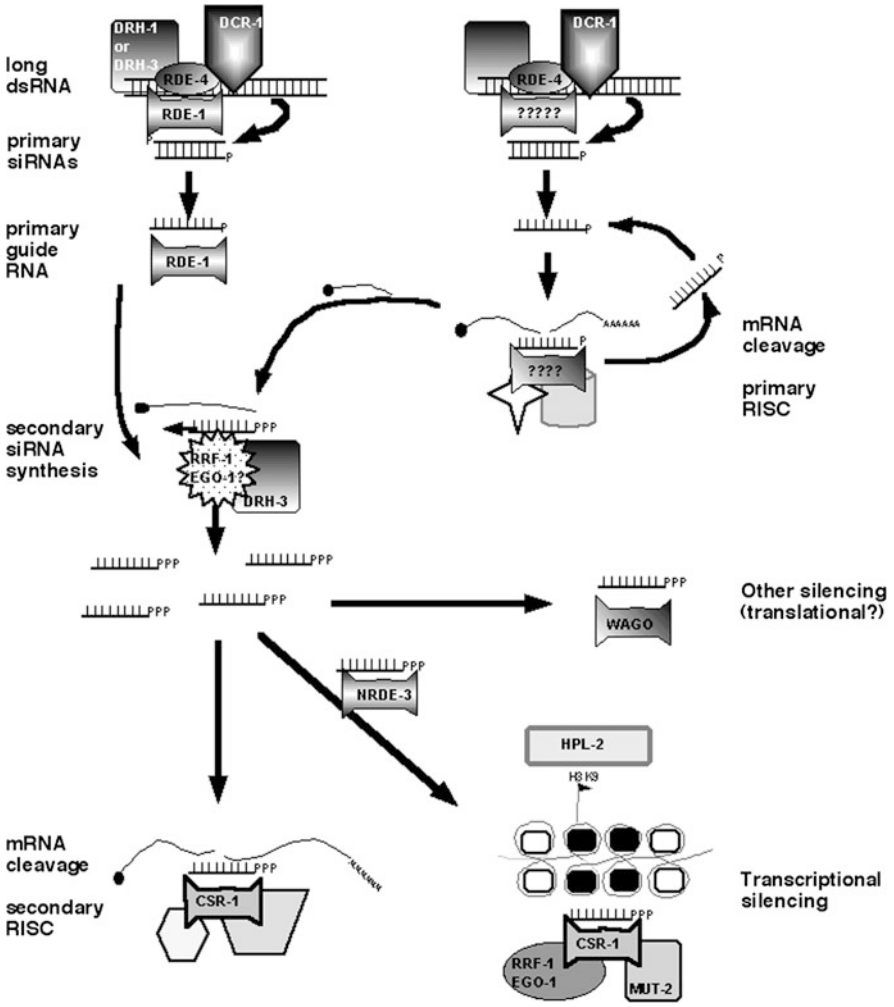

Fig. 6.3 A model of a small part of the broad set of RNAi mechanisms in *C. elegans*, adapted from references herein. The *leftmost part* of the figure is the classical RNAi pathway that responds to exogenous delivery of dsRNAs, leading first to the production of primary siRNAs from the input long dsRNAs. RdRP-dependent de novo amplification into secondary siRNAs, by EGO-1 and RRF-1 in the germline and RRF-1 in somatic cells, is dependent upon an mRNA template, which is presumed to be selected by RISC; the Argonaute protein involved in mRNA selection and degradation has not been definitively identified. Secondary siRNAs are bound by the Argonaute protein CSR-1, which has the capacity to cleave mRNAs. CSR-1 has also been demonstrated to function in the nucleus in endogenous transcriptional silencing events; while the Argonaute protein NRDE-3 is believed to facilitate shuttling of silencing RNAs into the nucleus

Siomi 2009) (Fig. 6.3). The sequence information in the bound RNA imparts specificity with respect to the gene being regulated, while distinct protein components in the Argonaute complex determine the nature of the silencing action.

For example, some Argonaute proteins are components of RISC where they are responsible for mRNA cleavage, whereas other Argonaute proteins direct activities that include translational arrest and transcriptional inhibition. Most Argonautes and associated RNA silencing components respond both to environmental dsRNAs and to endogenous ncRNAs, whereas a few components respond exclusively to environmental dsRNA. For example, the *C. elegans* Argonaute protein CSR-1 responds both to exogenous and endogenous RNAs, whereas the Argonaute protein RDE-1 associates with Dicer and apparently responds exclusively to exogenous RNA (Aoki et al. 2007; Steiner et al. 2009; Tabara et al. 1999; Yigit et al. 2006). Dicer is another example of an RNA silencing component that functions in both exogenous and endogenous RNA pathways; Dicer complexes that contain RDE-1 respond to exogenous dsRNAs, whereas distinct Dicer complexes lacking RDE-1 are required for processing of ncRNAs from primary transcripts into functional forms.

6.3.1 The Nature of Silencing Molecules in C. elegans

A systemic response to dsRNA in *C. elegans* is facilitated by RNA silencing pathways that also respond to endogenous ncRNAs. dsRNA introduced into the organism is metabolized into different classes of silencing RNAs that may compete with endogenous RNAs for the attention of the RNAi machinery. A better understanding of the RNA silencing pathways that respond to dsRNA and of the nature of the RNA molecules that are utilized by the pathways is required in order to completely elucidate systemic RNAi processes.

6.3.1.1 RNA Silencing Molecules Associated with Experimental Delivery of dsRNA

Dicer converts long dsRNAs into primary siRNAs within the cells of the animal, and systemic RNAi has been observed in response to delivery of both long dsRNAs and primary siRNAs. These observations were made possible by the use of *rde-4* and *rde-1* mutants. RDE-4 and RDE-1 have both been isolated in a protein complex with Dicer (Tabara et al. 2002). *rde-4* mutants are defective in the processing of long dsRNAs into siRNAs, and *rde-1* mutants accumulate siRNAs but are nonetheless strongly RNAi-defective. When homozygous *rde-4* (or *rde-1*) animals are injected with long dsRNA or with siRNAs, an RNAi response is observed in heterozygous *rde-4 (*or *rde-1*) progeny (Timmons et al. 2003; Winston et al. 2002). Such a heritable response is observed even when the dsRNA is injected into the intestine. From these results, we can infer that both long dsRNAs and siRNAs can traffic from the site of injection and enter the developing germline cells resulting in a systemic RNAi phenocopy in the ensuing progeny.

While both strands of Dicer-generated siRNAs are capable of participating in RNAi, only the antisense strand is utilized (Schwarz et al. 2003). Furthermore, these small (~22 nucleotide) monophosphorylated antisense oligomers are capable of eliciting an RNAi response when injected into *C. elegans*. Unlike double-stranded siRNAs, the ability of antisense strands to elicit silencing responses does not depend upon *rde-1* or *rde-4*; however, other components that function downstream of *rde-1* and *rde-4* are required (Tijsterman et al. 2002). Antisense siRNAs can elicit systemic silencing in treated animals and in their progeny, albeit the effects are less robust than for double-stranded siRNAs. Factors that may influence the robustness of RNAi responses to antisense versus double-stranded siRNAs may include the stability of the molecule, especially as it traffics from cell-to-cell.

Two distinct populations of siRNAs are observed in animals undergoing RNAi: primary siRNAs and secondary siRNAs (Aoki et al. 2007; Pak and Fire 2007). These molecules differ in chemical composition, functional usage, and abundance. Primary siRNAs have the characteristic hallmarks of a Dicer cleavage product; they are small (~22 bp), double-stranded RNA molecules with a 2-base overhang at each 3′ end and a monophosphate at each 5′ end. Secondary siRNAs were isolated as a population of antisense RNA molecules that are 21–25 nucleotides in length; and more importantly, they harbor a 5′ triphosphate at the 5′ end. In animals treated with dsRNA and displaying RNAi, secondary siRNAs are more abundant than primary siRNAs. Secondary siRNAs are synthesized de novo by RNA-dependent RNA polymerases (RdRPs) that utilize mRNAs as template, thereby amplifying the population of molecules that can trigger RNAi responses (Aoki et al. 2007; Sijen et al. 2007) (Fig. 6.3). Exactly how the mRNAs are selected by RdRPs is not known. Presumably, the association of mRNA with RISC or the aberrant nature of the cleaved mRNA serves as a signal for RdRP.

6.3.1.2 Endogenous Silencing RNAs and Associated RNA Silencing Mechanisms

Because the RNAi machinery responds to both exogenous dsRNAs and to endogenous ncRNAs, it is important to understand the interrelatedness of the mechanisms that utilize them. Our understanding of how ncRNAs can direct diverse silencing events has improved vastly from our knowledge of Argonaute proteins and their functions (Hutvagner and Simard 2008; Joshua-Tor 2006; Siomi and Siomi 2009). Argonaute proteins are crucial mechanistic components in RNA silencing mechanisms, and collectively they respond to both to exogenous delivery of dsRNA and to endogenously expressed ncRNAs. Argonaute proteins function in multiple steps of various RNA silencing mechanisms, from mechanisms that allow for the production and accumulation of silencing RNAs to downstream activities that utilize these silencing RNAs (Farazi et al. 2008). While Argonaute proteins are well conserved, they are somewhat structurally diverse with respect to conserved domains. Four distinct domains can be observed in Argonaute proteins. Included

among these is a PAZ domain, which has been demonstrated in biochemical and crystallographic analyses to bind ssRNA, and a PIWI domain, which assumes an RNaseH-like conformation. Indeed, some, but not all, Argonaute proteins harbor functionally conserved residues that allow for RNA cleavage, and cleavage activities can be demonstrated in in vitro assays using purified protein or *C. elegans* extracts.

Argonaute proteins can bind to and discriminate between ncRNA classes based, in part, on structural features at the 5′ end. Argonaute proteins function in multiprotein complexes. The nature of the interacting proteins in the Argonaute complex and the type of ncRNA bound to the Argonaute determines the functionality of the multiprotein complex in RNA silencing. For example, Argonaute proteins are components of RISC. Argonaute proteins are also associated with silencing activities in the nucleus that contribute to the establishment and maintenance of expression domains in chromatin (Gu et al. 2009; Guang et al. 2008).

The genome of *C. elegans* encodes 27 different Argonaute proteins. A number of strategies are currently being utilized in order to elucidate their functions. These include comparisons of deep sequencing data of ncRNA populations sequenced from wild-type, mutant, and dsRNA-treated animals; phenotypic analysis of Argonaute mutants; identification of interacting proteins; identification of bound ncRNAs isolated by immunoprecipitation; and in vitro assays for RNaseH-related activities. Single and multiple Argonaute mutant strains have been generated. These strains are collectively defective for all the AGO genes in *C. elegans*, and phenotypic analyses of these strains have revealed distinct and sometimes overlapping roles (Yigit et al. 2006). For example, the Argonaute protein RDE-1 is apparently exclusively required for an RNAi response to exogenously delivered dsRNA; others (for example, ALG-1 and ALG-2) are required for processing and function of miRNAs (Grishok et al. 2001); while yet others can participate in both pathways (R06C7.1, F55A12.1, PPW-2, F58G1.1, ZK1248.7, sago-2, ppw-1, sago-1, C16C10.3, T22H9.3, Y49F6A.1, and NRDE-3) (Gu et al. 2009; Yigit et al. 2006). The latter class of Argonautes represents an expanded clade of Argonaute proteins found in worms (the WAGOs). Analyses of various combinations of different WAGO mutants revealed partially overlapping roles for these genes in the germ line for an efficient response to exogenous dsRNAs; however, precise roles and expression patterns in somatic tissues have not been established. The multifunctional Argonaute CSR-1 is also required for an efficient RNAi response to exogenous dsRNA and has been observed to direct the cleavage of mRNAs in vitro in the presence of secondary siRNAs (Aoki et al. 2007) (Fig. 6.3). CSR-1 also has important endogenous roles in chromosome function; roles that apparently do not result in cleavage of target RNAs (Claycomb et al. 2009).

There are several classes of endogenous ncRNAs in *C. elegans*, and each class is associated with distinct Argonautes. The small ncRNAs that have been identified include endogenous siRNAs, 21U-RNAs, 21G-RNAs, and 22G-RNAs (Ambros et al. 2003; Batista et al. 2008; Gu et al. 2009; Ruby et al. 2006). The 21U-RNAs and 21G-RNAs are 21 nucleotides in length; their sequences begin with a Uridine- or Guanosine-monophosphate residue at the 5′ end, respectively, and both classes

have a modified 3′ hydroxyl residue in the penultimate ribose. The mechanism that leads to their biogenesis is incompletely elucidated. 22G-RNAs have a 5′ Guanosine residue that is polyphosphorylated, and they do not have 3′ end modifications. Analysis of ncRNA accumulation in *C. elegans* mutants demonstrated that the accumulation of 22G-RNAs is dependent upon two RNA-dependent RNA polymerases (EGO1 and RRF-1) that have partially overlapping functions in the germ line. Thus, biochemical analyses of the 22G-RNAs along with analysis of mutants suggests that the endogenous 22G-RNAs are amplified products of RdRP, which is reminiscent of the biochemical origin of secondary siRNAs.

While it is still early in the field for generalizations, the emerging picture is that the Argonautes (WAGOs, CSR-1 and others) that participate in the response to experimental delivery of dsRNAs are the same Argonautes that function in the 22G-RNA pathway (Gu et al. 2009; Yigit et al. 2006). A common feature of these pathways is that the secondary siRNAs and 22G-RNAs require RdRP function for their biogenesis and have similar structural characteristics: a polyphosphate residue at the 5′ end and an unmodified 3′ end. An important piece of experimental evidence for this hypothesis comes from the observation that CSR-1 binds both 22G-RNAs and secondary siRNAs (Aoki et al. 2007; Gu et al. 2009). These observations highlight the importance of secondary siRNAs in experimental RNAi responses. While expression pattern information has not been determined for each of the Argonaute proteins in detail, many of them are widely expressed and have overlapping functions. Thus, the cumulative effects of their activities in distinct cells contribute to a fully systemic response in animals treated with dsRNA.

The Argonaute protein RDE-1 is also required specifically for an RNAi response to exogenous dsRNA, yet RDE-1 does not bind secondary siRNAs. RDE-1 and DRH-1 (a DExH box helicase) proteins are both required for RNAi and are found in physical association with Dicer (Tabara et al. 2002). Initially, RDE-1 was thought to provide the Slicer activity that leads to mRNA cleavage; however, biochemical testing revealed that RDE-1 is required for cleavage of the "passenger" strand of the siRNA duplex and transfer of the guide strand to RISC (Sijen et al. 2007; Steiner et al. 2009). The Argonaute in RISC that recognizes primary siRNAs and executes Slicer activity on the complementary mRNA has not been identified (Fig. 6.3). Slicing activity is required for the further production of secondary siRNAs as a cleaved mRNA is likely to be recognized as a template by RdRPs.

The exact nature of the molecule, or molecules, which traffic from cell-to-cell has yet to be elucidated. *C. elegans* harbors four different RdRPs, allowing for secondary siRNA production in somatic as well as germline tissue. Thus, even though secondary siRNAs appear to play a critical role in RNAi, this does not mean that secondary siRNAs must be the mobile signal, as they may be synthesized in a cell that receives a long dsRNA, a primary siRNA, a guide RNA, or other metabolic form of the input dsRNA that has yet to be identified. Indeed, recent work has identified a new class of unusually small ncRNAs of human and viral origin, which highlights the potential for missing pieces in our puzzle of non-coding RNAs (Li et al. 2009).

6.3.2 Mechanisms that Inhibit RNAi Responses

Over one hundred *C. elegans* genes have been implicated in RNAi. Many of these genes are expressed in a tissue-specific manner; therefore, *C. elegans*, like other multicellular organisms, is naturally mosaic with respect to the robustness of RNAi activity in different tissues. As a consequence, even in a hypothetical situation where RNA silencing molecules are distributed uniformly to each cell of the animal, a nonuniform, or incompletely systemic, RNAi response may be observed.

Indeed some endogenous RNA silencing mechanisms compete with mechanisms that respond to exogenous dsRNA and, in effect, limit the RNAi response in certain tissues. Most tissues of the animal are able to mount a robust RNAi response to exogenous dsRNA with the notable exception of neuronal cells. Transgene-driven expression of dsRNA directly in neurons is not always sufficient to overcome RNAi intransigence, which indicates that cellular delivery of dsRNAs may not be part of the RNAi problem in neurons. *C. elegans* mutants that have a general enhancement in RNAi activity are capable of mounting an RNAi response in neurons. Mutant screens designed to uncover animals with RNAi activity in neurons is a strategy that has been employed to uncover the nature of the RNAi resistance in this tissue.

rrf-3 mutants have an enhanced RNAi response in most tissues, especially in neurons (Simmer et al. 2002). RRF-3 is an RdRP that is expressed in the nervous system and other tissues. Analysis of *rrf-3* mutants provides clues that exogenous dsRNAs are in competition with limiting components of endogenous RNA silencing pathways (Hutvagner and Simard 2008; Lee et al. 2006; Sijen et al. 2007; Tabara et al. 1999). While *rrf-3* mutants that are exposed to exogenous dsRNA have high levels of secondary siRNAs, they fail to accumulate some endogenous ncRNAs (Duchaine et al. 2006; Lee and Ambros 2001). Such disparate effects on the two RNA silencing pathways may indicate that common proteins in different multiprotein complexes are in limited supply. It has been hypothesized that when RRF-3 protein is present, it could bind a low-abundance protein that would otherwise be used in protein complexes with those RdRPs that facilitate secondary siRNA production in a wild-type animal; thus, the absence of RRF-3 in mutants leads to enhanced activity of the other RdRP complexes and therefore an elevated level of secondary siRNAs (Lee et al. 2006). Support for this hypothesis also comes from transgene expression experiments: overexpression of some Argonaute proteins gives rise to animals that have an improved RNAi response to exogenous dsRNA, indicating that the Argonaute proteins are normally limiting (Yigit et al. 2006). This supports the notion that RNAi pathways share complexes that contain limiting components.

eri-1 mutants display RNAi phenotypes that are similar to those of *rrf-3* mutants (Kennedy et al. 2004). ERI-1 proteins have a SAP domain that is found in DNA-binding proteins as well as a DEDDh-like 3′→5′ exonuclease, and ERI-1 proteins can be co-isolated with Dicer (Duchaine et al. 2006). Purified ERI-1 enzymes can partially degrade siRNAs, and *eri-1* mutants treated with dsRNA accumulate a higher level of antisense siRNAs.

Mutants defective for the Argonaute gene *ergo-1* also display enhanced RNAi activity in response to exogenous dsRNA, as well as defects in endogenous RNAi (Yigit et al. 2006). RRF-3, ERGO-1, and ERI-1 interact with Dicer and likely function in a RdRP pathway to produce endogenous ncRNAs. This function may indirectly suppress the production of mobile RNAi silencing signals, and thereby limit systemic RNAi responses.

Additional mutants in the *eri* class have been isolated based on enhanced RNAi phenotypes, and another member of this class has been recently characterized. eri-*6/7* mutants display enhanced exogenous RNAi activity; the *eri-6/7* gene encodes a Superfamily I helicase (Fischer et al. 2008).

Additional control over RNAi activities comes from genes with long-appreciated roles in development. These genes likely contribute to misexpression or misregulation of RNA silencing components. For example, *lin-35* mutants are hyperactive for RNAi in a variety of tissues, including the nervous system. *lin-35* encodes the worm ortholog of the p105Rb tumor suppressor gene that transcriptionally regulates a number of downstream targets. P-granule compartments and RNAi components overlap spatially in the germ line, yet in *lin-35* mutants, P-granule components are no longer localized exclusively in the germ line of early embryos; they also appear in somatic lineages, a factor that may contribute to the enhancement of RNAi in these tissues (Lehner et al. 2006).

Other cellular activities attenuate the RNAi response through modifications of the trigger dsRNA. Adenosine deaminases are enzymes that recognize dsRNA and convert adenosine residues to inosines. As a consequence, the altered RNA no longer assumes a perfect double-stranded conformation, thereby reducing the effectiveness of the molecule as a substrate in RNAi reactions. Thus, ADARs may particularly reduce the effectiveness of long dsRNA molecules, in particular, those that are expressed from transgene sources, as ADAR proteins localize to the nucleus (Knight and Bass 2002).

6.3.3 *Mechanisms that Facilitate Spreading of RNA Silencing Molecules*

The plasma membrane is a formidable barrier to the delivery of polynucleic acid, providing a line of defense against invading genomes. Paradoxically, the first step in the RNAi response to extracellular dsRNA involves entry of dsRNA into the cell. This is followed by recognition and loading of the dsRNA onto a multiprotein complex containing Dicer (Knight and Bass 2001). Experiments described above (see Sect. 6.3.1) highlight the fact that treatment of animals with long dsRNAs, siRNAs, as well as antisense RNAs can elicit a systemic RNAi response. Thus, we can infer that all these forms of silencing RNAs must have the capacity to enter cells. However, the precise trafficways from membrane transit to Dicer loading have yet to be clearly defined. Also remaining to be determined is how cellular entry of RNA silencing molecules leads to a systemic RNAi phenocopy: What kind

of molecules spread to distal cells? What are the precise mechanism(s) that facilitate such spreading? Do all delivery methods depend upon the same set of systemic dissemination mechanisms? Is a relay transfer mechanism involved or a direct transfer of RNAs from cell to cell?

Approaches to answer some of these questions have involved analyses of RNAi defective mutants coupled with direct testing of candidate genes. Other approaches involve analyses of cellular structures and mechanisms that are known to allow for entry of macromolecules, features that include cytonemes (tunneling nanotubes), gap junctions, endocytosis, channel and transporter-mediated import, and gap junctions. So far, little data exists that implicates a function for most of these in systemic RNAi.

Some evidence for the presence of cytonemes in *C. elegans* comes from observations of tentacle-like membranous arms that extend from the somatic Distal Tip Cell located at the distal end of each gonad arm (Hall and Altun 2008). The DTC guides the migration of the gonadal arms during development and promotes mitosis (prevents meiosis) of the dividing germline cells located in the distal region. The cytoplasmic extrusions stretch out over the gonad, covering a distance of 8–20 germline nuclei. In general, while cytonemes may provide opportunities for intercellular trafficking of macromolecules, their existence and functions are not well characterized in *C. elegans*. Furthermore, these structures have not yet been implicated in trafficking of RNAs in *C. elegans*.

Cells in the gonad are also connected to somatic cells by gap junctions (Greenstein 2005; Hall et al. 1999). Ten somatic myoepithelial sheath cells surround each arm of the *C. elegans* gonad. The gap junctions observed in electron micrographs provide connections between two sheath cells and between sheath cells and oocytes. Potentially, such junctions could provide a pathway connecting somatic and germline RNA mechanisms by allowing transfer of small molecules; however, roles for gap junctions in systemic movement of RNA silencing molecules from somatic cells to germline cells have not been established in *C. elegans*.

6.3.3.1 Endocytosis

Endocytosis has been implicated in the uptake of dsRNA molecules in a number of organisms, including *C. elegans*. Endocytosis is an essential process, and most of the mutant strains of *C. elegans* that harbor defects in genes required for endocytosis are hypomorphic. As a consequence, endocytic processes are reduced but not completely eliminated. For those mutants that are defective for genes clearly implicated in general endocytosis pathways, none have been observed to display defects in RNAi (Tijsterman et al. 2004; Timmons et al. 2003). However, because most of the mutants analyzed were not null mutants and because of the redundancy in endocytic pathways, the experiments performed have a high likelihood of eliciting false negative results.

Additional experimental approaches have been taken to address the role of endocytosis in the trafficking of RNA silencing molecules (Saleh et al. 2006).

RNAi methodology was used to uncover such roles for endocytosis genes: Bacterial feeding was used to knock down the function of conserved candidate genes with known functions in endocytosis, and the surviving animals were then placed on food designed to knock down a second *C. elegans* gene with a well-established RNAi phenocopy. RNAi of several of the endocytosis gene candidates displayed RNAi defects in this assay including: *chc-1* (the clathrin heavy chain), *dpy-23* (AP-50, an ortholog of the mu2 subunit of the Adaptor Protein Complex 2), *rab-7* (a GTPase required for endosome and endosome–lysosomal trafficking), *arl-1* (ADP-ribosylation factor), *cogc-2* (Conserved Oligomeric Golgi Component), *ZK1098.5* (related to human Trapping Protein Particle Complex 3, involved in tethering transport vesicles to the cis-Golgi membrane), and *vps-41* (related to yeast Vacuolar Protein Sorting factor).

C. elegans has a well-established track record in uncovering gene functions through forward genetics screens, and such screens for RNAi defective mutants have also hinted at roles for endocytosis in RNA silencing. Among the genes uncovered are *rsd-2, rsd-3,* and *rsd-6* (Han et al. 2008; Tijsterman et al. 2004). These are the better-studied group of putative endocytosis mutants simply because null mutant strains are available, they display obvious RNAi defects, and they are viable as homozygotes. *rsd-2, rsd-3,* and *rsd-6* are all required for RNAi in the germ line in response to ingested dsRNAs. Some somatic cells in the mutants retain the ability to respond to ingested dsRNAs, an indication that RNA can traffic from the intestine to somatic cells. Furthermore, injection of dsRNA into the mutants can elicit an RNAi phenocopy in the germ line.

RSD-2 is a nematode-specific protein with conserved domains. RSD-6 has a Tudor domain, a domain commonly found in DNA- and RNA-binding proteins that interact with other proteins, particularly proteins that are methylated (Charier et al. 2004; Cote and Richard 2005; Huyen et al. 2004; Zgheib et al. 2009). The RSD-3 protein is weakly expressed in most cells of the animal and is concentrated in the six coelomocyte cells that lie in the pseudocoelomic cavity, Coelomocytes have a high degree of endocytosis, and RSD-3 protein harbors an ENTH domain. The conserved Epsin (Eps15 interactor) N-terminal homology (ENTH) domain is found in eukaryotic proteins that participate in clathrin-mediated endocytosis. ENTH domains bind other proteins and inositol phospholipids and contribute to the membrane recruitment of clathrin (Grant and Caplan 2008). Epsin-mediated organization of intracellular membranes can impinge on other cellular processes as well, including mitotic spindle integrity (Liu and Zheng 2009). This latter study highlights the possibility that endocytosis/vesicle trafficking defects may indirectly affect RNAi mechanisms due to perturbations of intracellular membranes and RNAi-relevant structures.

A working hypothesis for *rsd-2, rsd-3*, and *rsd-6* function is that these genes are not required for uptake of dsRNA by intestinal cells, rather they facilitate systemic RNAi responses that are directed toward the germline after the dsRNA has entered intestinal cells. Even though screens for RNAi defective mutants have taken place in many different laboratories, few genes have been identified that are specifically required in responding to exogenous dsRNA. Mutants in this class include *rde-1,*

rde-4, sid-1, and possibly *sid-2* – these mutants are severely RNAi defective and lack other phenotypes associated with defects in the endogenous RNAi machinery. In contrast, *rsd-2, rsd-3,* and *rsd-6* mutants do display additional phenotypes that are more typical of RNAi defects and not endocytosis defects (Han et al. 2008; Tijsterman et al. 2004). This observation may indicate an intimate connection between endocytosis and endogenous RNA silencing mechanisms. However, when a GFP-tagged protein that normally undergoes receptor-mediated endocytosis is used to study trafficking, endocytosis defects in the mutants are not obvious (Han et al. 2008; Tijsterman et al. 2004). An alternative, but not mutually exclusive, working hypothesis for *rsd-2, rsd-3*, and *rsd-6* function is that the mutations render the animals dosage-sensitive with respect to the amount of dsRNA, and this defect is more obvious when the feeding method is used to deliver dsRNA to the worms. A number of different mutant strains that presumably do not have defects in endocytosis also have dosage-sensitive RNAi defects, including those defective in Argonaute genes from the WAGO class.

Lastly, biochemical approaches have revealed hints that endocytosis mechanisms are linked to RNAi. In a study to identify *C. elegans* proteins that interact with Dicer, a high confidence list of 20 interacting proteins was generated based on reproducibility of the interactions. Included in the list is the protein F38E11.5, a Beta-prime subunit of the vesicle COPI coatomer (Duchaine et al. 2006).

6.3.3.2 Transmembrane Proteins

Several transmembrane proteins that are localized to the plasma membrane are implicated in systemic RNAi. Included among these proteins are SID-1 and SID-2.

Several lines of evidence point to a role for SID-1 in order for *C. elegans* to mount a systemic RNAi response to experimental delivery of dsRNA. Initially, the *sid*-1 gene was identified based on the absence of a systemic RNAi response in transgenic animals. These experiments made use of a transgene configured for tissue-specific expression of dsRNA in the pharynx and a reporter transgene that could detect systemic RNAi phenocopies in the muscle. Transgene expression in this tissue led to systemic RNAi phenocopies in wild-type animals, but not *sid-1* mutants (Winston et al. 2002). The *sid-1* gene encodes a conserved transmembrane protein that is not found in all species. For example, humans and mice harbor two *sid-1*-like genes, whereas the *Drosophila* genome does not encode a *sid-1* gene.

Experiments using *Drosophila* cells have helped to elucidate possible roles for SID-1 in cellular import of dsRNAs (Feinberg and Hunter 2003). The *Drosophila* genome does not have *sid-1* genes, and systemic RNAi responses are much weaker in *Drosophila* in comparison to *C. elegans*. SID-1 expression in *Drosophila* S2 cells resulted in a dramatically enhanced RNAi response in individual cells, implying that the cells acquired the ability to take up dsRNA. More direct observations of dsRNAs using radiolabeled molecules in the S2 cell system showed the uptake in the SID-1-expressing cells to be cold-sensitive and ATP-independent. Furthermore,

RNAi responses were more robust when longer dsRNAs (100–500 bp) were used (Feinberg and Hunter 2003). SID-1 is therefore proposed to serve as a passive channel for uptake of longer dsRNA molecules. However, later studies that utilized the same *Drosophila* S2 cell culture system noted that SID-1-dependent intracellular accumulation of dsRNAs into cells was not size-restricted (Shih et al. 2009).

sid-1 mutants are insensitive to injected dsRNAs. The RNAi defects can be bypassed in *sid-1* mutants when dsRNAs are directly injected into the syncytial gonad, but not when the dsRNA is injected into the intestine (Winston et al. 2002). Furthermore, *sid-1* mutants proved to be severely RNAi-defective when ingestion methods (bacterial feeding) were used to deliver dsRNAs (Han et al. 2008; Tijsterman et al. 2004; Winston et al. 2002). Taken together, these results have revealed roles for SID-1 in various cell types. The model that emerged for SID-1 function is that SID-1 is required for uptake of dsRNAs: uptake of transgene-expressed dsRNAs into muscle cells, uptake of intestinally injected dsRNAs into the gonad, uptake of dsRNA from culture media into *Drosophila* S2 cells, and uptake of ingested dsRNAs into intestinal cells.

Later, the role for SID-1 in systemic RNAi was observed to be surprisingly cell autonomous, as revealed using transgenic strains and *green fluorescent protein* as both trigger and target (Jose et al. 2009). As mentioned previously, transgenic strains can be engineered to express *gfp* dsRNA from various different tissue-specific promoters, and these strains display systemic RNAi responses that are dependent upon *sid-1*. When wild-type SID-1 was added back to *sid-1* mutants by driving its expression in a specific target tissue (a tissue different from that expressing the *gfp* dsRNA), only the SID-1(+) target tissue regained the ability to display an RNAi response. When expression of both SID-1(+) and the *gfp* dsRNA were driven in the same tissue in a *sid-1* mutant background, systemic RNAi remained defective. These results highlight the importance of SID-1 function in RNA silencing mechanisms in the target tissues, and not for the "trigger" cells that are expressing dsRNA, which is consistent with a role for SID-1 in import, and not export, of RNA silencing molecules into somatic cells.

When bacterial food is used to deliver dsRNAs, additional information is learned regarding SID-1 and systemic RNAi responses in intestinal cells. Experiments that utilize animals that are mosaic for SID-1 expression, along with feeding methodology to deliver dsRNA, have revealed that some systemic RNAi responses to ingested dsRNAs are not dependent on *sid-1*. For example, *sid-1* mutants that specifically express wild-type SID-1(+) protein in the muscle display RNAi only in the muscle, and not other somatic cells, when the mosaic animals ingest dsRNA. These results highlight a SID-1-independent trafficking route for the ingested dsRNAs – a route that takes RNA silencing molecules from the intestinal lumen into intestinal cells and presumably into the muscle cells that are expressing SID-1 (+) protein. However, in these *sid-1* mutant animals, RNAi is defective in the intestinal cells themselves when the animals ingest dsRNAs. To resolve these paradoxical observations, it is proposed that a trans-cytosis mechanism in intestinal cells allows for uptake and basal secretion of ingested RNA silencing molecules

and dissemination to distal somatic cells, while SID-1 allows for uptake of RNAs into distal somatic cells (Jose et al. 2009).

Another transmembrane protein, SID-2, is an intestinal protein that has been implicated in systemic RNAi (Winston et al. 2007). *sid-2* mutants do not display RNAi responses when dsRNA is delivered by feeding or soaking, but do display systemic RNAi when dsRNA is delivered by injection or transgene delivery. Unlike the results obtained for SID-1 mutants, expression of SID-2 in *Drosophila* S2 cells does not lead to an enhancement in RNAi. SID-2 is a novel transmembrane protein. Amongst the collection of sequenced genomes, SID-2-related proteins can be observed in a few other nematode species. A closely related species, *Caenorhabditis briggsae,* has a *sid-2* gene that is somewhat diverged, and this species does not mount a systemic RNAi response. When the *C. elegans sid-2* gene was transformed into *C. briggsae,* the animals were able to mount a systemic RNAi response to ingested dsRNAs (Winston et al. 2007).

The current working model for SID-1- and SID-2-dependent RNAi activity is that RNA silencing molecules are trafficked in relay fashion by the SID proteins, with SID-2 allowing for import into intestinal tissues and SID-1 for import of RNA silencing molecules from the pseudocoelom into other somatic cells (Winston et al. 2007).

Our lab has identified additional transmembrane proteins from the ABC transporter superfamily that are required for RNAi (Sundaram et al. 2006; Timmons 2007). Out of the approximately sixty ABC transporter genes in *C. elegans*, nine of them are involved in RNAi mechanisms that respond to extracellular delivery of dsRNAs. We do not have evidence that the transporters are actively involved in the trafficking of RNA silencing molecules; furthermore, most of the transporters are localized to intracellular organelles and membranes. Rather, our current working model is that the RNAi machinery requires a substrate that is trafficked through subcellular compartments in order for the substrate to serve as an effective cofactor for a protein in the RNAi mechanism. It may be the case that this ABC transporter function is nematode-specific, as *C. elegans* is unable to biosynthesize a number of essential molecules and must have the capacity to gain or rebuild those molecules from starting material derived from the diet. Nonetheless, these findings highlight requirements for two distinct intercellular trafficking pathways that impinge upon RNAi: pathways that traffic RNA silencing molecules and ABC transporter substrates.

6.4 Conclusions

Distinct RNA species can elicit systemic RNAi responses when they are introduced into *C. elegans*. These molecules include long dsRNAs, siRNAs, and antisense RNAs. When these RNAs gain entry into cells, they are likely to be metabolized into an amplified pool of secondary siRNAs by RNA-dependent RNA polymerases. It may be the case that such an activity operating in most cells is sufficient to maintain a robust RNAi response; however, expression of hairpin dsRNA

molecules in a tissue-specific manner provides clues that RNA silencing molecules can also be distributed from a small number of dsRNA-expressing cells to nonexpressing cells.

A number of mechanisms have been implicated in cellular import of RNA silencing molecules in *C. elegans*. These include endocytosis mechanisms, the *sid* class of plasma membrane proteins, and the *rsd* class of proteins. However, the presence of *sid* and *rsd* genes in the genome of various organisms does not allow predictions as to whether the organism will have an ability to mount a systemic RNAi response (Tomoyasu et al. 2008). A requirement for trafficking of additional molecules that affect RNAi function is revealed through analyses of ABC transporter function in *C. elegans*. It remains to be seen whether *sid, rsd,* and ABC transporter functions are required for nematode-specific RNAi mechanisms or to RNA silencing mechanisms that are more generally conserved.

The ability to traffic silencing RNAs allows the animal to convey developmentally relevant information from cell to cell. RNA-based instructions that are transferred to the germ line could lead to alterations in gene expression that are particularly important to the survival of the species when animals are experiencing stress.

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Chapter 7
Circulating Nucleic Acids in Health and Disease

E.Y. Rykova, P.P. Laktionov, and V.V. Vlassov

Contents

Abstract Significant changes of the extracellular DNA and RNA concentration and composition have been found in the blood from patients with several pathologies compared with healthy individuals, indicating their potential as the disease biomarkers. Tumor-specific genome and mitochondrial DNA changes, such as mutations, microsatellite instability, aberrant methylation, as well as mRNA and microRNA

E.Y. Rykova (✉), P.P. Laktionov, and V.V. Vlassov
Institute of Chemical Biology and Fundamental Medicine, SB RAS, 8, Lavrentjeva Prosp., Novosibirsk 630090, Russia
e-mail: rykova@niboch.nsc.ru

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have been detected in cancer plasma and serum. Several studies provided evidence for the circulating nucleic acid's involvement into the disease development, which could be valuable for the development of novel therapeutic approaches. In the chapter, the usefulness of serological DNA and RNA markers in diagnosis and prognosis of cancer and other pathological states, including trauma, stroke, myocardial infarction, hematological disorders, complications in organ transplantation, and diabetes are discussed.

7.1 Introduction

Extracellular nucleic acids (exNA) in tissue cultures (Gartler 1959) and in human blood serum were discovered half a century ago (Mandel and Metais 1948). Later on, their prominent role in human health care was recognized, and now, exNA, which are present in blood plasma and other body fluids, are considered as valuable materials for diagnostics and moreover, are supposed to play roles in the development of some diseases (Fleischhacker and Schmidt 2007; Vlassov et al. 2007).

Intensive investigations of circulating nucleic acids (cirNA) were performed in order to develop low-invasive tests for cancer (Anker et al. 2003; Tong and Lo 2006a; Holdenrieder et al 2008). Leon et al. were the first to show plasma cirDNA elevated levels in cancer patients (Leon et al. 1977). CirDNA bearing the same genetic and epigenetic changes as in the tumor cells was shown to be detectable in cancer patients, indicating their potential value as tumor markers (Fleischhacker and Schmidt 2007; Anglim et al. 2008). Circulating tumor-derived mRNAs were found to be potential cancer biomarkers. Recent reports establish the quantification of circulating microRNAs (miRNAs) as a promising approach for cancer diagnostics (Cortez and Calin 2009).

Analysis of the cirNA in plasma of pregnant women was shown to provide important diagnostic possibilities for the pregnancy-associated and fetal disorders, reviewed by Tsang and Lo in detail in the next section. The usefulness of cirDNA and cirRNA has also been investigated in other pathologic states, including trauma, stroke, myocardial infarction, hematologic disorders, complications in organ transplantation, and diabetes (Butt and Swaminathan 2008; Gahan 2008). Despite the fact that the cirNA changes have been less investigated in other diseases, compared with cancer and fetal disorders, significant cirNA concentration and composition changes have been reported.

7.2 Circulating NA in Cancer

Late tumor detection is one of the main factors limiting the survival of cancer patients. Commonly used serological tumor protein markers were shown to be useful for the monitoring of treatment efficacy; however, they lacked sensitivity for the

primary tumor diagnostics. Malignant cell transformation involves an accumulation of genome and mitochondrial DNA (mt DNA) mutations, microsatellite instability (MSI), and aberrant methylation, leading to the abnormal mRNA levels. Stroun et al. were the first to demonstrate tumor-associated DNA changes in the plasma of cancer patients (Stroun et al. 1989). Detection of the same alterations in the tumor DNA and RNA and plasma cirNA in different studies has led to the arguments regarding the tumor origin of cirNA changes, which can potentially be used as the markers for the low-invasive cancer diagnostics. Other bodily fluids have been found to provide the source for the detection of tumor-derived NA, as urine in bladder, kidney, and prostate cancer, sputum and bronchial lavage in lung cancer, peritoneal washings in gastric and ovarian cancer, ductal lavage and nipple aspirate fluid in breast cancer, etc (Fleischhacker and Schmidt 2007; Phé et al. 2010).

7.2.1 Genomic and Mitochondrial DNA Mutations

Mutated ras genes were the first tumor-associated genetic alterations, which were found to be detectable in the cirDNA (Sorenson et al. 1994; Vasioukhin et al. 1994). Thus far, K-ras and p53 gene mutations remain the most frequently analyzed and valuable markers in detection, monitoring, and prognosis of colorectal, pancreatic, lung, breast, and other cancers (Anker et al. 2003; Fleischhacker and Schmidt 2007) (Table 7.1).

Detection frequency of mutated K-ras in the cirDNA was found in the range 20–24% for lung cancer (Pathak et al. 2006) and 27–40% for colorectal cancer (CRC), being strongly increased in urine of CRC patients (95%) (Su et al. 2008). The sensitivity of pancreatic cancer diagnostics increased up to 90% when the combination of plasma K-ras mutation and CA 19-9 tumor protein level was used (Dianxu et al. 2002). K-ras mutation alone or in combination with p53 and APC gene mutations in plasma was found to correlate with clinicopathological characteristics (tumor burden, TNM stage, metastasis) and with the disease prognosis (Trevisiol et al. 2006; Hsieh et al. 2005). However, another group reported no significant difference in the frequency of K-ras mutations in plasma DNA between nonsmall cell lung cancer (NSCLC) patients and cancer-free subjects (Trombino et al. 2005).

p53 mutations were detected with high rates in plasma from breast (24%) and lung cancer patients (40%) (Shao et al. 2001; Andriani et al. 2004). The sensitivity of the stage I lung cancer patient detection increased up to 68% when a combination of cancer genetic markers (p53 mutations, FHIT and 3p LOH) in plasma DNA was used (Andriani et al. 2004). The prognostic significance of p53 mutations for the poor overall survival was found in breast cancer (Garcia et al. 2006a).

The presence of tumor-derived mutations in the circulation may be indicative for the increased risk of cancer, especially for individuals exposed to carcinogens (Hosny et al. 2008; Gormally et al. 2007). p53 mutations were found in plasma from cancer-free individuals exposed to carcinogens of tobacco smoke but not in

Table 7.1 Detection of gene mutations in cancer patient cirDNA

Gene	Cancer	Method	Tumor detection	CirDNA detection	References
p53 249^{ser}	Hepatocellular carcinoma (HCC)	PCR – RFLP Restriction fragment polymorphism	ND	74/186 (40%) – HCC 15/98 (15%) – Liver cirrhosis	Kirk et al. (2005)
p53 codon 249^{ser}	HCC	PCR – RFLP, sequencing	2/20 (10%) – Tumor samples	1/76 (1.3%) – HCC 6/36 (17%) – Chronic liver disease (HCV)	Hosny et al. (2008)
p53 5–8 exon	Breast cancer	PCR – SSCP, sequencing	46/126 (36%)	30/126 (24%)	Shao et al. (2001)
p53 5–8 exon and MSI	Breast cancer	PCR – SSCP, sequencing	104/142 (72%)	61/142 (43%)	Garcia et al. (2006a)
p53	Lung cancer	Mutation load assay, based on PCR, enzyme digestions and quantification	13/38 (34%) (by sequencing of 3–5 exon)	12/80 (15%) – in lung cancer (over 2×10^{-4} p53 mutation load)	Hagiwara et al. (2006)
p53 5–8 exon	Lung cancer	PCR and sequencing, REA, MASA	26/64 (41%)	19/64 (40%)	Andriani et al. (2004)
p53	CRC	PCR – SSCP, DNA sequencing	78/104 (75%)	36/78 (46.2%)	Hsieh et al. (2005)
K-ras	CRC	PCR – RFLP	28/85 (33%)	10/38 (36%) – Serum	Trevisiol et al. (2006)
K-ras	Lung cancer	PCR, DHPLC analysis	ND	15/36 (42%) – NSCLC 12/40 (30%) – Control	Trombino et al. (2005)
K-ras and CA 19-9	Pancreatic cancer	PCR – RFLP	ND	29/41 (71%) – Plasma DNA 37/41 (90%) – DNA and CA 19-9	Dianxu et al. (2002)
K-ras	CRC	Restriction enriched real-time PCR, gel electrophoresis	ND	7/20(35%) – Serum 8/20 (40%) –Plasma 19/20 (95%) –Urine	Su et al. (2008)
K-ras, p53, APC, PIC3CA	Colorectal cancer (CRC)	Digital PCR with magnetic beads (BEAM-ing)	25/25 (100%)	8/16 (50%) – Plasma 23/25 (92%) – Stool	Diehl et al. (2008)
EGFR	Lung cancer	SARMS analysis and EGFR sequencing	23/46 (50%)	17/18 (94%) – Circulating cells 7/18 (39%) – Plasma	Maheswaran et al. (2008)

EGFR	Lung cancer	Microfluidics digital PCR	16/35 (46%) – Exon 19 or L858R mutation	6/35 (17%) – Exon 19 del. 9/35 (26%) – L858R mut	Yung et al. (2009)
B-RAF V600E	Cutaneous melanoma	qPCR using PNA clamp and FRET LNA probe	8/14 (57%) In melanoma cell lines	11/34 (32%) – Stage I–II 27/69 (39%) – Stage III–IV	Shinozaki et al. (2007)
B-RAF V600E	Cutaneous melanoma	Allele specific qPCR, MASA-PCR	ND	8/13 (61%) – Stage IV 4/24 (61%) – Stage III 0/4 (0%) – Stage I–II	Daniotti et al. (2007)
Mitochondrial DNA (MtDNA) mutations	Breast cancer	Sequencing	ND	25/25 (100%) – Polymorphisms in Plasma	Losanoff et al. (2008)
MtDNA	Breast cancer	Amplification, sequencing	14/15 (93%)	10/15 (67%) – Nipple aspirate fluid	Zhu et al. (2005)
MtDNA	Malignant melanoma	Amplification, sequencing	5/12(45%)	9/44(20%)– Plasma	Takeuchi et al. (2004)
MtDNA	HCC	Amplification, sequencing	13/19(68%)	8/10(80%)	Nomoto et al. (2002)
MtDNA	Prostate	Amplification, sequencing	3/16 (19%)	3/3(100%) – Plasma	Jerónimo et al. (2001)
MtDNA	Lung, bladder, and kidney cancer	MitoChip and capillary electrophoresis sequencing	18/24 (75%)	8/22 (36%) – BALs from lung, urine from bladder and kidney	Jakupciak et al. (2008)

the plasma of nonsmokers, indicating the association of the mutation with high risk of lung cancer development (Hagiwara et al. 2006). In areas with high risk for Hepatitis B virus (HBV) infection and heavy exposure to aflatoxin B1, p53 mutations were detected with high prevalence in plasma from patients with chronic liver disease and infection by HBV (16.7%) (Hosny et al. 2008).

Mutations of other tumor-specific genes were examined in the circulation recently (Table 7.1). The presence of T790M EGFR mutation was identified in plasma from 33% of NSCLC patients, which correlated with significantly reduced progression-free survival (Maheswaran et al. 2008). B-RAF V600E mutation was detected in serum from 37% of patients with cutaneous melanoma and had a predictive value in the biochemotherapy response (Daniotti et al. 2007; Shinozaki et al. 2007). Nucleophosmin (NPML1) gene mutations were revealed in plasma of 24% of acute myeloid leukemia patients and were associated with a more favorable clinical outcome (Ma et al. 2009).

mtDNA mutations were reported to have prognostic and diagnostic value in cancer (Jakupciak et al. 2008; Takeuchi et al. 2004). D-loop region mutations were detected in 20% of melanoma plasma samples and were more frequent in advanced disease (Takeuchi et al. 2004) (Table 7.1). In prostate cancer, mtDNA mutations were also found to be a relatively rare event (19%), inspite of which mutant mtDNA could be detected in all of the paired plasma samples (Jerónimo et al. 2001). In most of breast tumor samples, D-loop and coding region mtDNA mutations were detectable; however, in serum samples, only several D-loop region polymorphisms were found by DNA sequencing (Losanoff et al. 2008). In breast cancer, hepatocellular carcinoma, lung, bladder, and kidney cancer mtDNA mutations were detected with high frequency in blood plasma and other body fluids as nipple aspirate fluid, bronchial lavage, and urine (Nomoto et al. 2002; Zhu et al. 2005; Jakupciak et al. 2008).

Technical difficulties have been limiting the use of mutations as cancer markers, one of them being their wide heterogeneity. Mutations of p53 and K-ras genes could be successfully screened in plasma cirDNA because they have hot-spots at certain codons. Another challenge is the low relative amount of tumor-derived cirDNA: the median percentage of mutant DNA fragments in plasma from CRC patients was found to be 0.18% of the total cirDNA (Diehl et al. 2008). In order to reach the required sensitivity, different PCR-based methods were developed including the procedures, aimed at the enrichment of mutant sequences. One of the enrichment approaches is based on the restriction nuclease digestion, as restriction site mutation (RSM) and restriction fragment polymorphism (RFLP) (Jenkins et al. 2003; Kirk et al. 2005).

High-throughput microfluidic digital PCR was recently shown to allow for the precise mutation quantification by separation of the cirDNA sample into many reaction chambers, containing single molecules (Diehl et al. 2005; Yung et al. 2009). Using this technique, a panel of somatic mutations (TP53, KRAS, APC and PIC3CA) was quantified in plasma of CRC patients and EGFR mutations in plasma from NCSC patients (Diehl et al. 2008; Yung et al. 2009). This advanced approach could be widely applicable for the genetic testing based on the detection of not only

mutations but any other genetic alterations as well(Fleischhacker and Schmidt 2008).

7.2.2 Microsatellite Alterations

Changes in DNA microsatellite regions, namely loss of heterozygosity (LOH) and MSI, are frequently found in neoplastic tissues. LOH, characteristic for tumor DNA from head and neck squamous cell carcinomas (HNSCC) and small cell lung cancers (SCLC), were the first detected in plasma of patients with these tumors (Nawroz et al. 1996; Chen et al. 1996).

Due to the low proportion of tumor DNA in total cirDNA, the LOH detection rates by the allele shift in plasma were decreased compared with the rates in tissue samples, for example, in breast cancer, they were 9.0% and 27.5%, respectively (Schwarzenbach et al. 2007). In contrast, MSI assay has a higher sensitivity than LOH as far as it measures additional PCR signals in a background of unchanged DNA.

A panel of MSI markers is usually required for the development of a serological test with high sensitivity. In oral squamous cell carcinoma, presence of at least one from the panel of LOH alterations at nine microsatellite loci was detected in 90% of serum samples (Kakimoto et al. 2008). Using a panel of 12 MSI markers, high detection rates were achieved for lung cancer (88%) (Beau-Faller et al. 2003) and esophageal adenocarcinoma (81%) (Eisenberger et al. 2006). Five markers from the panel of 13 polymorphic MSI were relevant for discriminating between prostate cancer and benign prostate hyperplasia (BPH) (Schwarzenbach et al. 2008). MSI alterations were detected in blood plasma from I stage esophageal carcinomas and I stage lung cancer patients, as well as benign lung diseases (COPD and asthma), indicating that further research is needed to define their early diagnostic meaning in lung cancer (Samara et al. 2006; Eisenberger et al. 2006). Serum LOH detection has been correlated with disease recurrence and survival rates in breast cancer (Garcia et al. 2006a), oral squamous cell carcinoma (Kakimoto et al. 2008), and CRC (Denlinger and Cohen 2007), demonstrating the prognostic significance of LOH detection.

Taback et al. increased the rates of LOH detection in CRC patients by taking blood from the direct venous drainage system as compared with systemic circulation (Taback et al. 2006). Tumor-derived LOH was detected more frequently in blood from the mesenteric/portal veins of patients compared to peripheral vein (33% compared to 27%), supporting the origin of tumor-associated exDNA in the circulation.

High concordance of the allelic imbalance was not found in cirDNA and tumor DNA in contrast with high concordance of gene point mutations and epigenetic changes (Eisenberger et al. 2006). In breast cancer patients, D3S1255 marker was found with highest frequency in serum samples, whereas in paired tumor samples,

other LOH markers (D13S218 and D17S855) were more frequent (Schwarzenbach et al. 2007). These findings might be due to heterogenic tumor cell clones within the primary tumor followed by the development of different cell clones during their dissemination and metastasis.

The data accumulation demonstrates the limited sensitivity of the existing microsatellite markers in serum and plasma and bring up the need for the search of the novel markers. One critical point in MSI and LOH detection stays the use of mono- and di- versus polynucleotide for MSI detection (Laghi et al. 2008). Another point is that potential MSI markers have been usually detected first in the cancer tissue DNA, and it seems reasonable now to use the plasma and serum DNA as the source for the direct search of these markers.

7.2.3 Aberrant DNA Methylation in Cancer

Epigenetic alterations, which lead to the inactivation of tumor suppressor genes, were shown to be involved in the onset and progression of different cancers; thus, they may serve as biomarkers for the early cancer diagnostics. Aberrant methylation of the p16 gene was first detected in the serum of patients with lung cancer (Esteller et al. 1999).

As far as the tumor-derived methylated DNA amount in the circulation is low (mean 1.9% in prostate cancer patients) (Ellinger et al. 2008a), the sensitive and specific test should be based on a panel of markers. Notably, only two genes are now known to show tissue-specific methylation: BRCA1 in breast and ovarian cancer, GSTP1 in prostate, breast, renal, and hepatic cancers (Table 7.2). Methylated markers have been selected either by targeted approach, focused on the known genes potentially related to cancer (Barton et al. 2008) or by genome-wide search, which provide new DNA methylation markers by means of the comparative genome profiling between tumor and nontumor tissues (Radpour et al. 2009).

The selected methylated markers undergo further validation for their diagnostic potential in the targeted studies of the plasma samples. Carvalho et al. demonstrated low specificity for the panel of six novel selected methylated markers when they were detected by quantitative methylation-specific PCR (qMSP) in serum from head and neck squamous carcinoma patients, which can result from the compartment-specific methylation as a normal physiologic state in different tissues (Carvalho et al. 2008). The complex methylation profile of the cirDNA was recently shown by the direct analysis of four selected cancer-associated genes in serum of breast cancer patients by parallel bisulphite pyrosequencing (Korshunova et al. 2008). The authors have found no tumor-specific methylation patterns for any of the four tested loci and concluded that the direct search of biomarkers is needed that have no background in normal serum. In contrast, Lofton-Day et al. achieved the successful identification of two specific blood-based CRC marker candidates (NGFR and SEPT9) using the stringent criteria at all steps of the selection and validation process (Lofton-Day et al. 2008).

Table 7.2 Detection of gene hypermethylation in cancer patient cirDNA

Gene	Cancer	Method	Tumor detection	CirDNA detection	References
p 16 (CDKN2A)	Lung cancer	MSP	12/30 (40%)	14/91 (15%)	Fujiwara et al. (2005)
p 16	Lung cancer	Nested MSP	34/72 (47%)	29/72 (40%) – Sputum 15/72 (21%) – Serum	Belinsky et al. (2007)
p 16	Lung cancer	Quantitative MSP	33/63 (53%)	24/63 (38%)	Hsu et al. (2007)
p 16	Lung cancer	Quantitative MSP, p16 to MYOD ratio	ND	Total blood DNA 25/95 (26%) – Cancer	Suga et al. (2008)
p 16	Lung cancer and benign disease	Quantitative MSP	ND	Bronchial aspirates 18/75 (24%) – Cancer 0/64 (0%) – Benign	Grote et al. (2005)
p 16	Ovarian	MSP	89/215 (42%)	ND	Wiley et al. (2006)
p 16	Gastric cancer	MSP	24/44 (54%)	20/109 (18%)	Ikoma et al. (2007)
p 16	Hepatocellular carcinoma	MSP	30/45 (67%)	14/45 (31%)	Wong et al. (2000)
p 16	Prostate cancer	Quantitative MSP	ND	Circulating blood cells 4/20 (20%) – relapse 2/22 (9%) – no relapse	Rouprêt et al. (2008)
GSTP1	Prostate cancer	Fluorescent MSP	18/20 (90%)	23/32 (72%)	Goessl et al. (2001)
GSTP1	Hormone refractory Prostate cancer (HRPC)	Restriction endonuclease quantitative PCR	ND	5/18 (28%) – HRPC 10/85 (12%) – Localized cancer	Bastian et al. (2008)
GSTP1	Early and Hormone Refractory Prostate cancer (HRPC)	Conventional MSP	ND	20/62 (32%) – HRPC 3/14 (21%) – Early prostate cancer	Reibenwein et al (2007)
GSTP1	Prostate cancer	Quantitative MSP	ND	Circulating blood cells 20/20 (100%) –relapse 20/22 (91%) – no relapse	Rouprêt et al. (2008)
GSTP1	Breast cancer	Quantitative MSP GSTP1 to β-actin ratio	ND	12/47 (26%)	Hoque et al. (2006)
GSTP1	Bladder cancer	Restriction endonuclease, QMSP	ND	25/45 (59%)	Ellinger et al. (2008b)

(*continued*)

Table 7.2 (continued)

Gene	Cancer	Method	Tumor detection	CirDNA detection	References
GSTP1	Ovarian cancer	MSP	0/234 (0%)	ND	Wiley et al. (2006)
APC	Lung cancer	MSP	95/99 (96%)	42/89 (47%)	Fujiwara et al. (2005)
APC	Lung cancer	Quantitative MSP APC to MYOD ratio	95/99 (96%)	42/89 (47%) –Serum/plasma	Usadel et al. (2002)
APC	Breast cancer	Quantitative MSP	ND	23/80 (29%)	Van der Auwera et al. (2009)
APC	Breast cancer	MSP	15/34 (44%)	10/34 (29%)	Dulaimi et al. (2004)
APC	Breast cancer	Quantitative MSP	ND	8/47 (17%)	Hoque et al. (2006)
APC	Colorectal cancer	Quantitative MSP	ND	3/49 (6%)	Leung et al. (2005)
APC	Renal carcinoma	Quantitative MSP	5/17 (29%)	1/18 (6%)	Hoque et al. (2004)
APC	Bladder cancer	Restriction endonuclease, QMSP	ND	25/45 (59%)	Ellinger et al. (2008b)
RASSF1A	Lung cancer	Nested MSP	30/63 (48%)	25/63 (39%)	Hsu et al. (2007)
RASSF1A	Lung cancer	MSP	9/30 (30%)	11/91 (12%)	Fujiwara et al. (2005)
RASSF1A	Breast cancer	MSP	17/20 (85%)	15/20 (75%)	Shukla et al. (2006)
RASSF1A	Breast cancer	Quantitative MSP	ND	15/47 (32%)	Hoque et al. (2006)
RASSF1A	Breast cancer	MSP	22/34 (65%)	18/34 (56%)	Dulaimi et al. (2004)
RASSF1A	Breast cancer (pre- and postadjuvant therapy)	Quantitative MSP	145/148 (98%)	29/148 (20%) – Pretherapeutic sera 33/148 (22%) – 1-year-after sera	Fiegl et al. (2005)
RASSF1A	Breast cancer and benign tumors	MSP	ND	Plasma and cell-surface- bound cirDNA 15/20 (75%) – Cancer 8/15 (53%) – Benign	Skvortsova et al. (2006)
RASSF1A	Breast cancer	Quantitative MSP	ND	28/80 (35%)	Van der Auwera et al. (2009)
RASSF1A	Hepatocellular carcinoma (HCC) and HBV carriers	Restriction endonuclease, QMSP	ND	59/63 (93%) – HCC 36/63 (58%) – HBV carriers	Chan et al. (2008a)
RASSF1A	Melanoma (stage IV)	Fluorescent MSP	9/18 (50%)	13/50 (26%)	Mori et al. (2005)
RASSF1A	Renal carcinoma	Quantitative MSP	15/17 (88%)	2/18 (11%)	Hoque et al. (2004)

RASSF1A	Prostate cancer	Quantitative MSP	ND	Circulating blood cells 20/20 (100%) –relapse 21/22 (95%) – no relapse	Rouprêt et al. (2008)
RAR β2	Lung cancer and benign disease	Quantitative MSP	ND	Bronchial aspirates 42/75 (56%) – Cancer 8/64 (13%) –Benign	Grote et al. (2005)
RAR β2	Lung cancer	Nested MSP	34/63 (54%)	23/63 (37%)	Hsu et al. (2007)
RAR β2	Breast cancer	Quantitative MSP	ND	12/47 (26%)	Hoque et al. (2006)
RAR β2	Breast benign tumors	MSP	ND	Plasma and cell-surface- bound cirDNA 3/48 (6%) – Benign	Rykova et al. (2008b)
RAR β2	Renal cancer	Quantitative MSP	9/17 (53%)	1/18 (6%)	Hoque et al. (2004)
RAR β2	Head and neck squamous cell carcinoma	Quantitative MSP	39 /44 (90%)	2/13 (15%)	Carvalho et al. (2008)
RAR β2	Melanoma (stage IV)	Fluorescent MSP	11/18 (61%)	13/50 (26%)	Mori et al. (2005)
RAR β2	Gastric cancer	MSP	ND	26/109 (24%)	Ikoma et al. (2007)
RAR β2	Hormone refractory Prostate cancer (HRPC)	Restriction endonuclease quantitative PCR	ND	7/18 (39%) – HRPC 0/136 (0%) – localized cancer	Bastian et al. (2008)
RAR β2	Prostate cancer	Quantitative MSP	ND	Circulating blood cells 18/20 (90%) – relapse 15/22 (68%) no relapse	Rouprêt et al. (2008)

Recent reports provide evidence that the early cancer and premalignant disease detection by the MSP in plasma or serum cirDNA can become possible (Risch and Plass 2008; Barton et al. 2008). The promoter hypermethylation rates were significantly increased in the progression from normal lung tissue to atypical adenomatous hyperplasia and to lung adenocarcinoma for the marker genes (p16, TIMP3, DAPK, MGMT, RARβ2, RASSF1A, and hTERT) (Licchesi et al. 2008). p16 gene methylation was found to have a high value in predicting lung cancer individually and in combination with other genes when detected in plasma of cancer-free individuals (Table 7.2) (Hsu et al. 2007; Suga et al. 2008). Methylation for at least two of the six genes (p16, BLU, CDH13, FHIT, RARβ2, and RASSF1A) indicated an elevated risk of lung cancer with a sensitivity of 73% and specificity of 72% (Hsu et al. 2007). A panel of six methylated genes, including p16, detected in sputum, could predict incident lung cancer in a group of high-risk smokers with chronic obstructive pulmonary disease 3–18 months prior to clinical diagnosis (Belinsky et al. 2007).

RASSF1A hypermethylation was proposed as a risk assessment marker for hepatocellular carcinoma (HCC) in HBV carriers, for whom the RASSF1A methylation detection rate was 58% (Chan et al. 2008a). Promoter hypermethylation of at least one of RASSF1A, APC, BRCA1, p16, and DAPK genes could be detected in the serum or plasma of stage I ovarian cancer patients with 100% specificity and 76% sensitivity (Ibanez de Caceres et al. 2004). Methylation of at least one from the panel of four genes (RASSF1A, APC, GSTP1, and RARβ2) in blood plasma resulted in successful detection of 33% of early-stage (I–II) breast cancer patients (Hoque et al. 2006). Thus, methylated markers seem to be promising for the early cancer detection. However, their presence was found frequently in the sera from patients with benign disease (Table 7.2), requiring the methylation cutoff level evaluation by qMSP for the robust discrimination of the benign and malignant disorders (Rykova et al. 2008b; Martínez-Galán et al. 2008).

Several follow-up studies were designed to determine whether gene methylation rates can be useful for the cancer survival prognosis and therapeutic responsiveness. A defined panel of methylated genes (MDR1, RAR β2, EDNRB) in serum was shown to be associated with prostate cancer progression and to be a useful biomarker in hormone refractory prostate cancer (Bastian et al. 2008). Bladder cancer-specific mortality was significantly increased in patients with APC hypermethylation. APC, GSTP1, and TIG1 hypermethylation correlated with prognostic unfavorable clinicopathologic parameters, including tumor stage, multifocal bladder cancer, and surgical margin positivity (Ellinger et al. 2008b). RASSF1A gene methylation in plasma significantly correlated with decreased overall survival in metastatic melanoma (Mori et al. 2005) and poor outcome in breast cancer patients (Fiegl et al. 2005). Methylation of hMLH1 gene alone or in combination with APC and HLTF methylation in plasma was the prediction factor for poor overall survival in ovarian and CRC (Gifford et al. 2004; Leung et al. 2005; Wiley et al. 2006).

Most of the currently available DNA methylation detection methods are based on the sodium bisulphite-mediated (BIS) DNA conversion followed by quantitative real-time MSP (qMSP) (Kagan et al. 2007). A number of new techniques with

increased sensitivity now complement the traditional PCR-based methods, such as methylation-specific fluorescent amplicon generation (MS-FLAG), sensitive melting analysis after qMSP (SMART-MSP), etc (Kristensen and Hansen 2009). Genome-wide amplification of BIS-modified DNA followed by pyrosequencing (qMAMBA) was developed for the quantitative methylation detection of the minute quantities of cirDNA (Vaissière et al. 2009). As the alternative for the techniques, based on the BIS reaction, novel approaches were introduced leading to the methylated allele enrichment, based on the methylation-sensitive restriction enzymes processing and employing of methylated DNA binding proteins (Rauch et al. 2006; Kimura et al. 2009).

Another promising way to increase the sensitivity of the cirDNA-based assay was recently developed, based on the observation that part of the extracellular DNA in blood is bound to the surface of blood cells (Kolesnikova et al. 2008). Presence of tumor-associated methylated markers was demonstrated in the cell-surface-bound cirDNA (csb cirDNA) fractions, which were obtained by successive treatment of blood cells with EDTA-containing PBS and trypsin solution (Fig. 7.1). It was shown that using csb cirDNA as a template for MSP increased the rates of

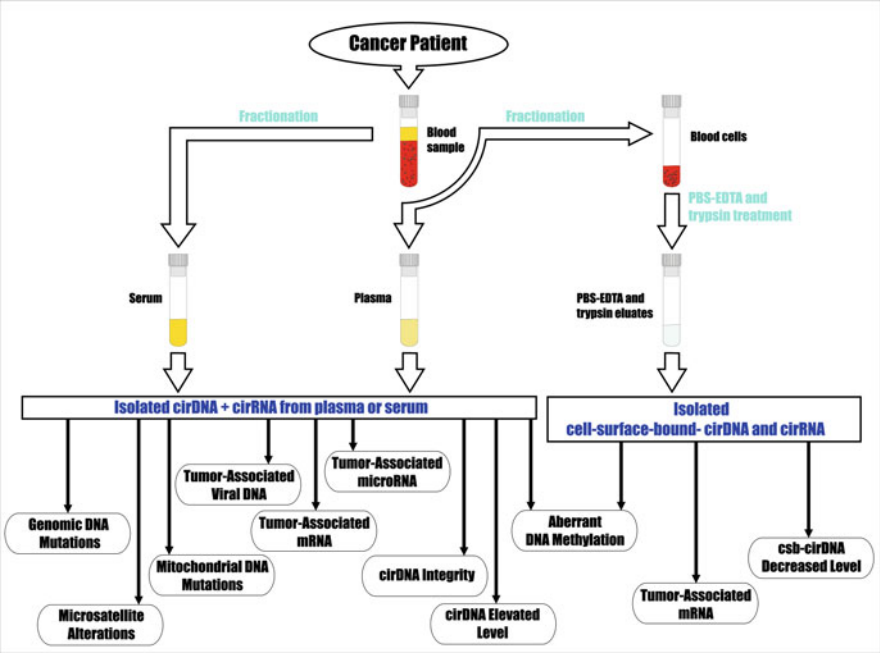

Fig. 7.1 General scheme of circulating nucleic acids preparation and their use for the detection of cancer-associated changes. Blood serum and plasma samples are the sources of the cirNA. A fraction of cirNA is tightly associated with the cell surface and can be isolated by successive treatment of the blood cells by EDTA-containing phosphate-buffered solution (PBS-EDTA), followed by mild trypsin treatment (Skvortsova et al. 2006)

gastric and breast tumor detection compared with the using of plasma cirDNA only (Skvortsova et al. 2006; Kolesnikova et al. 2008).

7.2.4 Viral DNA in the Circulation

Lo et al. were the first group who reported the presence of tumor-associated Epstein–Barr virus (EBV) DNA in plasma of 96% of nasopharyngeal carcinoma (NPC) patients (Lo et al. 1999a). EBV DNA in blood plasma has been the most extensively studied phenomenon regarding its application for the NPC detection and therapy monitoring (Chan and Lo 2007). EBV DNA molecules in plasma were found to be short DNA fragments of less than 200 bp and not associated with virions, which is consistent with the hypothesis that cell-free viral DNA may be shed from malignant cells (Suwiwat et al. 2007).

Detection of plasma EBV DNA concentration in combination with plasma anti-EBV IgA level possessed the NPC diagnostics with high sensitivity (up to 99%) and specificity (more than 90%) (Leung et al. 2004; O et al. 2007). Plasma EBV DNA levels were found to correlate to tumor burden, therapeutic treatment, disease recurrence, and survival (Chan and Lo 2007). Patients who responded to a therapy demonstrated a significant reduction of plasma EBV DNA, whereas there was no change in nonresponders (To et al. 2003; Ozyar et al. 2007). Lin et al. demonstrated that 5-year overall survival and relapse-free rates were significantly different between three groups of NPC patients, which had different values of both pre- and posttreatment EBV DNA concentrations in blood plasma (Lin et al. 2007b). A positive correlation between plasma and urine EBV DNA concentration was found, indicating that urine EBV DNA analysis may potentially be applicable as an ultra-noninvasive test (Chan et al. 2008b). Thus, quantitative analysis of EBV DNA in plasma can become a robust test for screening, staging, monitoring, and prediction of relapse in patients with NPC.

In addition to NPC, EBV DNA in blood plasma was detected with high rates in patients with T-cell, NK-cell lymphomas and peripheral T-cell proliferative diseases (Suwiwat et al. 2007). Cell-free circulating EBV DNA was present in the plasma of 43% of Hodgkin's lymphoma patients, correlated with advanced disease and was a valuable marker of chemotherapy response (Gandhi et al. 2006).

In contrast to EBV DNA in NPC, the potential application of circulating human papillomavirus (HPV) DNA in cervical cancer (CC) has not been investigated systematically. Probably due to the low level of cell-free HPV DNA in the circulation (median 202 copies/ml plasma), the incidence of circulating HPV DNA in CC patients was found to vary from 12% to 65% in the reports (Yang et al. 2004). The sensitivity of HPV DNA assay in blood plasma was shown to be increased to 65% using nested PCR method, which detected at least ten genital types of HPV with a sensitivity of one viral copy (Wei et al. 2007). Plasma HPV DNA level was found to be related to clinical response, therapeutic treatment, and CC recurrence.

7.2.5 Tumor-Associated mRNA Detection in Blood

Tumor-derived RNA was first reported to be detectable in plasma or serum of cancer patients with NPC and melanoma using reverse transcriptase PCR (RT-PCR) (Lo et al. 1999b; Kopreski et al. 1999). Detection of circulating RNA was proposed to have advantages over DNA due to the fact that RNA is more tumor-specific (Fleischhacker and Schmidt 2007; Chan and Lo 2007). However, the main limitation for the applicability of mRNA markers can be the low cell-free RNA abundance in plasma and lack of integrity (El-Hefnawy et al. 2004). Plasma cirRNA is degraded compared with the pool of cellular RNA; however, the amplification of at least 310-bp-long products is allowed (Cerkovnik et al. 2007). Circulating RNA was shown to be protected from degradation by nucleases either due to being packaged in apoptotic bodies (El-Hefnawy et al. 2004) or inside the small (50–90 nm) membrane-bound particles (exosomes) (García et al. 2008).

The most common tumor markers are considered to be the components of human telomerase reverse transcriptase (hTERT) mRNAs, which were shown to be detectable in serum and plasma of patients with breast cancer (44%), CRC (82%), follicular lymphoma (100%), lung cancer (89%), and HCC (90%) (Chen et al. 2000; Khair et al. 2007; Tong and Lo 2006a, b; Miura et al. 2006). hTERT mRNA was superior to other tumor markers in detection and prognosis of lung, gynecological, hepatocellular, and gastric cancer diagnosis (Miura et al. 2006, 2007; Tani et al. 2007).

Other commonly used RT-PCR targets in cancer studies in blood cells and plasma are cytokeratins CK19, CK20, and carcinoembryonic antigen (CEA). Cytokeratin 19 (CK19) in combination with mammaglobin mRNA was associated with poor prognosis in breast cancer (Silva et al. 2001). CK19 and CEA mRNA in plasma was shown to be associated with advanced stages of CRC and to be a prognostic factor of the distant metastases development (Silva et al. 2002). The main limitation of using cytokeratin mRNAs as tumor markers is their high rates of positive results in control (Khair et al. 2007).

A number of tissue-specific markers were selected on the basis of targeted approach, such as beta-catenin and CD133 for CRC, mammaglobin for breast cancer, tyrosinase for melanoma, and hnRNP B1 ribonucleoprotein for lung cancer (Lin et al. 2007a; Wong et al. 2004; Sato et al. 2008). In colon cancer patients, two plasma markers LISCH7 and thymidylate synthase (TS) mRNA were recently found to be strongly associated with tumor staging and were considered to be poor prognosis factors (Garcia et al. 2006b, 2007). The elevated levels of ki-67 and RASSF1A mRNAs were found in plasma and cell surface-bound cirRNA fraction in breast cancer patients (Fig. 7.1) and were valuable for the discrimination of breast malignant and benign tumors (Rykova et al. 2008a).

Differentially expressed genes were identified in serum from breast cancer patients (O'Driscoll et al. 2008).Three selected markers were further confirmed by quantitative RT-PCR to be significantly increased in cancer patient serum. In another study, five biomarkers selected from 335 transcripts, differentially expressed in serum, yielded sensitivity 91% and specificity 71% in oral cancer diagnostics

(Li et al. 2006). Among six genes, which were considered candidate neuroendocrine tumors (NET) marker genes, Tph-1 mRNA in plasma was 100% specific for detecting small intestinal-NET disease (Modlin et al. 2009).

Recent studies demonstrate miRNA in plasma as an extremely promising marker of human cancer, which is described by Tewari and Mitchell in the next section.

7.2.6 CirNA Elevated Levels in Cancer

Elevated levels of cirDNA were found in cancer and some other pathologies associated with the increase of the cell death originated products, coming into circulation (autoimmune disorders, trauma, stroke, etc). As far as investigators used different techniques for the sample processing, DNA extraction, and analysis, for healthy group the mean concentration of cirDNA varied from 1 to 27 ng/ml between the studies. The mean cirDNA concentrations were elevated up to several hundreds of ng/ml in some cancer groups (Pathak et al. 2006; Fleischhacker and Schmidt 2007). Highly sensitive techniques based on the intercalating dyes fluorescence and quantitative PCR have been used in recent studies. However, the data obtained by different qPCR assays are not always being in agreement, due to the different size and gene location of the PCR amplification target (Ellinger et al. 2008a).

In prostate cancer, plasma cirDNA was shown to be elevated compared with that in BPH (Chun et al. 2006). However, similar amounts of cirDNA in prostate cancer and healthy plasma and even higher amounts in that from BPH compared with cancer (Boddy et al. 2005). In esophageal cancer, β-actin gene level in plasma was found to be increased (Banki et al. 2007). In contrast, Herrera et al. found no difference between plasma cirDNA β-actin levels in esophageal cancer patients versus controls (Herrera et al. 2005).

In gastric cancer, the significant increase of the cirDNA level was detected in plasma and serum using fluorescence-based assay and qPCR (Kolesnikova et al. 2008; Sai et al. 2007). In patients with breast cancer and benign breast tumors, cirDNA levels were elevated compared with healthy women (Skvortsova et al. 2006; Catarino et al. 2008; Kohler et al. 2009). Moreover, benign and malignant breast tumors can be discriminated by the changes of cell-surface-bound cirDNA amount (Fig. 7.1) (Skvortsova et al. 2006). Patients with benign breast tumors had the same amount of csb cirDNA as healthy women and the significantly decreased level of csb cirDNA was in breast cancer patients (Skvortsova et al. 2006).

A return of plasma cirDNA level to normal after the surgery indicated the complete resection of the tumor in esophageal, colorectal, breast, and lung cancer (Banki et al. 2007; Frattini et al. 2008; Catarino et al. 2008). Increase in plasma cirDNA level during follow-up in cancer patients after surgery was significantly associated with development of recurrences or metastases (Banki et al. 2007; Frattini et al. 2008). Hence, the post-operative cirDNA level can be a valuable factor for therapy response monitoring and cancer relapse detection.

A part of cirDNA was found to be present in plasma and serum as DNA complexed with histones as nucleosomes, which may come from damaged and stressed cells (Holdenrieder et al. 2008; Holdenrieder and Stieber 2009). Enzyme-linked immunosorbent assays (ELISAs) have been developed for the circulating nucleosome quantification, which detected significant changes of their concentration in malignant and benign diseases. Nucleosome concentration was found to be a valuable marker for the early estimation of therapy outcome during the first week of chemo- and radiotherapy in lung, pancreatic, and CRC patients (Holdenrieder et al. 2008; Holdenrieder and Stieber 2009).

Several studies indicate that the elevated levels of mtDNA in plasma are of diagnostic significance in prostate and testicular germ cell cancers (Mehra et al. 2007; Ellinger et al. 2009). Elevated mtDNA in plasma was associated with poor prognosis and survival, being independent of current prognostication factors (Mehra et al. 2007). Circulating mtDNA levels were significantly lower in patients with breast tumors (benign and malignant) compared with healthy donors (Kohler et al. 2009).

Concentration of cirRNA in cancer patients stays less investigated. Some reports indicated that cancer patients might have significantly increased cirRNA levels in plasma and serum (Goebel et al. 2005; Feng et al. 2008). Others did not find the significant difference between malignant patients and control group (Sueoka et al. 2005; Cerkovnik et al. 2007).

7.2.7 DNA and RNA Integrity

CirDNA represents a population of heterogenic size molecules and several investigations have shown that cirDNA fragmentation is less pronounced in cancer patients, which can result from decreased activity of DNA-hydrolyzing enzymes in the blood (Vlassov et al. 2007). An increase in cirDNA integrity (the ratio of longer to shorter DNA fragments) was shown in ovarian and esophageal cancer patients using β-actin gene (Wang et al. 2003; Tomita et al. 2007) and in NPC using leptin gene as targets with different amplicon sizes (Chan et al. 2008c). Umetani et al. proposed to measure DNA integrity in serum by qPCR of Alu repeats with two sets of primers (115 and 247 bp) as a potential biomarker for patients with breast and CRC (Umetani et al. 2006a, b). Sunami et al. have shown the elevation of long LINE-1 (300 bp) copy number in cancer patient serum as the result of total cirDNA quantity and DNA integrity increase (Sunami et al. 2008).

Plasma RNA integrity was also proposed to be a potential tumor marker, as far as 3′ to 5′ GAPDH mRNA ratio was significantly lower in the NPC plasma compared with healthy controls (Wong et al. 2006).

7.2.8 Technical Aspects of cirNA Detection

As mentioned above, disagreements between cirNA studies have come from the use of a wide variety of protocols for the sample preparation, nucleic acids extraction, and analysis techniques (Fleischhacker and Schmidt 2007; Tong and Lo 2006a, b). The sample processing and storage temperature were found to have an influence on the DNA and RNA yield (Chan et al. 2005; Holford et al. 2008). CirDNA amount in the stored plasma was declined depending on the time and the number of freezing and thawing, while no influence of the storage time and temperature was detected on the amount of the extracted cirDNA samples (Chan et al. 2005).

Plasma and serum samples have been used as the source of cirNA for the assays. Notably, serum samples yield up to six times higher DNA levels than those obtained from plasma (Thijssen et al. 2002). Compared with plasma,serum samples were highly variable between patients (Boddy et al. 2005). The use of paired plasma and serum samples for the qPCR GAPDH gene quantification have led to different results of the benign and malignant breast lesions comparison (Zanetti-Dällenbach et al. 2008). It was generally assumed that extraneous DNA release into serum occurs through the process of coagulation during cell clotting.

One of the critical factors is the preparation of samples free from contaminating blood cells, which could be achieved by additional centrifugation or filtration. Plasma samples subjected to two centrifugation steps were more reliable, compared with one-spin plasma and serum (Boddy et al. 2005; Chiu et al. 2001). The protocols used for the DNA and RNA isolation as well as the sample volume used for extraction vary markedly. The commonly used commercial DNA isolation kits have different DNA extraction efficiencies especially for the low-molecular weight fragments (lower 150 bp), which poorly bind to the extraction columns (Kirsch et al. 2008). The higher amount and greater range of fragment sizes of the isolated cirDNA was obtained using either salting out or phenol–chloroform extraction procedures (van der Vaart and Pretorius 2008). Using plasma concentrating methods, higher amounts of cirRNA were isolated from normal plasma, while its integrity was not affected (El-Hefnawy et al. 2004; Cerkovnik et al. 2007).

It is necessary to develop the standard cirNA-based assays, which could be acceptable for clinical use. The accuracy of the assays can be increased by the automated rather than manual cirNA isolation and detection. The robust assays should use the sensitive high throughput techniques as MALDI-TOF Mass Spectrometry-based genotyping of single nucleotide polymorphisms (SNPs) (Ding and Lo 2006) and digital PCR-based gene mutation detection (Lo et al. 2007; Zimmermann et al. 2008). The second generation DNA sequencing methods have been recently used for the cirDNA sequence analysis and methylation profile investigation (Fan et al. 2008; Beck et al. 2009; Korshunova et al. 2008). Due to high costs, the cirNA diagnostic applications using massively parallel sequencing will not come to medical practice soon; nonetheless, the great potential of their future development is emerging (Lo and Chiu 2009).

7.3 Circulating Nucleic Acids in Other Diseases

7.3.1 CirNA in Diabetes Mellitus

Diabetes mellitus is one of the major health problems all over the world. In diabetic patients, plasma cirDNA concentration, measured by means of β-actin qPCR, is significantly changed compared with healthy subjects (Butt et al. 2006). Certain mRNA species can become more specific plasma markers for assessing beta cell function in vivo, as Pdx1, Egr1, and Chgb, which were found to be reproducibly detected in the cultivation medium of insulin-producing cells (Fig. 7.2) (Rani et al. 2007).

Diabetic retinopathy, nephropathy, and neuropathy are the major complications of the disease, leading to blindness, renal failure, and amputations, respectively. Swaminathan et al. have shown that retina-specific rhodopsin, RPE65, and retinoschisin mRNAs were significantly associated with severity of retinopathy (Shalchi et al. 2008; Butt and Swaminathan 2008). Renal damage in diabetic patients was found to be associated with the nephron-specific nephrin mRNA increase in patient plasma (Butt et al. 2006). Significant differences of the nephrin mRNA level in plasma were found between normoalbuminuric and macroalbuminuric diabetic patients and the control subjects. Enolase mRNA increase in plasma can become an important marker for the discrimination of diabetic patients with neuropathy from diabetic controls (Sandhu et al. 2008).

Specific miRNA expression patterns were identified in serum from diabetes, lung cancer, CRC, and healthy donors, providing evidence that serum miRNAs contain fingerprints for various diseases (Chen et al. 2008). Notably, diabetes patients and lung cancer patients share a number of common serum miRNAs that are not found in

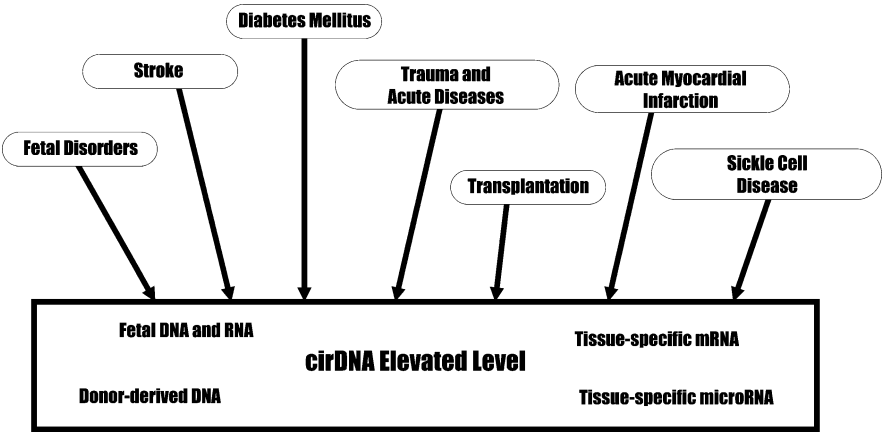

Fig. 7.2 Potential applications of the cirNA-based assays for detection and prognosis of different disorders

healthy subjects. Whether these miRNAs reflect a general inflammatory response shared by various diseases should be further investigated.

7.3.2 Trauma and Acute Diseases

Plasma cirDNA concentration was shown to increase in patients with massive tissue destruction, suggesting that cell death is one of the mechanisms leading to the exNA release into the circulation. The levels of genomic DNA and mtDNA in plasma of patients with blunt and burn injuries were significantly related to the severity of injury and the outcome (Lam et al. 2004; Chiu et al. 2006). Remarkably, plasma cirDNA concentrations were significantly higher in patients who subsequently developed acute lung injury, acute respiratory distress syndrome, or died than those without complications (Chiu et al. 2006). Thus, plasma cirDNA elevation had a predictive value for posttraumatic organ failure and multiple organ dysfunction syndrome.

Plasma cirRNA level, measured by means of GAPDH qRT-PCR, was significantly higher in the filtered plasma portions (but not in the unfiltered plasma) from trauma patients as opposed to controls (Rainer et al. 2004). Furthermore, increased plasma cirRNA levels were associated with severity of injuries and death.

Changes of plasma cirDNA concentrations were found to have predictive significance for patients admitted to the intensive care unit (ICU) with different pathologic states, for example, in patients with acute abdominal pain (Rainer et al. 2008). Median plasma cirDNA concentrations were threefold higher in patients with systemic inflammatory response syndrome, fivefold higher in patients who died within 28 days, and eightfold higher in patients admitted to the ICU (Rainer et al. 2008). Rhodes et al. demonstrated strong association of the increased plasma cirDNA concentrations with morbidity in ICU patients and with the requirement of renal support (Rhodes et al. 2006). CirDNA had a sensitivity of 92% and specificity of 80% when a concentration higher than 127 ng/ml was chosen as a cutoff level in ICU patient death prediction.

Severe sepsis and septic shock represent the major causes of death in patients admitted to the ICU. Nucleosomal DNA level in plasma increased with severity of sepsis (Zeerleder et al. 2003), as well as the elevated level of the first-day plasma cirDNA was found to be an independent predictor of ICU mortality of patients with severe sepsis (Saukkonen et al. 2008). The authors found no predictive value of cirDNA for hospital mortality and concluded that a panel of different markers should be evaluated to predict the outcome of a patient in a complicated disease like sepsis.

A number of miRNA expression profiles found in peripheral blood leukocytes were found to differentiate sepsis patients from healthy controls (Vasilescu et al. 2009). Moreover, in sepsis patient, plasma miR-150 levels were significantly reduced and correlated with the level of disease severity measured by SOFA score. Plasma levels

ratio for miR-150/interleukin-18 were found to be valuable for assessing the severity of sepsis.

7.3.3 Exhaustive Exercise

Hypoxya and increased radical production during exhaustive exercise are known causative factors for DNA degradation and apoptosis. Plasma cirDNA concentration was shown to be increased up to 17.5-fold immediately after long-distance running, both half-marathon and ultra-marathon distance and returned to preface levels 24 h later (Atamaniuk et al. 2008). Fatouros et al. demonstrated that plasma cirDNA concentration may serve as a sensitive marker for the overtraining-induced inflammation in subjects during a chronic excessive resistance exercise (Fatouros et al. 2006).

7.3.4 Acute Myocardial Infarction

Tenfold increase of cirDNA levels were found in blood plasma from patients with acute myocardial infarction (AMI) compared with healthy donors (Chang et al. 2003), which correlated with the traditional biochemical markers of AMI as CK and troponin (Antonatos et al. 2006). Patients with complicated post-AMI course have shown 3.5-fold increase of cirDNA level compared with patients without complications (Destouni et al. 2009). Rainer et al. investigated plasma cirDNA concentration, measured using β-globin-specific qPCR, as a prognostic marker in patient chest pain as probable cardiac cause (Rainer et al. 2006). Increase of plasma DNA concentration was found to be related to the development of complications including early cardiac arrest, cardiac failure, 6-month readmission, and 2-year mortality.

Ji et al. (2009) miR-208, specifically produced in heart, as a candidate marker for the AMI diagnostics. They demonstrated plasma miR-208 level increase in the isoproterenol-induced myocardial injury in rats, which correlated with the concentration of plasma cardiac troponin I, a classic AMI biomarker.

7.3.5 Stroke

Stroke results in central nervous system injury and release of the neuropathological markers into the circulation through the disruption of the blood–brain barrier. Plasma cirDNA elevation was an independent predictor of stroke outcome in patients with negative neuroimaging results and had the same value as S100 protein in predicting poststroke morbidity and mortality in the first 24 h since symptom onset (Lam et al. 2006). Combination of plasma cirDNA and S100 protein was found to be

useful for diagnosing hemorrhagic stroke within 6 or 24 h of symptom onset (Rainer et al 2007). Increased nucleosomal cirDNA levels were also correlated with morbidity and mortality in the early stroke patients (Geiger et al. 2006).

Tissue-specific mRNAs seem very promising as plasma markers in different pathologic conditions. Chan et al. proposed that S100 calcium-binding protein B (S100B) mRNA could be used as brain-specific plasma marker of stroke (Chan et al. 2007). Notably, using bone marrow transplantation model, they found that plasma S100B was predominantly of hematopoietic origin, indicating that caution is required in the interpretation of the presumed origin of plasma RNA markers.

A few miRNAs (miRNA-298, miRNA-155 and miRNA-362-3p) were upregulated and downregulated more than twofold in both brain and blood of experimental rats after several different injuries, such as ischemic strokes, brain hemorrhage, and kainate seizures (Liu et al. 2010). Increase of miR-133 (but not miR-122 and miR124) level in plasma was specifically detected in an experimental rat surgical model of stroke (Laterza et al. 2009). These data propose possible use of blood miRNA as biomarkers for human brain injury.

7.3.6 Transplantation

Donor-derived DNA presence in plasma of recipients, the so-called DNA microchimerism, has been detected in plasma of liver, heart, and kidney transplantation recipients (Hubacek et al. 2007; Fu et al. 2006). Lui et al. showed that a median 59.5% of the cirDNA in the plasma of hematopoietic stem cell transplant (HSCT) recipients was of donor origin, shedding light on the origin of the donor-derived cirDNA (Lui et al. 2002). Recently, investigators demonstrated the presence of donor DNA in the urine of HSCT patients, which probably originated from donor-derived cytokeratin-producing epithelial cells, appearing in the renal tubules of recipient, rather than from the transrenal passage of plasma cirDNA (Hung et al. 2009).

DNA microchimerism was positively correlated with the acceptance of the transplanted organs in renal, heart, and liver transplant recipients (Fu et al. 2006; Araújo et al. 2004). The survival time of transplanted kidneys was significantly higher in the microchimerism-positive recipients (8.7 years) than in microchimerism-negative recipients (5.4 years). Y chromosome-based assays are generally used for microchimerism detection in female transplant recipients; however, the background microchimerism should be taken into account resulted from the male pregnancies and/or previous blood transfusions. Thus, more than 50% of female recipients were found to carry chimeric DNA in their plasma before transplantation (Vymetalova et al. 2008).

Organ-specific RNAs were investigated as potential markers of graft rejection, which could predict the renal transplantation outcome after an episode of acute rejection. The mean ratio of a functional factor for regulatory T lymphocytes (FOXP3) mRNA copies to 18 S rRNA copies was significantly increased in urine

from patients with acute rejection (3.8) compared with chronic allograft nephropathy or normal biopsy (1.3 and 1.6, respectively) (Muthukumar et al. 2005). Acute rejection of renal allograft was recently associated with alterations in miRNA expression within allografts and was accompanied with the same miRNAs over-expression in the donor peripheral blood mononuclear cells (PBMCs) (Anglicheau et al. 2009).

7.3.7 Sickle Cell Disease

The most recent observation of the cirDNA level changes was in the sickle cell disease (SCD) (Vasavda et al. 2007). Acute painful crises in SCD were associated with several fold increases in the cirDNA when compared with steady state, indicating that cirDNA may have potential as a biomarker in SCD patients' crises. Recently, cirDNA was also found to be useful in monitoring response to hydroxyurea therapy in SCD (Ulug et al. 2008).

7.4 Potential Therapeutic Implications of the cirNA

Over the past decade, cirNAs have been increasingly studied as valuable diagnostic tools. In contrast, their roles in the normal and pathologic states of the organism have not been established. Some indications of the cirNA biological significance were highlighted in separate studies, which showed their potential for the development of the novel therapeutic approaches in cancer and other diseases.

The concept of messenger DNA was first proposed by Gahan and Chayen (1965) and was recently supported by the observation that transfer of tumor DNA from dying to living cells is possible via the uptake of apoptotic bodies (Bergsmedh et al. 2001). The hypothesis of "genometastasis" was suggested to describe the possibility that tumor-derived cirDNA may transform normal susceptible cells located in distant target organs with dominant oncogenes, which result in formation of metastases (García-Olmo et al. 2005). Based on the genometastase hypothesis, the research for the metastases prevention using DNAse in vivo was stimulated. DNase I injections significantly reduced metastases development in the mice model of Lewis lung carcinoma, indicating the potential application of the DNAse-based therapy in human cancer (Shklyaeva et al. 2008).

Recent studies provide evidence that extracellular RNA, present at the sites of cell damage or vascular injury, can induce vascular endothelial growth factor (VEGF)-dependent hyperpermeability of brain-derived microvascular endothelial cells in vivo and in vitro (Fischer et al. 2009). exRNA coming from necrotic cells in site of ischemic stroke was shown to impair the brain–blood barrier and contribute to vasogenic edema formation. Application of ribonuclease I (RNase I) diminished

edema-formation and also reduced lesion volume in experimental stroke (Walberer et al. 2009).

Numerous groups have identified miRNAs as the molecules involved in cancer progression (Zoon et al. 2009). Because of their size, abundance, tissue specificity, and relative stability in plasma, circulating miRNAs hold promise as unique accessible biomarkers to monitor pathologic states. Tumor-derived miRNAs were found in plasma, leading to the proposal that these molecules could be involved in a systemic environment creation that is conducive to disease progression (Jackson 2009). This hypothesis was supported by the recent findings that mRNA and miRNA can be transferred from one cell to another by the naturally cell-derived exosomes and be functional in this new environment (Yuan et al. 2009; Skog et al. 2008). Moreover, glioblastoma exosomes stimulated proliferation of a human glioma cell line, indicating a self-promoting aspect (Skog et al. 2008). As far as miRNAs are continuously being shown to act as both oncogenes and tumor suppressors, exosomes containing tumor suppressor miRNAs may serve as novel therapeutic molecules, which has advantages over siRNA, such as in vivo stability and no evident toxicity (Wang et al. 2009; Zhang and Farwell 2008).

Tumor-derived DNA-containing nucleosomes in plasma were proposed to be important for evasion of tumor cells from immunological surveillance. Nucleosomes incorporated by immune cells were found to inhibit natural killer cell-mediated lysis of tumor cells (Hengartner 2001). Since tumor-derived RNA-containing exosomes in plasma were established to have a high concentration of tumor antigens, Ichim et al. proposed that exosomes may induce an abortive T cell activation, leading to immunological anergy (Ichim et al. 2008). Thus, the removal of these immunosuppressive microvesicles from circulation by an extracorporeal filtration could be useful in anticancer therapy.

7.5 Conclusion

Potential of cirNAs for the development of the low-invasive tests for cancer diagnostics have been suggested by a number of studies. Changes of the cirDNA and cirRNA in plasma and serum can be used as sensitive and specific markers for cancer diagnostics, staging, prognosis, and monitoring of the response to therapy. Other applications are the disease prognosis and therapy monitoring following trauma, stroke, myocardial infarction in diabetes and some other disorders. However, detailed studies should be performed for these potential biomarkers to become a routine tool in the laboratory medicine. To become valuable in the clinical setting, cirNA assays should be standardized, automated, and certified in order to enable rapid and reliable detection and quantification in clinical laboratories. The use of high throughput and robust methodologies for diagnostic laboratories should bring rapidly forward the selection of the informative biomarkers. Additional research is needed in the area of the pathologic significance of the exNA, coming out from tumor cells or damaged tissues and entering the circulation.

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Chapter 8
Circulating MicroRNAs in Cancer

Patrick S. Mitchell and Muneesh Tewari

Contents

Abstract MicroRNAs (miRNAs) are small (~22 nucleotide) non-protein-encoding RNAs that posttranscriptionally regulate gene expression via suppression of specific target messenger RNAs. Aberrations in miRNA expression have been associated with cancer and numerous other diseases. Recently, extracellular miRNAs have been found to be present in the circulation, where they exist in a stable, ribonuclease-resistant form. Preliminary reports suggest that tumor-derived circulating miRNAs may serve as a new class of blood-based biomarkers for cancer. Here, we describe early advances in the research field of circulating miRNA, review the possibilities and challenges associated with the development of miRNAs as biomarkers for

P.S. Mitchell
Human Biology Division, Fred Hutchinson Cancer Research Center, 1100 Fairview Avenue North, Mailstop D4-100, Seattle, WA 98109, USA
Molecular and Cellular Biology Program, University of Washington, Seattle, WA, USA
M. Tewari (✉)
Human Biology Division, Fred Hutchinson Cancer Research Center, 1100 Fairview Avenue North, Mailstop D4-100, Seattle, WA 98109, USA
Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, WA, USA
Public Health Sciences Division, Fred Hutchinson Cancer Research Center, Seattle, WA, USA
e-mail: mtewari@fhcrc.org

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cancer and other disease states, and discuss the possible biological roles of this newly discovered species of circulating nucleic acid.

8.1 MicroRNAs: History, Biogenesis, and Mechanism of Action

MicroRNAs (miRNAs) are a class of small (~22 nucleotide) non-protein-encoding RNAs that posttranscriptionally regulate gene expression via suppression of specific target messenger RNAs (mRNAs) (Flynt and Lai 2008). In 1993, Lee, Feinbaum, and Ambros demonstrated that the small noncoding RNA *lin-4* regulates developmental timing by an antisense RNA:RNA interaction in the nematode *Caenorhabditis elegans* (Lee et al. 1993). MiRNAs are now recognized as a major mode of posttranscriptional gene regulation conserved from many single cell eukaryotes to humans (Christodoulou et al. 2010), with widespread regulatory functions in diverse biological processes, including development (Kloosterman and Plasterk 2006; Stefani and Slack 2008), apoptosis (Jovanovic and Hengartner 2006), and immunity (Taganov et al. 2007). The current version of miRBASE v14.0 (Griffiths-Jones et al. 2008) contains over 700 human miRNA sequences comprising ~1% of all transcribed genes in the human genome; these miRNAs are estimated to regulate at least 30% of all mRNAs via interactions with partially complementary nucleotide sequences in the 3′UTR of targeted mRNAs (Rajewsky 2006).

MiRNAs are encoded by much longer primary miRNA (pri-miRNA) transcripts that may encode either single or multiple miRNAs. The mature miRNA sequences in these transcripts exist in the context of a hairpin structure, in which the miRNA represents either the 5′ or 3′ arm of the hairpin stem within the pri-miRNA transcript (Fig. 8.1). In the nucleus, the RNase III endonuclease Drosha cleaves at a site adjacent to the hairpin to produce a ~60–100 nucleotide precursor miRNA (pre-miRNA), which is exported to the cytoplasm for further processing by the Dicer/TRBP complex (Fig. 8.1). Processing by Dicer produces the mature ~22 nucleotide miRNA duplex, from which the active, mature miRNA strand is subsequently loaded into a protein complex called the miRNA-induced silencing complex (miRISC) (Kim et al. 2009; Rana 2007). MiRNA-mediated posttranscriptional repression occurs by miRNA binding to sites predominately located within the 3′UTR of its mRNA target(s) with perfect or partial complementarity, leading to mRNA cleavage or translational block, respectively (Valencia-Sanchez et al. 2006). MiRNA targeting to specific mRNAs is largely governed by sequence complementarity of nucleotides 2–8 at the 5′ end of the mature miRNA sequence (known as the "seed" sequence), although sequence motifs in the 3′ end of the miRNA have also been shown to influence the efficiency of miRNA-mediated repression of target mRNAs (Bartel 2009).

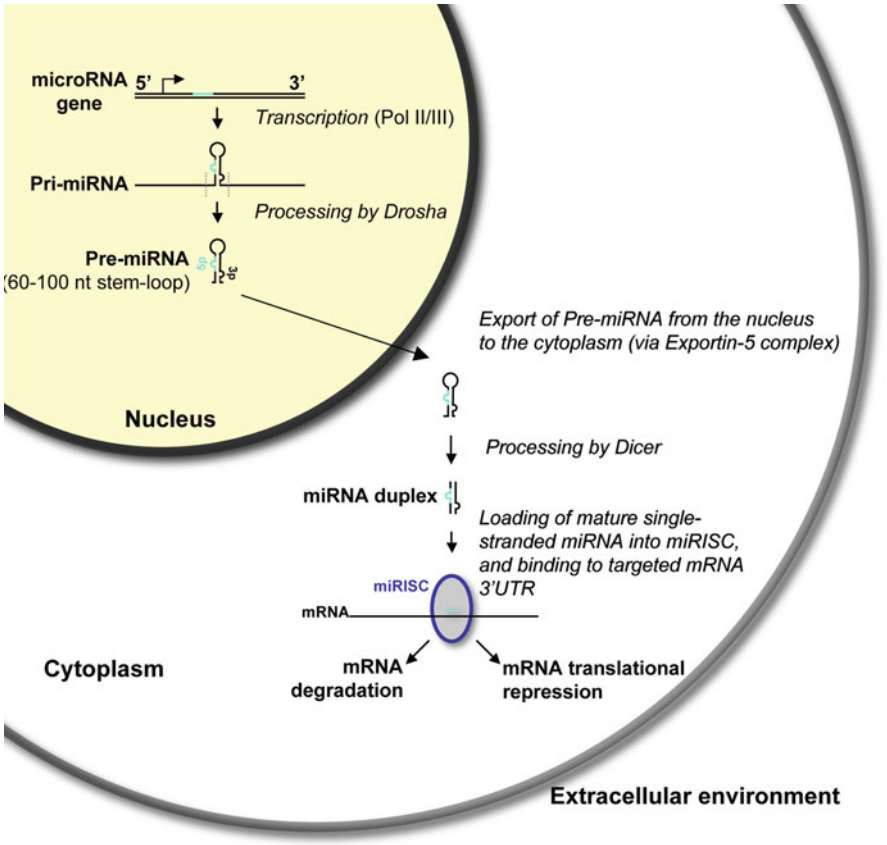

Fig. 8.1 A simplified model of miRNA biogenesis and targeting. *Pri-miRNA* primary microRNA; *Pre-miRNA* precursor microRNA; *miRISC* miRNA-Induced Silencing Complex; *UTR* untranslated region

8.2 Tumor Cell miRNAs in Cancer Biology and as Tissue-Based Disease Biomarkers

Altered expression and/or activity of cellular miRNAs is believed to contribute to cancer and other human diseases (Esquela-Kerscher and Slack 2006; Lu et al. 2005). One of the early reports implicating miRNAs in tumorigenesis came from Calin et al. who demonstrated that the genomic loci encoding miR-15a and miR-16-1 are deleted in chronic lymphocytic leukemia cells and that these miRNAs normally target the antiapoptotic protein BCL2 (Cimmino et al. 2005). A multitude of studies have since shown that miRNAs can contribute to both epithelial and hematopoietic cancers by acting as either tumor suppressors or oncogenes (also known as "oncomiRs") (Calin and Croce 2006; Esquela-Kerscher and Slack 2006). Two such examples in cancer are perturbations in the expression of let-7, a miRNA

targeting the oncogene Ras (Johnson et al. 2005), and the aberrant overexpression of miR-21 and subsequent downregulation of its target, the tumor suppressor PTEN (Meng et al. 2007). Abnormal miRNA expression appears to be subject to the same genomic alterations (e.g., mutation, chromosomal rearrangements, and amplification/deletion (Calin and Croce 2006; Giannakakis et al. 2007)) and regulatory processes (e.g., promoter methylation, RNA editing (Lujambio and Esteller 2007; Schmittgen 2008)) as protein-coding genes associated with cancer. Disruption of miRNA biogenesis has also been suggested to contribute to tumorigenesis (Merritt et al. 2008), although this is less clearly understood.

Global profiling of miRNAs in malignant compared to nonmalignant tissue has demonstrated that miRNA expression profiles can distinguish cancer types as well as developmental lineage and differentiation state (Lu et al. 2005), suggesting that miRNAs may serve as cellular prognostic and diagnostic biomarkers. MiRNA expression signatures in cancer tissue have been shown to be capable of identifying the tissue of origin of the cancer as well as to predict prognosis (Calin et al. 2005; Rosenfeld et al. 2008).

Aside from cancer, dysregulation of miRNA expression and/or function has been shown to be important in the pathogenesis of a number of diseases, including congestive heart failure, numerous autoimmune disorders, and diabetes (Latronico and Condorelli 2009; O'Connell et al. 2010; Poy et al. 2007). Taken together, the literature indicates that aberrant miRNA regulation has a broad impact on human disease.

8.3 Circulating MicroRNAs as a Novel Class of Blood-Based Biomarker for Cancer Detection: Potential Utility and Current Challenges

Evidence of miRNAs present in the circulation was first published by Chim et al. (2008) who reported the detection by quantitative reverse-transcription PCR (qRT-PCR) of miRNAs of apparent placental origin in the plasma of pregnant women. Shortly thereafter, Lawrie et al. (Lawrie et al. 2008) reported elevated levels of specific miRNAs in serum from lymphoma patients, and our group described the direct cloning and sequencing of miRNAs from human plasma-derived small RNA cDNA libraries (Mitchell et al. 2008). Using a human prostate cancer cell line mouse xenograft system, we demonstrated that human-specific, tumor-derived miRNAs enter the circulation and are robustly detected in plasma from tumor xenografted animals but not from mock-injected animals. Extending this observation to human cancer of non-hematopoietic origin, we showed that serum levels of an epithelial-specific miRNA, miR-141, could distinguish healthy individuals from patients with advanced prostate cancer (Fig. 8.2) (Mitchell et al. 2008).

Since these original reports, multiple studies (summarized in Table 8.1A) have demonstrated unique miRNA signatures in the plasma or serum of patients relative to healthy individuals for a wide array of malignancies. Additionally, several reports

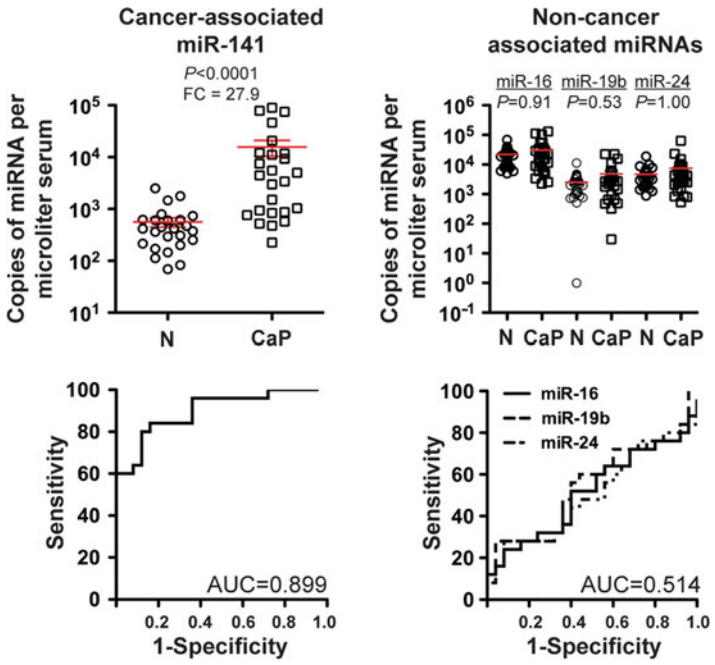

Fig. 8.2 Circulating miR-141 as a prostate cancer biomarker. *Upper*: Serum levels of miR-141 (*left column*) or noncancer associated miRNAs miR-16, miR-19b, and miR-24 (*right column*) were measured in individual serum samples by TaqMan miRNA qRT-PCR, where miRNA abundance is given in terms of miRNA copies/μl serum. Red bars, mean +/− SEM of miRNA copies/μL serum for each group. *P* values were assigned by Wilcoxon signed-rank test. *Lower*: Receiver operating characteristic (ROC) curves plot sensitivity vs. (1 – specificity) to assess the ability of each miRNA biomarker to distinguish CaP and normal (i.e., healthy control) sera. *AUC* area under the curve; *CaP* prostate cancer patient sera; *FC* fold-change; *N* normal sera (i.e., from individuals with normal PSA and normal digital rectal exam). The figure shown is adapted from Mitchell et al. (2008)

indicate that circulating miRNAs as biomarkers may be applicable to other pathophysiologic states as well, such as diabetes, myocardial infarction, and liver damage (summarized in Table 8.1B), as well as pregnancy (summarized in Table 8.1C). Taken together, these studies provide proof-of-principle for the development of noninvasive, blood-based miRNA tests for the detection and monitoring of cancer and other disease states.

However, research in this area is at an early stage from the perspective of the development of near-term clinical diagnostics for cancer as there are many unknowns and challenges that need to be resolved. One important unknown is whether tumor-derived miRNA markers can detect early-stage cancer, and more specifically detect cancer before it would otherwise be diagnosed based on symptoms or currently available screening approaches. Most studies to date have examined plasma or serum samples from patients with well-established, clinically apparent cancers. In our own laboratory, we have noted that the serum miRNA

Table 8.1 A review of the circulating miRNA literature

Title	Disease type	Comments	References
A. Cancer-associated			
Detection of elevated levels of tumor-associated microRNAs in serum of patients with diffuse large B-cell lymphoma	B-cell lymphoma	Serum levels of miR-21, miR-155, and miR-210 were associated with patients with diffuse large B-cell lymphoma in comparison to healthy controls.	Lawrie et al. (2008)
Circulating microRNAs as blood-based biomarkers for cancer detection	Prostate cancer	miRNAs from plasma were identified via cloning and sequencing and measured by qRT-PCR. Initial characterization of circulating miRNAs demonstrated that plasma miRNAs are cell-free and highly stable (e.g., resistance to extended room temperature incubation and multiple freeze–thaw cycles). This study established proof-of-concept that tumor-derived miRNAs enter the circulation using a xenograft model of prostate cancer, and that specific miRNAs (e.g., miR-141) are enriched in the serum of patients with prostate cancer relative to matched, healthy controls.	Mitchell et al. (2008)
MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer	Ovarian cancer	A subset of miRNAs previously described to be elevated in ovarian cancers was enriched in immunocaptured circulating $EpCAM^+$ exosomes of presumed tumor origin.	Taylor and Gercel-Taylor (2008)
Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases	Lung and colorectal cancer, diabetes	Next-generation sequencing of miRNAs from plasma and serum revealed that specific circulating miRNAs vary between disease types — authors suggest that serum miRNAs contain "fingerprints" for various diseases. Candidate lung cancer miRNA biomarkers (miR-25 and miR-223) identified by Solexa sequencing were shown to be elevated on average in serum from patients with nonsmall cell lung cancer relative to healthy controls using qRT-PCR.	Chen et al. (2008)
Glioblastoma microvesicles transport RNA and proteins that promote tumor growth and provide diagnostic biomarkers	Glioblastoma	Microvesicles derived from glioblastoma cells were shown to transfer both protein and mRNA to recipient cells in a cell culture system. Exosome-associated miRNA profiles were found to be similar to those in the parent glioblastoma cells.	Skog et al. (2008)

The detection of differentially expressed microRNAs from the serum of ovarian cancer patients using a novel real-time PCR platform	Ovarian cancer	Using miRNA TaqMan Low-Density Arrays to profile serum miRNAs, an aberrant circulating miRNA profile was observed in patients with ovarian cancer relative to healthy controls.	Resnick et al. (2009)
Differential expression of microRNAs in plasma of patients with colorectal cancer: a potential marker for colorectal cancer screening	Colorectal cancer	Circulating levels of miR-17-3p and miR-92 in the setting of colorectal cancer. Comparison of preoperative and postoperative plasma levels of miRNA biomarkers were significantly reduced following tumor resection, suggesting that these miRNAs were tumor-derived, and that circulating miRNAs may be utilized to monitor treatment response and remission of disease.	Ng et al. (2009)
Mature miR-184 as potential oncogenic microRNA of squamous cell carcinoma of tongue	Squamous cell carcinoma (tongue)	miR-184, which is aberrantly expressed in squamous cell carcinoma of the tongue, was detected in patient plasma at levels elevated relative to healthy individuals. For the majority of patients observed, miR-184 plasma levels were significantly reduced following surgical resection of the primary tumor.	Wong et al. (2009)
Exosomal microRNA: a diagnostic marker for lung cancer	Lung adenocarcinoma	Circulating exosome-associated miRNAs were elevated on average in 28 lung cancer patients versus eight healthy individuals. Authors suggest that the exosome-miRNA signature is reflective of the tumor miRNA signature.	Rabinowits et al. (2009)
Downregulation of miR-92 in human plasma is a novel marker for acute leukemia patients	Acute myeloid leukemia and acute lymphoblastic leukemia	MiRNA microarray profiling of plasma derived from a small number of healthy individuals and AML or ALL patients found a marked decrease in the plasma level of miR-92a in cancer patients.	Tanaka et al. (2009)
Circulating microRNAs in breast cancer and healthy subjects	Breast cancer	qRT-PCR measurement of serum miR-145 and miR-155 in a small cohort of patients with breast cancer demonstrated no difference between these miRNAs relative to healthy controls.	Zhu et al. (2009)
Detection of cancer with serum miRNAs on an oligonucleotide microarray	Ovarian, lung, colorectal, breast, and prostate cancers	Global profiling of serum miRNAs using a novel microarray platform identified a set of 28 miRNA biomarkers that could classify individuals with cancer from healthy donors.	Lodes et al. (2009)

(*continued*)

Table 8.1 (continued)

Title	Disease type	Comments	References
MicroRNAs in plasma of pancreatic ductal adenocarcinoma patients as novel blood-based biomarkers of disease	Pancreatic cancer	Multivariate analysis indicated that plasma miR-21, miR-210, miR-155, and miR-196a discriminates pancreatic ductal adenocarcinoma patients from healthy controls at greater specificity and sensitivity than of the miRNA markers singly.	Wang et al. (2009a)
Plasma microRNAs are promising novel biomarkers for early detection of colorectal cancer	Colorectal cancer	Plasma levels of miR-29a and miR-92a are modestly elevated in patients with advanced adenomas relative to normal controls, suggesting that small, localized tumors may release sufficient copies of miRNA into the blood for detection of early-stage disease.	Huang et al. (2010)
Circulating microRNAs as novel minimally invasive biomarkers for breast cancer	Breast cancer	Authors measured circulating miRNAs, selected on the basis of previous association with breast cancer, in whole blood as well as in serum and plasma. Blood levels of miR-195 and let-7a were significantly increased in breast cancer patients relative to controls, and were significantly reduced following surgical resection. Additionally, circulating miR-21 and miR-10b were correlated with hormone receptor status.	Heneghan et al. (2010)
B. Tissue injury-associated			
Circulating microRNAs, potential biomarkers for drug-induced liver injury	Liver injury	Using a mouse model, authors demonstrated that circulating miRNAs could be used as biomarkers to detect drug-induced liver injury. The plasma level of liver-specific miRNA miR-122 was shown to be a more sensitive indication of liver-injury than existing markers aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Interestingly, miRNAs that were observed to decrease in expression in the liver of drug-induced animals were observed at higher concentration in corresponding plasma; however, whether this represents selective export of injury-induced miRNAs or general diffusion of cellular miRNAs from damaged cells could not be distinguished.	Wang et al. (2009b)

Plasma miR-208 as a biomarker of myocardial injury	Myocardial infarction	The plasma level of the heart tissue-specific miR-208 was elevated in rats with chemical-induced myocardial injury relative to sham-treated animals. The concentration of circulating miR-208 was not affected by other forms of tissue injury (e.g., renal infarction) and showed general agreement with a known marker of myocardial infarction, plasma cardiac toponin I (cTnI).	Ji et al. (2009)
Plasma microRNAs as sensitive and specific biomarkers of tissue injury	Tissue injury	Authors demonstrate that increases in plasma levels of tissue-specific miRNAs miR-122 (liver), miR-133a (muscle), and miR-124 (brain) specifically and robustly reflect tissue injury induced in rat models of disease. Importantly, these miRNAs were found to be at uniformly low concentration (or absent) in sham-treated animals, suggesting that circulating miRNAs represent a potentially highly sensitive and specific biomarker for tissue injury.	Laterza et al. (2009)
MicroRNA fingerprints identify miR-150 as a plasma prognostic marker in patients with sepsis	Sepsis	In concurrence with miRNA profiling from peripheral leukocytes, plasma level of miR-150 was observed to be decreased in sepsis patients relative to healthy controls. Plasma miR-150 did not correlate with the number of WBCs, such that circulating level of miR-150 is independent of absolute leukocyte count. The distribution of miR-150 levels across patients correlated with disease severity.	Vasilescu et al. (2009)
Circulating microRNA-1 as a potential novel biomarker for acute myocardial infarction	Myocardial infarction	Muscle-specific miR-1 was found to be elevated in the plasma of acute myocardial infarction (AMI) patients, relative to non-AMI control subjects. At time of discharge, plasma miR-1 levels in AMI patients were indistinguishable from that of control subjects (although it is unclear whether this effect was a response to therapeutic intervention or normal miRNA half-life), suggesting that miR-1 may be a useful marker for clinical triage of patients presenting with AMI-like symptoms.	Ai et al. (2010)

(*continued*)

Table 8.1 (continued)

Title	Disease type	Comments	References
C. Pregnancy-associated			
Detection and characterization of placental microRNAs in maternal plasma	–	Tissue-profiling and subsequent qRT-PCR analysis of maternal plasma pre- and 24h-post delivery identified a panel of miRNAs that were enriched in the setting of pregnancy. Of these, the concentration of plasma miR-141 increased with time of pregnancy. miRNAs were demonstrated to be cell-free by virtue of passage through pore sizes $\geq$ 0.22 micron, suggesting that circulating miRNAs may be protected from endogenous RNases by $<$ 0.22 micron subcellular particles.	Chim et al. (2008)
Serum microRNAs are promising novel biomarkers	–	A number of placenta-specific miRNAs located on chromosome 19 were found to be highly enriched in maternal plasma relative to nonpregnant women as measured by qRT-PCR. Plasma miRNAs were shown to be stable after short-term ambient incubation and over the course of multiple freeze–thaw cycles.	Gilad et al. (2008)
Human villous trophoblasts express and secrete placenta-specific microRNAs into maternal circulation via exosomes	–	Next-generation sequencing of placental tissue identified novel placenta-specific miRNAs mapping to a single locus on chromosome 19. These miRNAs were present in maternal plasma during pregnancy and cleared rapidly postdelivery. In-situ hybridization demonstrated colocalization of some, but not all, miR-517b with the exosome marker CD63 in placenta tissue and the BeWo trophoblast cell line. miR-517b was strongly enriched in anti-CD63 immunoprecipitated lysate from exosomes produced in BeWo culture.	Luo et al. (2009)

marker miR-141, which is effective at distinguishing patients with advanced (metastatic) prostate cancer from healthy controls (Mitchell et al. 2008), is not effective at distinguishing patients with early-stage prostate cancer from healthy controls (P. S. Mitchell and M. Tewari, unpublished observations). One explanation for this is that small, early-stage prostate cancers may not release sufficient miRNA into the circulation to be measurable from a small (0.4 mL) blood sample using current methods. An alternative explanation, however, is that since tumor cells undergo tremendous genetic and epigenetic evolution in response to selective pressures during tumor progression (Merlo et al. 2006), the most prominent markers released into the blood by a more advanced cancer may be different than those released in more nascent tumors. In addition, there are profound differences in host response and the tumor microenvironment (including angiogenesis, immune infiltration, and systemic response) present at later stages of tumor progression relative to earlier stage tumors. Thus, one could expect that the blood profile of molecular markers in the setting of larger, postdiagnosis cancer may be substantially different than that in the setting of earlier, prediagnosis cancers. To that end, miRNA biomarkers associated with late-stage disease may prove useful in disease monitoring and assessment of treatment response but not necessarily for early detection. The question of whether plasma or serum miRNAs will be useful markers for early detection of cancer thus remains an open one that may be resolved by studies ongoing in this area.

An important milestone that has not yet been reached in most studies of circulating miRNA markers in cancer is the independent validation of markers across different patient specimen sets. As has been seen in studies of other blood-based disease biomarkers (i.e., proteins), many markers that are initially discovered fail validation in specimens from independent patient populations, either because of biases in the population or methods used in initial discovery, or because of heterogeneity in the intended target population of patients for a given marker.

An additional hurdle is the lack of a rational and robust approach for data normalization across specimens that can correct for differences in miRNA abundance due to factors other than the disease state being assessed. More detailed study of biologic factors and preanalytic factors that affect circulating miRNA abundance (e.g., time of day of blood collection, method of blood collection and processing, etc.) is expected to be useful in identifying the optimal protocol for blood collection and processing, as well as for identifying a robust panel of small RNA endogenous controls that may be used to normalize results between individuals. Such advances are likely to be critical for translating the concept of circulating miRNAs as cancer biomarkers to the clinic.

Although the circulating miRNA biomarker field is at an early stage, research in this area will benefit from the availability of powerful approaches for global RNA characterization (i.e., next generation high-throughput sequencing platforms and nanofluidic qRT-PCR platforms) and simple, universally applicable assays for amplification and quantitative detection of the RNA signal (e.g., qRT-PCR). These techniques will likely enable the discovery–validation pipeline for miRNA

biomarkers to be more efficient than traditional proteomic biomarker discovery–validation pipelines, which typically encounter bottlenecks at the point of antibody generation and quantitative assay development for validation of biomarker candidates. We can expect that the development of improved miRNA assays and a better understanding and standardization of preanalytic methodologies will lead to substantial improvements in the effectiveness of the circulating miRNA biomarker approach. Moreover, a deeper appreciation of the biophysical nature of circulating miRNA and the mechanism(s) that protects miRNAs from endogenous ribonucleases (for more discussion, see Sect. 8.4) is also likely to improve the effectiveness of this approach.

8.4 Circulating MicroRNAs: Biological Mechanisms

An obvious question that arises when considering the mechanisms underlying circulating miRNAs is whether the detected signal might be due to contamination of plasma or serum specimens with cells or large cell fragments. Several lines of evidence suggest that circulating miRNAs exist as cell-free entities in the bloodstream: miRNAs derived from plasma or from cell culture supernatant pass through 0.22 μm filters (Chim et al. 2008; Mitchell et al. 2008), and serial centrifugation experiments have shown that circulating miRNAs do not sediment at speeds adequate to clear whole cells (500 × *g*) and large cellular debris (16,000 × *g*) (Mitchell et al. 2008). Although these experiments do not rule out the possibility that cell-free miRNAs in plasma or serum are secondary to cell lysis in the circulation or during sample collection, the miRNAs found in clinical specimens appears to be present largely in a cell-free state.

Although miRNAs in plasma and serum exist in a cell-free form, we and others have found that they are highly stable in these body fluids, despite the presence of high levels of ribonuclease activity in blood (Chen et al. 2008; Chim et al. 2008; Gilad et al. 2008; Mitchell et al. 2008). For example, incubation of plasma at room temperature for up to 24 h led to no decrease in the level of three circulating miRNAs we examined, nor did multiple cycles of freeze-thawing (a condition to which clinical specimens may be routinely subjected) lead to a significant decrease in miRNA abundance (Fig. 8.3) (Mitchell et al. 2008). In addition, Chen et al. found that high (pH 13) or low (pH 1) pH caused no decrease in circulating miRNAs in serum samples (Chen et al. 2008).

The unexpected stability of circulating miRNAs in the face of high endogenous ribonuclease activity in blood begs the question of what mechanism protects circulating miRNAs from degradation. When we compared the stability of endogenous miRNAs present in plasma to that of exogenously introduced synthetic miRNAs, we found that exogenous miRNAs were completely degraded when added directly to plasma, whereas endogenous miRNAs were highly stable (Mitchell et al. 2008). Additionally, when "naked" miRNAs purified from plasma are "added back" to an aliquot of the same plasma source sample in vast excess

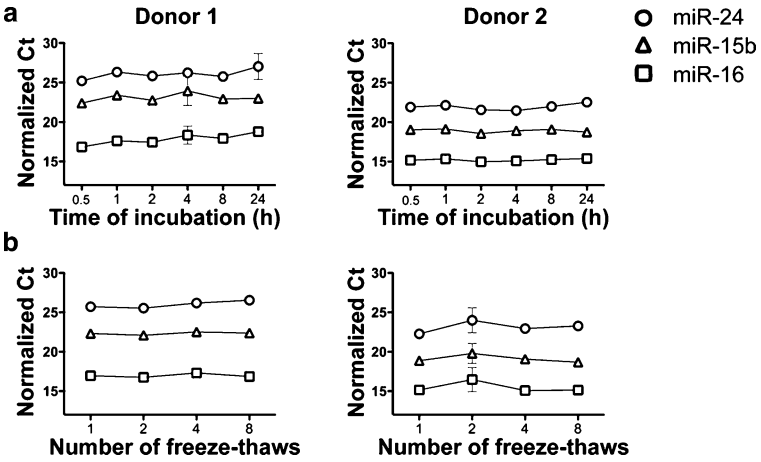

Fig. 8.3 Stability of circulating miRNAs in plasma. Plots show normalized Ct values for the indicated miRNAs measured in parallel aliquots of human plasma samples that were either *A:* incubated at room temperature for 0.5, 1, 2, 4, 8, or 24 h, or *B*: subjected to 1, 2, 4, or 8 cycles of freeze–thawing. Both experiments were carried out using plasma from two different individuals as indicated. Normalization of raw Ct values across samples is based on the measurement of three nonhuman synthetic miRNAs spiked into each sample at known molar amounts after initial plasma denaturation for RNA isolation (described in detail in (Kroh et al. 2010; Mitchell et al. 2008)). Error bars represent standard deviation (SD) of triplicate experiments, where $SD < 1$ is not depicted. The figure shown is adapted from (Mitchell et al. 2008)

of endogenous levels, the naked miRNA is rapidly degraded (P.S. Mitchell and M. Tewari, unpublished observations). Barring the possibility of some novel, stabilizing covalent modifications of miRNA that are lost during the RNA isolation process, we interpret these findings to indicate that the stability of circulating miRNAs is not due to an intrinsic property of the miRNA molecule but is rather acquired in vivo and requires additional, extrinsic factors. We note that there is one report indicating that miRNAs isolated from serum may be resistant to RNase A-treatment (Chen et al. 2008), which supports the alternative hypothesis that circulating miRNAs are intrinsically resistant to ribonuclease activity. Although future work will be needed to reconcile these disparate observations, one possible explanation is that the ribonuclease activity in plasma and serum is distinct from the RNase A treatment used by the authors of that study.

Currently, the leading extrinsic mechanism for miRNA stability in the circulation is postulated to be miRNA containment within exosomes. Exosomes are membrane-bound vesicles of 50–110 nm diameter that are secreted from a diverse array of cell types and are present in biological fluids (i.e., blood, urine, and saliva) and cell culture supernatant (Keller et al. 2006; Thery et al. 2002). Tumor-derived exosomes have been proposed to be a rich and accessible source of diagnostically relevant miRNAs (Rabinowits et al. 2009; Skog et al. 2008; Taylor and Gercel-Taylor 2008; Valadi et al. 2007). Taylor et al. demonstrated that members of the human miRNA family miR-200 and other cancer-associated miRNAs (miR-21, miR-205) are

enriched in $EpCAM^+$ exosomes isolated from the plasma and serum of patients with ovarian cancer (Taylor and Gercel-Taylor 2008). Additionally, exosomal miRNAs have also been indicated as putative biomarkers in cancers of the lung and brain (Rabinowits et al. 2009; Skog et al. 2008) as well as markers of gestation (Luo et al. 2009). Although exosomes appear to generally harbor a similar distribution of miRNAs relative to that of the parent cell, exosome-enriched or -depleted miRNAs have also been reported, implying the existence of a miRNA-sorting mechanism (Rabinowits et al. 2009; Skog et al. 2008; Taylor and Gercel-Taylor 2008; Valadi et al. 2007), perhaps analogous to that of ESCRT-mediated sorting of proteins in exosomes (van Niel et al. 2006).

An intriguing hypothesis stemming from the discovery that miRNAs can be exosomal cargo is that exosomes mediate the exchange of miRNAs between cells. MiRNA-mediated cell-to-cell communication was postulated by Valadi et al., who demonstrated that mRNA and miRNA is present within mast cell exosomes, and that upon delivery of exosomes to recipient cells, the exosomal mRNA is actively translated to functional protein (Valadi et al. 2007). A subsequent report by Skog et al. (2008) provided additional evidence for the presence of mRNA and miRNA in exosomes and for the transfer of mRNAs to recipient cells. Transfer of miRNAs between cells has not yet been rigorously established, however, and whether such transferred miRNAs are functional in mammals is not yet known. It is worth noting, however, that miRNA transfer and subsequent noncell autonomous posttranscriptional regulation by small RNAs occurs in other organisms, albeit in a nonexosomal context. For example, systemic RNA interference (RNAi) in plants and *C. elegans* is well-documented (reviewed in (Yoo et al. 2004) and (Hunter et al. 2006)), and more recently, signaling to distant sites by RNAi in *Drosophila* was found to be a critical component of antiviral immunity (Saleh et al. 2009).

Although exosomes are currently the best-documented potential mechanism of protection of circulating miRNAs, extracellular miRNAs have also been associated with microvesicles (Hunter et al. 2006; Yuan et al. 2009) and apoptotic bodies (Zernecke et al. 2009) and could potentially be associated with circulating proteins as well. Future work is expected to clarify the various states in which miRNAs may circulate in the bloodstream. An enhanced understanding of the molecular forms in which circulating miRNAs exist will undoubtedly shed light on important aspects of miRNA biology.

An important unclarified topic in the field is how miRNAs are released from cells, and whether the release occurs in a regulated manner. At this time, it is not known whether miRNAs are released in a passive process, such as during apoptotic and/or necrotic cell death, or whether miRNA release occurs through active mechanisms that do not involve cell death. Similarly, knowledge of mechanisms that may regulate miRNA release is scant, which precludes an understanding of the possible biological function of circulating miRNAs. Nonetheless, the prospect of extracellular miRNAs as paracrine or hormonal signaling molecules in normal physiology and cancer is a fascinating postulate that deserves further investigation.

8.5 Concluding Remarks

Several questions regarding the basic mechanisms of release and stability of circulating miRNAs will need to be answered in order to elucidate potential biological functions of these extracellular molecules. From the standpoint of clinical cancer diagnostics, circulating miRNAs have the potential to yield broad utility at various points in the clinical course of cancer diagnosis and therapeutic intervention. Technological advances such as next generation sequencing are likely to enable more comprehensive circulating miRNA biomarker discovery. We can envision circulating miRNA applications ranging from use in early diagnosis, to monitoring of tumor response to therapy, and early detection of relapse. Realizing this potential, however, will require a better understanding of the preanalytic and biologic factors that affect circulating miRNA levels, as well as the careful design and implementation of studies that include patient populations for the independent validation of putative circulating miRNA biomarkers. Altogether, the discovery of circulating miRNAs has opened up a myriad of possibilities both for clinical applications and for new forays into basic biological mechanisms.

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Chapter 9
Biology and Diagnostic Applications of Cell-Free Fetal Nucleic Acids in Maternal Plasma

Jason C.H. Tsang and Y.M. Dennis Lo

Contents

Abstract The discovery of cell-free fetal nucleic acids in maternal plasma in 1997 has opened up new possibilities for noninvasive prenatal diagnosis. Since then, many of the fundamental biological parameters of this phenomenon have been elucidated, including the effects of gestational age, the size of the circulating nucleic acid molecules, and their rapid clearance following delivery. A variety of circulating fetal nucleic acid markers have also been developed, including fetal genetic, epigenetic, mRNA, and miRNA markers. A number of exciting new technologies have been developed for their analysis, including single molecule counting technologies, such as digital PCR and massively parallel sequencing. Together, these developments have culminated in a number of prenatal diagnostic applications, including

J.C.H. Tsang and Y.M. Dennis Lo (✉)
Li Ka Shing Institute of Health Sciences and Department of Chemical Pathology, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, New Territories, Hong Kong SAR, China
e-mail: loym@cuhk.edu.hk

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fetal sexing, RhD blood group genotyping, monogenic disease analysis, and the detection of fetal chromosomal aneuploidies. It is hoped that such advances will ultimately make prenatal testing safer for pregnant women and their fetuses.

9.1 Introduction

Prenatal diagnosis is an established practice in modern obstetrics. There are two major groups of genetic diseases in prenatal diagnosis, namely, chromosomal abnormalities and monogenic diseases. It has been found that around 1% of newborns are affected by chromosomal abnormalities (Jacobs et al. 1992) and monogenic diseases, such as thalassemia and cystic fibrosis, causing significant mortality and morbidities worldwide. Early prenatal diagnosis is therefore essential to offer parents an option to continue the pregnancy and also to allow better coordination of care for the affected babies after birth. Recently, the American College of Obstetrics and Gynecology has recommended Down syndrome screening for all pregnant women. This recommendation highlights the importance of prenatal diagnosis in current obstetric practice.

Conventional modalities in prenatal diagnosis include amniocentesis, chorionic villous sampling, and cordocentesis. They are well-established procedures with high accuracy because they provide fetal tissues for analysis by direct sampling. However, the major drawbacks of these methods are their invasive nature and the association of a small but finite risk of fetal loss. In view of this, several noninvasive screening strategies such as the quadruple test and the One-Stop Clinic Assessment of Risk (OSCAR) for Down syndrome have been developed to reduce the use of invasive tests by combining the maternal age, a number of serum markers, and characteristic ultrasound findings. Although several combinations have shown promising detection sensitivity and specificity (Leung et al. 2009), they are not diagnostic and require high operator expertise (e.g., for ultrasound scanning) and complicated statistical interpretation. Inconclusive results may even exacerbate the psychological stress of the testing couples. Moreover, similar, reliable noninvasive screening tests are still lacking for many common monogenic diseases.

As a result, the development of a prenatal test that is truly noninvasive, diagnostic, rapid, and operator-independent, remains a long sought goal.

9.2 From Circulating Fetal Cells to Circulating Fetal Nucleic Acids

In 1893, a German pathologist, Georg Schmorl, reported the presence of some "very peculiar" multinucleated cells in the pulmonary capillaries of a number of deceased pregnant women in his study on eclampsia, which he hypothesized to be

placental in origin (Lapaire et al. 2007). The implication of his first report of circulating fetal cells in the maternal body was not recognized until about 80 years later when Walknowska et al. detected XY lymphocytes in the peripheral blood of pregnant women and ignited research to develop noninvasive prenatal diagnosis by targeting circulating fetal cells in maternal circulation (Walknowska et al. 1969). Subsequently, investigators were able to isolate circulating fetal cells for aneuploidy detection such as chromosome 13, 18, 21, X, and Y (Bianchi and Lo 2001). Theoretically, circulating fetal cells are an ideal source for noninvasive prenatal diagnosis since the whole set of genetic materials and the karyotype of the fetus can be analyzed directly. Nevertheless, the widespread use of noninvasive prenatal diagnosis by analysis of circulating fetal cells was limited by the scarcity of these cells and their reported long-term persistence in the maternal circulation after delivery. Quantitative analysis of the cellular component of maternal blood by PCR showed that there are only some 19 cells in 16 mL of blood on average, and it has been reported that circulating fetal cells are detectable in the maternal circulation even 27 years after delivery (Bianchi et al. 1996, 1997). These have greatly affected the sensitivity and the specificity of prenatal tests based on circulating fetal cells.

Recognizing the pseudo-malignant nature of the placenta and inspired by previous reports of the presence of circulating tumor nucleic acids in cancer patients, Lo et al. (1997) hypothesized the presence of circulating cell-free fetal nucleic acids in maternal plasma (Lo et al. 1997). They were able to detect Y chromosome sequences in about 80% of male-fetus-bearing mothers with maternal serum and plasma but only in about 17% when nucleated blood cells were used, suggesting that a significant proportion of fetal nucleic acids exists in extracellular form in the maternal circulation. Following the same line of reasoning, Poon et al. detected circulating fetal RNA in maternal plasma in 2000 (Poon et al. 2000) and recently, placental miRNA was also detected in maternal plasma (Chim et al. 2008b). These findings have opened up new avenues to the development of noninvasive prenatal diagnosis and stimulated investigators to look at the biological significance of this phenomenon.

9.3 Characteristics of Circulating Fetal Nucleic Acids in Maternal Plasma

The presence of circulating fetal nucleic acids in maternal plasma was a surprising finding, since the conventional belief was that nucleic acids are too fragile to exist in extracellular form, especially with the presence of DNases and RNases in the circulation (Tsui et al. 2002). Therefore, a specific unknown protective mechanism or a continuous large-scale release of fetal nucleic acids into the maternal circulation must be present to account for their presence.

A number of studies have been carried out to further look into the kinetics of fetal nucleic acids in maternal plasma. Lo et al. developed a quantitative real-time PCR assay and found that circulating fetal DNA constitutes only 3–6% of the total

circulating DNA in the maternal plasma, and it can be detected as early as 18 days after conception and increases as gestation progresses (Lo et al. 1998b). More recent data obtained by more sophisticated digital PCR systems suggested that the fractional concentration may be some twofold higher, but it is still around 10% (Lun et al. 2008a). Despite that, the fractional concentration for circulating fetal nucleic acids is at least 970-fold higher than circulating fetal cells in early pregnancy (Lo et al. 1998b).

In addition, circulating fetal DNA was found to be rapidly cleared from the maternal circulation after delivery, with a half-life in the order of 16 min (Lo et al. 1999c). Despite the report from Invernizzi et al., consensus is that there is no long-term persistence after delivery (Invernizzi et al. 2002). Contamination of persisting fetal cells due to inadequate centrifugation during plasma separation is believed to account for the long-term persistence in the study by Invernizzi et al. (Benachi et al. 2003; Chiu et al. 2001; Smid et al. 2003). The exact clearance mechanism for circulating DNA is still uncertain, but the kidney has been proposed to be an important organ after the discovery of cell-free fetal DNA in maternal urine (Botezatu et al. 2000; Hung et al. 2009; Koide et al. 2005; Majer et al. 2007).

Similar kinetic studies on circulating fetal RNA also demonstrated complete clearance 24 h after delivery with a half-life of some 14 min (Chiu et al. 2006; Ng et al. 2003b). The majority of circulating fetal RNA was shown to have a 5′ preponderance, and filtration studies by Ng et al. suggested that circulating RNA is filterable (Ng et al. 2003b; Wong et al. 2005), implying protection by microparticulate matter. A number of studies also supported this argument (Gupta et al. 2004). Interestingly though, such filterable property cannot be detected in circulating fetal DNA and miRNA (Chim et al. 2008b).

These findings have several implications, (1) The presence of circulating fetal nucleic acids in maternal plasma has been confirmed by numerous groups; (2) Enrichment process and sensitive detection technology may further enhance the performance of prenatal tests based on this phenomenon; (3) Circulating fetal nucleic acids are specific to the current pregnancy; (4) The release of circulating fetal nucleic acids into the maternal circulation is a continuous process, and a specific protective mechanism for circulating fetal RNA is likely to exist; (5) Circulating fetal nucleic acids may be used in the monitoring of gene expression and the well-being of the fetus.

9.4 Origin of Circulating Fetal Nucleic Acids

The source of circulating nucleic acids in the plasma of normal individuals remained largely an enigma until Lui et al. demonstrated that the “sex” of the circulating nucleic acids in plasma changed in patients after sex-mismatched bone marrow transplantation (Lam et al. 2004a; Lui et al. 2002a). They estimated that about 60% of the circulating nucleic acids are of hematopoietic origin. Subsequent

studies on sex-mismatched organ transplantation patients concluded that the heart, liver, and kidneys account for only a minority of circulating nucleic acids in plasma (Lui et al. 2003).

There have been much discussion concerning the possible sources of circulating fetal nucleic acids, namely, (1) direct release from circulating fetal cells in the maternal circulation, (2) from the placenta, or (3) trafficking from other fetal organs or from the amniotic fluid to the maternal circulation. As discussed above, the rapid clearance of circulating fetal nucleic acids after delivery suggested that the release mechanism must be a continuous process to account for their persistence throughout pregnancy. Therefore, direct release from circulating fetal cells are less plausible due to their scarcity in the maternal circulation, and investigators are not able to detect significant correlation between the amount of circulating fetal cells and circulating fetal nucleic acids during pregnancy (Hyodo et al. 2007; Sekizawa et al. 2000). Nucleic acid trafficking from fetal organs or amniotic fluid is also not supported since the presence of circulating fetal nucleic acids has been demonstrated in maternal plasma well before the establishment of the fetal circulatory system and since there are no significant correlations between amniotic and circulating fetal DNA in maternal plasma (Bianchi et al. 2001; Zhong et al. 2006). There was a theoretical concern that circulating fetal nucleic acids are due to the ex vivo release from circulating fetal cells during venepuncture or centrifugation. However, a number of studies have dismissed such a concern (Lui et al. 2002b). To date, most evidence supports the view that the placenta is the major contributor of circulating fetal nucleic acids due to its close contact with the maternal circulation and its high cell turnover. More importantly, placental-specific mRNA and epigenetic markers have been detected successfully in maternal plasma, and there is a strong correlation between fetal nucleic acids and human chorionic gonadotropin (hCG) produced by the placenta (Ng et al. 2003a). It is possible that the circulating fetal nucleic acids are released from apoptotic placental trophoblasts during the continuous development of the placenta or due to pathological insults (Tjoa et al. 2006). Elevated levels of circulating fetal nucleic acids have been reported in a number of obstetric complications such as pre-eclampsia and preterm labor (Farina et al. 2004; Lau et al. 2002; Leung et al. 1998, 2001; Levine et al. 2004; Lo et al. 1999b; Ng et al. 2003a; Zhong et al. 2001). Characteristic patterns of "DNA laddering," similar to that present in apoptosis, has also been demonstrated in circulating fetal nucleic acids (Li et al. 2004b). Moreover, size fractionation analysis of circulating fetal nucleic acids also showed that circulating fetal DNA consists predominantly of short fragments, with 80% being less than 200 bp, resembling the shorter DNA fragments following apoptosis (Chan et al. 2004).

Despite numerous efforts to elucidate the biological characteristics of circulating fetal nucleic acids in maternal plasma, it is still an enigma as to whether they serve any biological function during pregnancy. The recent report of circulating fetal miRNA in maternal plasma is of special interest and opens up the possibility of potential maternal gene expression regulation by fetal miRNA (Chim et al. 2008b).

9.5 Clinical Application of Circulating Fetal Nucleic Acids

During the past decade, qualitative genetic analysis of circulating fetal nucleic acids has found many successful clinical applications in noninvasive fetal sex determination, RhD blood group typing, and prenatal analysis of a number of monogenic diseases. Quantitative analysis, in addition, has revealed elevated levels of circulating fetal nucleic acids in fetal aneuploidy and a number of obstetrics complications. Very recently, circulating fetal nucleic acids have been analyzed with unprecedented precision using single molecule counting technologies such as massively parallel sequencing and microfluidic digital PCR technology (Table 9.1). Here, we will discuss some of the key studies to illustrate the principle.

9.5.1 Qualitative Analysis of Circulating Fetal Nucleic Acids

In Mendelian genetics, an allele is said to be dominant if inheritance of only one copy of the allele predicts the manifestation of the characteristics of the allele. The allele is regarded as recessive if disease manifestation requires inheritance of both copies of the allele. Many biological characteristics such as sex, blood groups, and monogenic diseases in humans have been shown to obey this model. Therefore, noninvasive prenatal diagnosis can be achieved by detecting the paternally inherited allele of the fetus in maternal plasma, for instance, the positive detection of the Y-chromosome sequence in maternal plasma determines the sex of the fetus and absence of the Y-chromosome sequence means otherwise. Indeed, this principle was originally utilized to prove the presence of circulating fetal nucleic acids in maternal plasma (Lo et al. 1997). The same principle also applies to fetal RhD blood group typing. Recently, a systemic review on multiple studies concluded that circulating fetal nucleic acids analysis has a very high accuracy in noninvasive fetal RhCE genotyping (Geifman-Holtzman et al. 2009).

The interpretation of test results is more complicated in dealing with monogenic diseases, but the essence is still in the detection of the paternally inherited mutant allele in maternal plasma. In autosomal-dominant diseases, the positive detection of the paternally inherited mutant allele in an unaffected mother signifies the inheritance of the disease in the fetus. In autosomal-recessive diseases, negative detection of the paternally inherited mutant allele in a heterozygous mother excludes the inheritance of the disease. Nevertheless, if both of the parents are heterozygous for the mutation, such as in Southeast Asia where thalassemia trait is common, the maternal mutant allele and the paternally inherited mutant allele of the fetus are indistinguishable in the maternal plasma. To tackle this problem, an indirect detection of the paternally inherited mutant allele can be used. One approach that has been used is the detection of linked single nucleotide polymorphisms (SNPs) close to the mutation (Fig 9.1). In this approach, parental genotypes are first analyzed to look for heterozygous SNPs linked to the mutation between the couple,

Table 9.1 Table showing diagnostic applications of circulating fetal nucleic acids in maternal plasma

Marker	Principle	Application	Example	Reference
Fetal DNA	Qualitative analysis by real-time, fluorescent, nested or conventional PCR, and mass spectrometry-based methods	Diagnosis or exclusion of fetal genetic disorders	Fetal sex: X-linked diseases, congenital adrenal hyperplasia	Costa et al. (2002)
			Fetal rhesus blood group: prevention of maternal alloimmunization in pregnancies with rhesus incompatibility	Geifman-Holtzman et al. (2009), Lo et al. (1998a)
			Fetal HLA haplotyping and sexing: congenital adrenal hyperplasia	Rijnders et al. (2001), Chiu et al. (2002a)
			Monogenic diseases: autosomal-dominant: achondroplasia, Huntington disease	Amicucci et al. (2000), Bustamante-Aragones et al. (2008), Chiu et al. (2002b), Ding et al. (2004), Li et al. (2005, 2008), Lun et al. (2008b), Saito et al. (2000), Tsang et al. (2007)
			Autosomal-recessive: beta-thalassemia, hemoglobin E, cystic fibrosis, congenital adrenal hyperplasia, myotonic dystrophy	
	Quantitative analysis by real-time PCR	Assessment and monitoring of obstetric complications	Fetal complications: chromosomal aneuploidy, intrauterine growth restriction, fetomaternal hemorrhage, postexternal cephalic version	Lau et al. (2000), Lo et al. (1999a), Wataganara et al. (2004)
			Maternal complications: preeclampsia, preterm labor, ectopic pregnancy, invasive placentation, hyperemesis gravidarum, polyhydramnios	Hahn et al. (2005), Lazar et al. (2006), Leung et al. (1998), Lo et al. (1999a), Sekizawa et al. (2001), Zhong et al. (2000)
	Relative allelic and chromosomal ratio analysis by digital PCR or massively parallel sequencing	Fetal chromosomal aneuploidy detection	Trisomy 13, 18, and 21	Chiu et al. (2008), Fan et al. (2008)
			Beta-thalassemia and hemoglobin E disease	Lun et al. (2008b)

(*continued*)

Table 9.1 (continued)

Marker	Principle	Application	Example	Reference
		Diagnosis or exclusion of fetal genetic disorders		
Fetal RNA	Quantitative analysis by real-time PCR	Assessment and monitoring of obstetric complications	hCG: Gestational trophoblastic disease CRH: Preeclampsia	Farina et al. (2004), Masuzaki et al. (2005), Ng et al. (2003a)
	RNA–SNP allelic ratio analysis by mass spectrometer or digital PCR	Fetal chromosomal aneuploidy detection	PLAC4: Trisomy 21	Lo et al. (2007b)
Fetal epigenetic marker	Quantitative analysis by real-time PCR	Assessment and monitoring of obstetric complications	Hypomethylated SERPINB5, hypermethylated RASSF1A: Preeclampsia	Tong et al. (2007), Tsui et al. (2007)
	Epigenetic allelic ratio analysis	Fetal chromosomal aneuploidy detection	Hypomethylated SERPINB5: Trisomy 18	Tong et al. (2006)

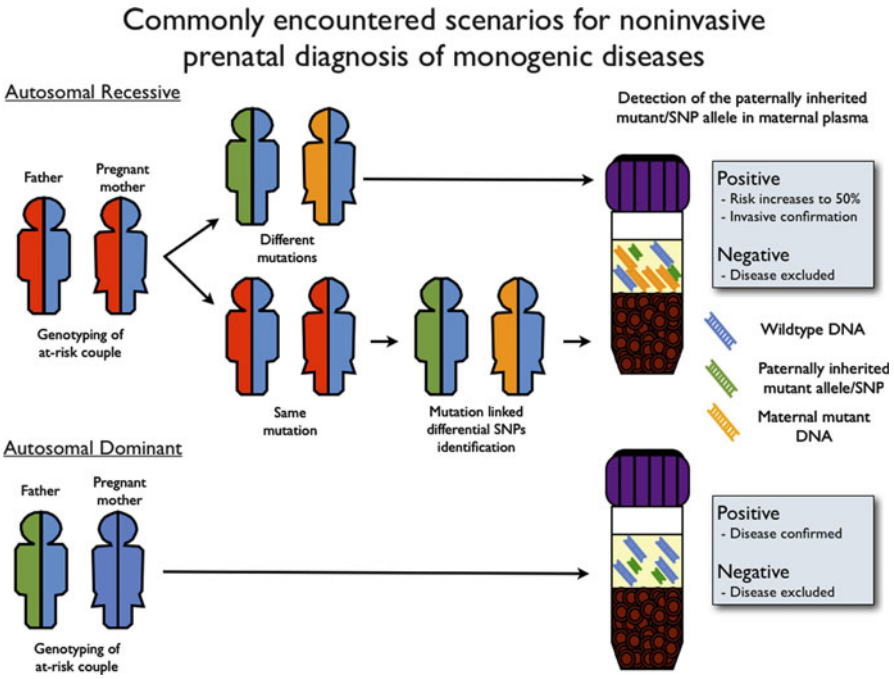

Fig. 9.1 Schematic diagram showing the principle of noninvasive prenatal diagnosis of Mendelian inheritance diseases by fetal DNA in maternal plasma

and then maternal plasma is analyzed to look for the paternally inherited SNPs. Negative detection implies negative detection of the paternally inherited mutant allele in the fetus, therefore excluding the inheritance of thalassemia. Using this approach, 50% of invasive procedures can theoretically be avoided.

Nevertheless, there are limitations in this approach: (1) excellent sensitivity and specificity of the detection system is required to differentiate the minority of circulating fetal nucleic acids from a large background of highly similar maternal nucleic acids; (2) linkage with heterozygous SNPs may not be present; (3) the parental genotype has to be available beforehand; (4) the risk would increase from 25% to 50% if the paternally inherited mutant allele is present in autosomal recessive diseases, and invasive procedures are still required to clarify the fetal genotype.

To improve the detection accuracy of differentiating circulating fetal nucleic acids from the background of highly similar maternal nucleic acids, quantitative fluorescent real-time PCR with allele-specific primers has been used (Chiu et al. 2002b). However, the specificity of this design depends on the allelic specificity of the amplification primer, which in turn depends on the number of base mismatches at the 3′ end of the primer. As a result, differentiating single base difference in diseases caused by point mutations can be a challenge, as once the allele-specific

primers are extended incorrectly, the product will amplify exponentially. In 2004, Ding et al. approached this difficulty by a mass spectrometry-based assay design called the single allele base extension reaction (SABER) (Ding et al. 2004). They first amplified the gene of interest in maternal plasma with PCR, and then a single base extension reaction was performed with an extension primer designed to end one base 5′ to the base of difference. Only one terminator nucleotide complementary to the fetal allele is used in the extension reaction. In the presence of the fetal allele, the primer will be extended and detected by mass spectrometry. Extension from the maternal allele should theoretically be minimal. A number of studies have shown promising results with the SABER design, yet there is also concern that SABER might be nonspecific in certain mutational context due to nucleotide misincorporation (Chow et al. 2007; Ding et al. 2004; Li et al. 2006, 2007, 2008; Tsang et al. 2007).

In addition to the detection of the paternally inherited mutant allele, investigators are searching for better alternatives such as detection of allelic imbalance. The concept of this approach will be discussed later in the session together with chromosomal and allelic dosage analysis.

9.5.2 *Quantitative Analysis of Circulating Fetal Nucleic Acids*

The level of circulating nucleic acids depends on the rate of production and clearance. As previously discussed, circulating fetal nucleic acids are believed to be released from apoptosis in the placenta and may be partly cleared by the maternal kidney through urine. It is logical to reason that any pathological insult to the placenta or the maternal kidney might affect the circulating concentrations. Actually, investigators have demonstrated that fetal aneuploidy, preeclampsia, hyperemesis gravidarum, preterm labor, polyhydramnios, intrauterine growth restriction, and procedures like external cephalic version are associated with higher levels of circulating fetal nucleic acids in maternal plasma. There has been much expectation to apply these findings to noninvasive prenatal monitoring and diagnosis. However, the diagnostic accuracy is imperfect due to the significant overlap in levels between normal and pathological pregnancies. Furthermore, these studies were typically based on the use of fetal Y-chromosomal markers, indicating that the application of this concept to pregnancies involving female fetuses require the use of new types of markers.

9.5.3 *The Development of Universal Fetal Markers*

As mentioned above, most quantitative studies of circulating fetal nucleic acids depend on the detection of Y-chromosome sequences in pregnant mothers bearing

male fetuses. In order to generalize the findings, a universal fetal marker is necessary. Such a marker should be highly fetal-specific, sex-independent, and ideally should be easily detectable.

One promising candidate is circulating fetal RNA in maternal plasma. Because of the unique function of the placenta during pregnancy, it is logical to believe that placental gene expression might exhibit unique features distinguishable from that of other maternal organs. Based on this line of reasoning, Ng et al. have demonstrated the presence of circulating placental mRNA in maternal plasma by detecting hCG and human placental lactogen (hPL) mRNA in the maternal plasma (Ng et al. 2003b). Circulating corticotrophin releasing hormone (CRH) mRNA has also been found to be elevated in preeclamptic patients (Farina et al. 2004; Ng et al. 2003a). With advances in microarray technology, discovery of more fetal specific mRNA is expected since placental gene expression can be analyzed in a large-scale and systemic fashion. One theoretical concern in using circulating fetal mRNA is its susceptibility to degradation. Prompt processing of blood samples with Trizol have been reported to allow the preservation of the circulating fetal mRNA.

Recognizing the importance of epigenetic modifications in the control of gene expression, a number of investigators have looked for fetal-specific epigenetically modified DNA sequences in maternal plasma. Poon et al. demonstrated the presence of differential methylation patterns of the imprinted human IGF-H19 locus in circulating fetal and maternal DNA (Poon et al. 2002). Chim et al. later furthered this area of research by identifying differential methylation patterns of the SERPINB5 sequence (coding for maspin) in the placenta and maternal blood cells (Chim et al. 2005). They found that the SERPINB5 sequence is hypomethylated in the placenta and is distinguished from hypermethylated SERPINB5 of maternal blood cell origin. Therefore, the hypomethylated SERPINB5 sequence can be used as a fetal-specific marker. Nonetheless, it is still not ideal since the analysis of the hypomethylated SERPINB5 sequence requires bisulfite treatment, which is a labor-intensive and inefficient process. To overcome this limitation, Tong et al. described another method by combining methylation-sensitive restriction enzymes with a stem–loop primer (Tong et al. 2007). In this method, hypomethylated fetal sequences are digested into short fragments selectively by the enzyme, and stem–loop primers specific to the restriction sites are designed to amplify such short fragments. This new method has another advantage over bisulfite treatment in that signal from the maternal hypermethylated sequences is suppressed. Investigators also tried to look for fetal-specific hypermethylated markers such that methylation-sensitive restriction enzymes, many of which cut unmethylated DNA while leaving methylated DNA intact, can be used. Later effort showed that the RASSF1A sequence is hypermethylated in the placenta, but hypomethylated in maternal blood cells, and can also be used as a universal fetal marker in maternal plasma (Chan et al. 2006). Systemic analysis of specific chromosomal methylation patterns of the placenta has been carried out and it can be envisioned that many more fetal epigenetic markers will be discovered in the future (Chim et al. 2008a; Old et al. 2007).

9.5.4 Chromosomal and Allelic Dosage Analysis

The development of universal fetal markers not only overcame previous limitation in the quantitative analysis of circulating fetal nucleic acids but also inspired researchers to develop a new approach to noninvasive prenatal diagnosis of fetal aneuploidy. In 2007, Lo et al. described a new approach named RNA–SNP allelic ratio analysis (Lo et al. 2007b). Lo et al. studied a SNP present in a placental specific mRNA called PLAC4. The PLAC4 gene is located on chromosome 21. The core of this approach assumed that the ratio of the fetal RNA–SNP alleles detected in the maternal plasma reflects the chromosome ratio of the fetus. In other words, in a fetus with trisomy 21 and heterozygous for the linked SNP in PLAC4, the ratio between the two alleles will be 1:2 or 2:1, but the ratio will remain 1:1 in a normal pregnancy (Fig 9.2). By detecting the presence of this difference in the allelic ratio in maternal plasma, noninvasive prenatal diagnosis of trisomy 21 can be achieved. In their first study, this approach has shown promising results of 90% sensitivity and 96.5% specificity.

Tong et al. showed that an allelic ratio-based approach could also have application to DNA methylation markers, the so-called epigenetic allelic ratio approach (Tong et al. 2006). They demonstrated this principle by using a SNP in the SERPINB5 gene on chromosome 18 and applied this strategy to the noninvasive prenatal diagnosis of trisomy 18.

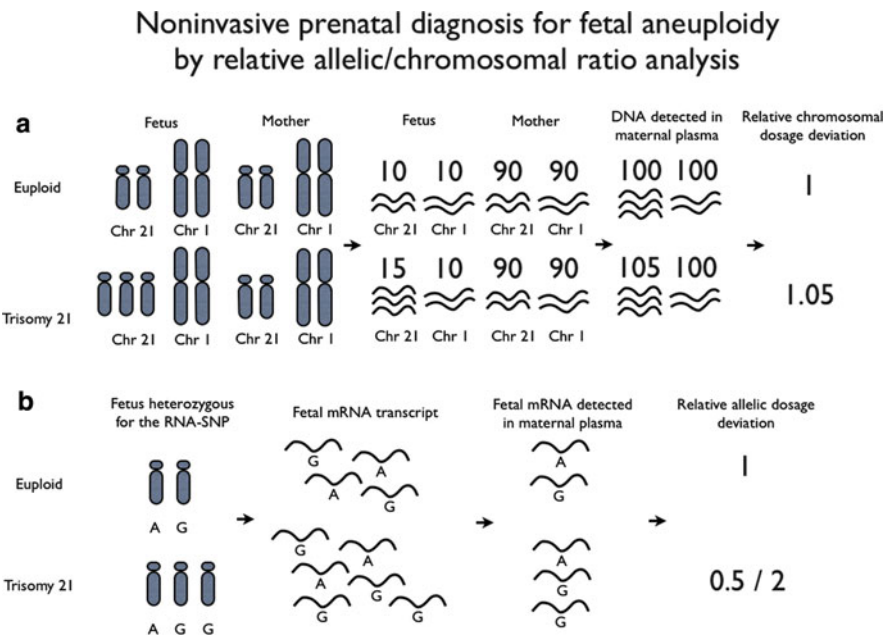

Fig 9.2 Schematic diagram showing the principle of relative chromosomal dosage and relative allelic dosage analysis in noninvasive fetal aneuploidy diagnosis

The idea of allelic ratio analysis appears to be a promising approach in noninvasive prenatal diagnosis of fetal aneuploidy, but one limitation of this approach is its dependence on the presence of fetal-specific marker and linked SNPs on the target chromosome. Combination of multiple SNPs is needed to maximize the population coverage of the test.

In view of these limitations, investigators have recently developed a new approach based on single molecule counting in which some variants are sex- and polymorphism-independent (Chiu et al. 2009). The first of such approaches is based on digital PCR (Fan and Quake 2007; Lo et al. 2007a; Lun et al. 2008b). In this technology, multiple PCRs were carried out in parallel on very diluted plasma nucleic acid samples, such that a positive reaction reflects the presence of a single template molecule. The number of template molecules in the original undiluted sample can then be estimated by Poisson distribution-based statistical analysis of the number of the positive reactions. Furthermore, the relative dosage of two DNA sequences in a mixture can also be estimated with an appropriate statistical model. Two groups of investigators have successfully applied this technology to determine the relative chromosomal dosage in the maternal plasma of trisomy 21 pregnancies based on the idea that the number of a particular sequence in the maternal plasma reflects the proportional contribution from the maternal and fetal chromosomes. For instance, in a case of normal pregnancy, for every 100 copies of a particular sequence from chromosome 21 detected in the maternal plasma, about 10 will be contributed by the fetus, but in a case of trisomy 21 pregnancy, there will be additional copies of the sequences contributed by the extra chromosome, meaning 15 copies from the fetus and 105 copies in total. By comparing the ratio of two particular sequences from chromosome 21 and a reference chromosome (e.g., chromosome 1), researchers can detect a change of ratio from 100/100 in normal pregnancy to 105/100 in trisomy 21 pregnancy (Fig 9.2). This degree of precision was difficult to be achieved prior to the use of digital PCR. Since the sensitivity of digital PCR ultimately depends on the number of reactions performed and the number of genome equivalents in the sample, one can improve the sensitivity of the assay by increasing the number of reactions for a fixed concentration of fetal nucleic acids. Lo et al. and Fan et al. both demonstrated that with the use of a few thousand digital PCRs, a diagnosis of trisomy 21 could be achieved in a sample containing 10% trisomic fetal DNA (Fan and Quake 2007; Lo et al. 2007a). In general, the lower the fractional concentration of fetal DNA, the higher is the number of digital PCRs that are needed to achieve robust detection of the trisomy. A similar principle has also been shown to be feasible in detecting allelic ratio imbalance of the PLAC4 mRNA sequence in the RNA–SNP approach discussed above, resulting in the so-called digital RNA–SNP approach (Lo et al. 2007a). To improve the throughput and reduce the cost and amount of plasma required in digital PCR, a microfluidics array chip platform has been developed. Recently, Lun et al. have applied this technology on the noninvasive prenatal diagnosis of monogenic diseases by detecting the allelic imbalance between the mutant and wild-type alleles in maternal plasma (Lun et al. 2008b). This approach is called the digital relative mutation dosage method. They showed that noninvasive fetal genotyping

is potentially feasible in all heterozygous mothers, which was previously possible in certain pregnancies only by detection of a linked SNP of the paternally inherited mutant allele.

As an even more powerful variant of the single molecule counting theme, two groups of researchers independently applied massively parallel genomic sequencing technology in the noninvasive prenatal diagnosis of fetal chromosomal aneuploidy (Chiu et al. 2008; Fan et al. 2008). In this approach, millions of plasma DNA molecules are sequenced simultaneously by a "next generation" DNA sequencer. Each molecule is sequenced at one end, and this sequence tag is then used to map the sequence back to the reference human genome. The proportional representation of each chromosome in the plasma can then be calculated. In maternal plasma from women carrying trisomy 21 fetuses, the proportional representation of chromosome 21 sequences in maternal plasma would be increased. Chiu et al. and Fan et al. both reported a high sensitivity and specificity in the prenatal detection of trisomy 21 using this approach (Chiu et al. 2008; Fan et al. 2008). The current shortcoming of this approach is its relatively high costs and the relatively low throughput in terms of the number of cases analyzed per run per machine.

9.5.5 Enrichment of Circulating Fetal Nucleic Acids in Maternal Plasma

As emphasized above, circulating fetal DNA only constitutes about 3–6% of the total circulating DNA in maternal plasma, and detection of these minor species requires highly sensitive technologies (Lo et al. 1998b). Besides technical advance of different detection systems, investigators have also tried to develop strategies to enrich the concentration of fetal nucleic acids in maternal plasma. This can theoretically be done either by suppressing the maternal background or by improving the recovery of the fetal portion. Dhallan et al. reported that the use of formaldehyde-treated plasma can increase the fractional concentration of fetal nucleic acids by preventing excessive maternal cell lysis after blood sampling (Dhallan et al. 2004). However, these results cannot be replicated in a number of independent studies (Chinnapapagari et al. 2005; Chung et al. 2005a, b). It is believed that the apparent improvement might be due to an artifact of the imprecise quantification methodology originally adopted by Dhallan et al. (Lo et al. 2004).

Another way to suppress the maternal contribution exploited the size difference between fetal and maternal circulating nucleic acids. As more than 85% of circulating fetal nucleic acids are short fragments ($<$ 200 bp), with sizes generally smaller than the maternally derived plasma DNA molecules, size fractionation can therefore be used to enrich the fractional concentration of the fetal nucleic acids either by physical or other means (Chan et al. 2004). Physical size fractionation by electrophoresis has been shown to be effective, but this method has been criticized as labor-intensive and contamination-prone since the band corresponding to the fetal

size range has to be sliced and reextracted after electrophoresis (Li et al. 2004a). Besides physical enrichment, investigators also tried to enrich the fractional concentration of fetal nucleic acids by designing different molecular methods that would allow the selective amplification or counting of the short DNA fragments. One example of the former is the use of a stem–loop primer (Tong et al. 2007). One example of the latter involves the use of a nested set of PCR primers in a digital PCR format, the so-called digital nucleic acid size selection (NASS) strategy (Lun et al. 2008b).

9.5.6 Preanalytical Issues in Circulating Fetal Nucleic Acid Analysis

With the rapid developments coming from many centers, investigators should be cautious in comparing studies from different groups, particularly with regard to the preanalytical details such as choice of anticoagulants, the time from venesection to blood processing, details of the centrifugation step of plasma separation, and sample collection time with respect to invasive procedures. All these factors have been found to affect the concentration of cell-free nucleic acids in the plasma samples.

EDTA has been found to be a better anticoagulant than heparin or citrate for delayed blood processing for plasma DNA analysis. It is recommended that blood should be processed as soon as possible, ideally within 6 h after venesection (Lam et al. 2004b). As discussed above in the controversy of long-term persistence of circulating fetal nucleic acids, the centrifugation step is crucial as inadequate separation may cause false positive detection of residual circulating fetal cells (Chiu et al. 2001). Submicron filtration or 16,000$\times g$ centrifugation are reported to be effective in producing genuine acellular plasma. Another important parameter is the sample collection time with respect to invasive procedures such as amniocentesis and chorionic villous sampling since it is associated with a significant increase in the fetal nucleic acid concentration.

It can be speculated that preanalytical standardization of circulating fetal nucleic acids analysis will be increasingly important as this technology is put in routine clinical practice.

9.6 Conclusion and Speculation

The history of the analysis of circulating fetal nucleic acids in maternal plasma is short. Over the last 12 years, analysis of circulating fetal nucleic acids has proven to be a practical approach for noninvasive prenatal diagnosis. For example, noninvasive fetal sex determination and Rh blood group typing by circulating fetal nucleic

acid analysis have been widely validated. Recent advances in digital PCR and massively parallel genomic sequencing have shown promise in noninvasive prenatal diagnosis of fetal chromosomal aneuploidy and monogenic diseases. Other potential applications such as noninvasive fetal gene expression profiling by circulating fetal mRNA analysis may ultimately lead to better understanding of fetal development and prediction of obstetrics complications. Research of circulating fetal nucleic acids has now entered a new era from feasibility and hypothesis testing to systemic and large-scale validation. It is hoped that this approach would ultimately make prenatal testing safer for pregnant women and their fetuses. The biology of maternal plasma nucleic acids, in addition, would be expected to keep workers in the field busy for many more years.

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Chapter 10
The Biology of Circulating Nucleic Acids in Plasma and Serum (CNAPS)

P.B. Gahan and M. Stroun

Contents

Abstract Although nucleic acids have been known to circulate in the blood since 1948, their biology has been studied only since the 1960s. This chapter contains discussion of (a) the presence of DNA and RNA circulating in human plasma and serum from both healthy individuals and patients, (b) the amounts of DNA/RNA

P.B. Gahan (✉)
King's College London, Anatomy and Human Sciences, London Bridge, London SE1 1UL, UK
e-mail: pgahan@aol.com

M. Stroun
OncoXL, Eclosion, 14m chemin des Aulx, 1228 Plan-les-Ouates, Switzerland
e-mail: Mauricestroun@bluewin.ch

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present together with the variables affecting these amounts, (c) possible sources of the DNA/RNA in blood, and (d) the ability of the circulating nucleic acids to enter other cells and to modify the biology of the recipient cells. The relationship of the DNA from cancer patients is considered with respect to the formation of metastases.

10.1 Introduction

The first evidence for the presence of nucleic acids in blood from healthy donors, pregnant women, and clinical patients was made by Mendel and Métais (1948). This study was largely forgotten due to (a) later studies raising doubts concerning the efficacy of the first demonstration, largely because of the uncertainties in the analytical methods available at the time (Stroun et al. 1977a) and (b) the fact that the structure of DNA was still awaiting to be determined (Watson and Crick 1953; Wilkins et al 1953) as was the identification of DNA as the gene (Sinsheimer 1957; Table 10.1).

As a result, the presence of DNA in the circulatory system was essentially ignored until high DNA levels were demonstrated in the blood of patients with systemic lupus erythematosus (Koffler et al. 1973). These data were challenged by Steinman (1975) who, working with only 2 ml blood samples, found no DNA in

Table 10.1 Some developments in understanding DNA and its cellular roles

Date	Authors	Discovery
1871	Miescher	Isolation of DNA (nuclein)
1929	Levene and London	Building blocks of DNA identified
1944	Avery et al.	DNA as genetic material
1948	Mandel and Metais	Circulating nucleic acids in blood
1949	Chayen and Norris	Cytoplasmic DNA localization
1953	Watson and Crick	DNA structure
1953	Wilkins et al.	DNA structure
1957	Sinsheimer	DNA-gene concept
1959	Gartler	DNA uptake by mammalian cells
1962	Gahan et al.	DNA mobility
1962	Stroun	DNA mobility
1962	Pelc	Metabolic DNA
1965	Gahan and Chayen	Messenger DNA
1969	Stroun et al.	Released bacterial DNA transcription in plants
1971	Stroun	Bacterial DNA-dependent RNA polymerase released from bacteria into plants
1972a, b	Stroun and Anker	Released nucleic acids with associated polymerases – a general phenomenon
1977a	Stroun et al	Circulating nucleic acids
1977	Leon et al.	Raised blood DNA levels in cancer
1989	Stroun et al.	Cancer derived blood DNA
2008	Gahan et al.	Metabolic DNA is source of released DNA

serum and only 2 μg/ml in plasma. However, Anker et al. (1975a) showed100 ml peripheral blood to contain 10–15 μg DNA that could explain the negative findings of Steinman who used small blood volumes. Subsequent observations demonstrated the presence of DNA for a range of illnesses (Tan et al. 1966) as well as in cancer patients (Leon et al. 1977). Differences were demonstrable between those cancer patients with primary tumors only and those with metastases (Leon et al. 1977). Extraction and purification of the DNA from cancer patients subsequently showed it to be tumor cell-derived (Stroun et al. 1989).

Such studies formed the basis for a broad range of analyses of nucleic acids circulating in plasma and serum (CNAPS) of patients suffering from a wide spectrum of disorders. Such studies included the increase in blood DNA levels in patients presenting with injury (Tan et al. 1966), stroke (Lam et al. 2003; Rainer and Lam 2006; Geiger et al. 2007), and acute myocardial infarction (Chang et al. 2003; Saukkonen et al. 2007; Antonatos et al. 2006). Successful application of circulating fetal DNA/RNA in pregnant mothers (Lo et al. 1989) has permitted the early detection of fetal sex (Vainer et al. 2008; Veecchione et al. 2008), pre-eclampsia in the first trimester (Lo et al. 1999a; Gupta et al. 2006; Lau et al. 2002), fetal blood genotyping, e.g., fetal Rh status (Finning et al. 2004; Zhang et al. 2000; Li et al. 2004), Down syndrome (Dhallan et al. 2007; Lo et al. 2007) and ß-thalassemia (Li et al. 2005; Papasavva et al. 2006).

This review will consider the biology of CNAPS including the possible cellular origins of the DNA/RNA found in blood, the mechanisms of release into the blood and their uptake into other cells, as well as their possible biological effects in the host organism.

10.2 Nucleic Acid and Nuclease Content

Both DNA (1.8–15 ng/mL) and RNA (2.5 ng/mL) are found in plasma and serum from healthy donors (Anker et al. 1975; Rykova et al. 2006). These levels rise in patients with various cancers, trauma, myocardial infarction, and stroke with values of up to 3,000 μg DNA per mL being recorded on occasions (Wu et al. 2002). The amount of DNA and RNA present in the plasma and serum will depend upon the health status of the individual and the level of nucleases present in blood. The average blood plasma concentration of DNAase I, which forms 90% of total blood DNase, is 41 ± 30 ng/mL for healthy men and 21 ± 21 ng/mL for healthy women with an activity of 0.307 ± 0.249 U/mL for men and 0.405 ± 0.509 U/mL for women. In contrast, values for gastrointestinal cancer patients were about 350 ng/mL (Tamkovich et al. 2006). The average serum RNAase value is 104 units per mL (Reddi and Holland 1976).

Hence, the relatively low levels of circulating DNA in healthy individuals may indeed be due partially to peripheral blood DNAase activity although DNA from cancer patients may be resistant to DNAase and the DNAase levels may have just been low. Similarly, high RNA levels may also be due to RNA resistance to

RNAase digestion especially when high RNAase and RNA levels are present together. The RNA may be protected with a glycolipid due to its apoptotic origin (Böttcher et al. 2006). Furthermore, an RNA fraction is associated with the released DNA-complex from living cells that appears to be protected from digestion by RNAase as is the DNA component from DNAase activity (Anker et al. 1975; Stroun et al. 1977a, 1978; Adams and Gahan 1982,1983).

10.3 Nucleic Acid Sources

There would appear to be seven sources of blood nucleic acids, namely (1) breakdown of blood cells; (2) breakdown of bacteria and viruses, (3) leucocyte surface DNA, (4) cell and tissue necrosis, (5) cell apoptosis, (6) cellular release of exosomes, and (7) spontaneous release of a newly synthesized DNA/RNA-lipoprotein complex from living cells.

10.3.1 DNA

10.3.1.1 Leucocytes

The amount of DNA released from the death of leucocytes is minimal and cannot account for the total amounts found in normal blood i.e., 1.8–36 ng/mL (Anker et al. 1975; Tamkovich et al. 2006; Chang et al. 2003) and certainly not the levels found during clinical disorders, although larger amounts have been reported for normal individuals and those suffering from, e.g., cancer (Wu et al. 2002).

10.3.1.2 Bacteria and Viruses

Few bacteria will be found normally in blood and so could not account for the levels of DNA seen. Some viral DNAs have been recorded in peripheral blood, specifically Epstein Barr virus (EBV) in patients with nasopharyngeal carcinoma (Lo et al. 1999b) and human papilloma viral carcinoma in about half of cervical carcinoma patients and hepatitis patients (Chan et al. 2003; Yang et al. 2004). Human herpes virus-6 (HHV-6) DNA has also been demonstrated in both plasma and serum. HHV-6 DNA in plasma was found to be readily attacked by DNAase and so is considered to be unencapsulated. HHV-7 was also determined and identified but only in P1 of the plasma subfractions, whereas HHV-6 appeared in P1, P2, and S subfractions, especially in P1 (Achour et al. 2007).

That bacteria and viruses form a very small part of CNAPS is confirmed by studies on blood from 51 apparently healthy individuals; 4.5×10^5 DNA sequences (7.5×10^7 nucleotides) were obtained of which 87% were attributable

to known database sequences. Of these, only 3% were found to be xenogeneic (Beck et al. 2009).

10.3.1.3 Cell-Surface DNA

The presence of DNA has been demonstrated on the surface of both leucocytes and erythrocytes (Tamkovich et al. 2005; Skvortsova et al. 2006). This has been interpreted as either DNA entering the cells or merely being associated with the surface. However, lipopolysaccharide from gram-negative bacteria can activate interleukin-5 (IL-5) – or interferon-gamma-primed eosinophils to release mitochondrial DNA (mtDNA) in a reactive oxygen species-dependent manner yet independently of eosinophil death. DNA release occurs rapidly in a catapult-like manner – in < 1 s. mtDNA and granule proteins form extracellular structures able to bind and kill bacteria both in vitro and in vivo under inflammatory conditions (Yousefi et al. 2008). In addition, during infection, neutrophils release sticky webs of chromatin (DNA + histones) that can trap invading microbes. These neutrophil extracellular traps contain granules laden with lytic enzymes and antimicrobial peptides resulting in the rapid killing of pathogens (Kessenbrock et al. 2009).

10.3.1.4 Necrosis

Necrosis has also been mentioned as a source of DNA in blood. However, during necrosis, the DNA is nonspecifically and incompletely digested, and when applied to gels and run electrophoretically, the DNA smears rather than forming a ladder (Nagata et al. 2003; Van der Vaart and Pretorius 2007; Wang et al. 2003). The DNA released from necrotic cells yields fragments in excess of 10,000 bp [66]. Indeed, it has been shown by electrophoresis and sucrose-gradient sedimentation that blood plasma samples contain high molecular weight DNA fragments ranging from 21 kb (Stroun et al. 1987) to 80 kb in length (Giacona et al. 1998) This would seem to make necrosis a contributor to the circulating DNA though not necessarily a major contributor, since agarose gel electrophoretic separation of the isolated DNA has revealed a ladder pattern (Nagata et al. 2003; Holdenrieder and Stieber 2004; Kessenbrock et al. 2009) with fragment sizes primarily ranging from 180 to 1,000 bp.

10.3.1.5 Apoptosis

This would appear to present one of the major contributors to CNAPS. DNA in blood was shown to be double-stranded using either hydroxyapatite chromatography or density gradient centrifugation (Anker et al. 1975; Stroun et al. 1977a, b). Agarose gel electrophoretic separation of the DNA isolated from blood has revealed a ladder pattern (Nagata et al. 2003; Nagata 2005; Holdenrieder and Stieber 2004) with fragment sizes ranging from 180 to 1,000 bp. The fragment

ends are capped so that they may be present in the form of nucleosomes or apoptotic fragments. This could be interpreted as the circulating DNA being the product of apoptosis from, e.g., tumors. The electrophoretic pattern of DNA from apoptotic cells is initially of 50–300 kb fragments that then break down into multiples of nucleosomal fragments of 180–200 bp to form the typical ladder pattern on the gel (Nagata et al. 2003; Nagata 2005; Holdenrieder and Stieber 2004). Thus, it appears that the circulating DNA fragments have their origin via apoptosis (Giacona et al. 1998; Holdenrieder and Stieber 2004; Jahr et al 2001). However, under normal circumstances, the fragments from cells undergoing apoptosis are phagocytosed by macrophages and dendritic cells at the final stage (Pisetsky 2004) and so should not be released into the blood. Nevertheless, there could be a release from solid tumors if there was a breakdown in the phagocytic process (Jahr et al. 2001; Atamaniuk et al. 2006; Viorritto et al. 2007).

Further evidence for the apoptotic origin of DNA comes from the identification of mtDNA in CNAPS (Viorritto et al. 2007). Both particle-associated and free mtDNA have been found in plasma, their respective concentrations being affected by the process used to derive plasma from whole blood (Goebel et al. 2005). mtDNA increases were found in trauma patients (Chiu et al. 2003; Lam et al. 2004), with the median plasma mtDNA concentrations being 15,105,000 copies per mL in the severely injured subgroup and 7,115,000 copies per mL in the minor/moderate subgroup. Similarly, Mehra et al (2007) found a 2.6-fold increase in mtDNA in patients who died from prostate cancer as opposed to those who survived. The median mtDNA copies per 100 μL plasma for prostate cancer patients were 49,193 (interquartile range, 18,683–109,485 copies) and 19,037 (interquartile range, 13,515–24,744 copies) for benign controls. However, there was no significant relation between mtDNA in plasma and patient age ($n = 69; r = -0.20, P = 0.10$).

10.3.1.6 Exosomes

Exosomes may be a low-level contributor to CNAPS. Although Ekstrom (2008) reported that no DNA was present in exosomes released from mouse mast cells, mtDNA has been reported to be present in exosomes released from glioblastoma cells and astrocytes (Guescini et al. 2009).

10.3.1.7 Spontaneously Released, Newly Synthesized DNA

A major contributor to CNAPS would appear to be newly synthesized DNA, which has been shown to be spontaneously released from both stimulated and nonstimulated human (Anker et al. 1975; Rogers et al. 1972; Olsen and Harris) and rat lymphocytes (Adams and Gahan 1982, 1983) in vitro. This process has been shown to be of general occurrence in vitro being extruded from bacteria (Ottolenghi and Hotchkiss 1960, 1962; Borenstein and Ephrati-Elizur 1969), human (Anker et al. 1975), and other mammalian (Olsen and Harris 1974; Adams et al. 1997), avian

(Adams and MacIntosh 1985), and amphibian (Stroun and Anker 1972a; Stroun et al. 1977a, b; Anker and Stroun 1977) cells, a process confirmed by in vivo studies on whole chick embryos (Challen and Adams 1987). This DNA is complexed with newly synthesized lipoprotein (Adams and Gahan 1982, 1983; Adams and MacIntosh 1985) and also has newly synthesized RNA associated with it (Adams and Gahan 1982, 1983), i.e., all of the components of this complex are newly synthesized. In addition to tumor cells releasing this newly synthesized DNA complex in vitro (Adams et al. 1997), whole tumors in vivo have also been shown to release DNA into the blood circulation (García-Olmo et al. 2010).

10.3.2 RNA

In a similar fashion to DNA, RNA can be released by (1) breakdown of blood cells, (2) bacteria and viruses, (3) necrosis, (4) apoptosis, (5) exosomes, and (6) spontaneous release of a newly synthesized RNA in the form of the DNA/RNA-lipoprotein complex from healthy cells.

10.3.2.1 Leucocytes

Clearly, the breakdown of leucocytes could release a range of RNAs into the blood, thus accounting for the levels of RNA already measured (Rykova et al. 2006).

10.3.2.2 Bacteria and Viruses

The presence of viral RNA has been demonstrated for hepatitis C RNA in the plasma and serum of European and African patients using an RNA-polymerase chain reaction and the isothermal NASBA nucleic acid amplification system encompassing a gel-based detection assay. The extraction method used has permitted the detection of hepatitis C RNA equally well in both serum and plasma with either heparin or EDTA (Cha et al. 1991). Majde et al (1998) have also shown the release of dsRNA into the extracellular medium from influenza virus-infected MDCK epithelial cells. Little information is available of the levels of RNA released from bacteria in the blood-stream.

10.3.2.3 Necrosis and Apoptosis

A spectrum of mRNAs has been demonstrated in plasma and extends from genes overexpressed in a variety of different tumors (Li et al. 2006; Garcia et al. 2006) to fetal genes in the blood plasma of pregnant women (Hung et al. 2009) and patients

with diabetic retinopathy (Shalchi et al. 2008) as well as housekeeping genes detected in the plasma of healthy persons (Holford et al. 2008).

QRT-PCR detectable fragments of ribosomal 18S rRNA were also found in the extracellular RNA pool circulating in blood plasma of healthy subjects and cancer patients (El-Hefnawy et al. 2004). Ribosomal 28S rRNA fragments secreted by primary and cultivated human cells into the culture medium have been demonstrated (Vlassov et al. 2007) as have exosomes in blood carrying mRNA for EGFRvIII (Skog et al. 2008).

Mitochondrial RNA (mtRNA) has also been reported to be present in plasma there being no significant relation between mtRNA in plasma and patient age ($n = 69$; $r = -0.16$, $P = 0.19$ for mtRNA). However, in a study on prostate cancer patients, nonsurvivors were found to have a 3.8-fold increase in mtRNA compared with survivors ($P = 0.003$; nonsurvivors: median copies, 16,038; interquartile range, 5,097–48,544 copies; survivors: median copies, 4,183; interquartile range, 2,269–8,579 copies) (Mehra et al. 2007).

10.3.2.4 Exosomes

In general, the RNAs released by cells, e.g., in exosomes, can be in a variety of forms including dsRNA, rRNAs, m-RNAs, and small RNAs. Thus, exosomes from both mouse and human mast cells have been shown to contain RNA as have primary bone marrow-derived mouse mast cells (Valadi et al. 2007). The presence of RNA in exosomes has also been reported for those released from tracheobronchial ciliated epithelial cells (Kesimer et al. 2009).

Importantly, exosomes released into the blood from glioblastoma patients have been shown to contain mRNA mutant/variants and miRNA characteristic of gliomas. In particular, tumor-specific EGFRvIII, containing a mutation specific for glioblastoma was detected in the serum microvesicles of seven out of twenty five glioblastoma patients (Skog et al. 2008).

Exosomes are known to contain RNAse that is employed to destroy unwanted RNA (Alberts et al. 2002). Hence, if such exosomes were to be released into the blood stream, they could contribute to the RNAse present.

10.3.2.5 Spontaneous Released, Newly Synthesised RNA

In addition, the RNA associated with the DNA/RNA-lipoprotein complex released from living cells will also be a source of blood RNA. When the DNA-lipoprotein is initially synthesized as a cytosolic complex, the RNA is not yet present but is associated with the complex on leaving the cell (Stroun and Anker 1972a). Hence, there appears to be a synthesis of the RNA fraction prior to leaving the cell and apparently involving the DNA-dependent RNA polymerase associated with the complex (Stroun et al, 1969; Stroun 1970, 1971; Adams and Gahan 1982).

10.4 Origin and Timing of Nucleic Acid Release

10.4.1 DNA

Newly synthesized DNA can be detected as ^{3}H-DNA released into the culture medium in vitro in as little about 3 h after removal of the ^{3}H-thymidine source after incubating either isolated frog auricles or lymphocytes (Stroun et al. 1977a, b; Adams and Gahan 1982, 1983). The DNA is released in small quantities as seen from studies of the release of DNA from frog heart auricles (Stroun et al. 1977b) in which 200 auricle pairs released 21.3 μg DNA in 4 h, i.e., 0.1 μg DNA per auricle pair in 4 h. When the medium was changed each 4 h for a total period of 24 h, 200 auricle pairs released similar amounts of DNA during each 4 h period as if following a homeostatic mechanism. The total yield in 24 h (6 × 4 h) reached 128 μg DNA, i.e., 0.64 μg DNA per auricle pair per 24 h. As the frog heart is syncytial and, moreover, not all nuclei are necessarily releasing DNA at the same time, no calculation was made concerning the release of DNA per cell. Similar data were obtained with lymphocytes (Anker et al. 1975) thus confirming the homeostatic nature of the DNA release.

Thus, the newly synthesized, released ^{3}H-DNA can be detected early (Table 10.2). It is not clear if the apparent limit to the amount of newly synthesized complex released is due to a feedback mechanism operating on achieving a certain extracellular level of the complex or if there is an equilibrium established between the amount released and the amount taken up by adjacent cells/tissues.

Adams and MacIntosh (1985) working with chick embryo fibroblasts showed that, on fractionating the DNA after labeling the cells for various periods of time with ^{3}H-thymidine, the first labeled DNA was found in the nuclear fraction. ^{3}H-DNA was found subsequently in the cytosol after 3 h and in the external medium after 5 h.

It has been argued that the initial sites of nuclear synthesis of this released DNA fraction are those originally designated as metabolic DNA (Pelc 1962). This concept was initiated after finding labeled nuclei in nondividing cell populations, which could be explained neither by synthesis for mitosis nor repair DNA. Pelc concluded that it consisted of "additional copies of cistrons that are active in the cell, that these copies perform the metabolic functions of the DNA, transcription to RNA for example, and that they are subject to deterioration while active and can be replaced" (Pelc 1968). Recently, arguments have been put forward that metabolic DNA and released newly synthesized DNA fractions could represent the same

Table 10.2 DNA release times from different cell sources

Human lymphocytes	3 h	Anker et al. (1975a), Rogers et al. (1972)
Chick embryo fibroblasts	5 h	Adams and MacIntosh (1985)
Rat lymphocytes	1–3 h	Adams and Gahan (1982, 1983)
Frog heart auricles	4 h	Stroun et al. (1977a, b)

DNA since they share common features in that (a) there is a synthesis of both forms of DNA in both actively dividing cell populations and nondividing, differentiated cell populations, (b) the two forms of DNA have lower molecular weights than that of the stable DNA fraction, and (c) the synthesis of these two DNA fractions in differentiated cells is paralleled by different metabolic processes, e.g., cardiac muscle function, stem rigidity by collenchymal cells, and metabolic functions in hepatocytes (Gahan et al. 2008).

The mechanism of CNAPS DNA release from the cell is not fully understood though preliminary in vitro studies have shown that protein secretion inhibitors reduce the release of LINE-1 fragments from HeLa cells and human endothelial umbilical vein cells (HUVEC) (both cell free and cell-surface bound DNA). Hence, in the case of HUVEC monensin, glyburide and methylamine reduced the DNA release by 30, 35, and 19%, respectively. Monensin reduced the release from HeLa cells by 15% though glyburide increased cell-surface-bound DNA by 50% 9 (Morozkin et al. 2008). Interestingly, the presence of both Alu repeat sequences and LINE-1 in serum from both normal and cancer patients (Stroun et al. 2001; Morozkin et al. 2008) implies the presence of transposons as a component of CNAPS.

10.4.2 RNA

The synthesis of the RNA fraction appears to be later than that of the DNA and lipoprotein in the complex. The lipoprotein is made in the cytosol at about 3 h after DNA synthesis is initiated in the nucleus (Adams and Gahan 1982). DNA-dependent RNA polymerase is present in the complex (Stroun 1970, 1971; Stroun et al 1969). The complex released from the cell 5 h after the initiation of DNA synthesis contains newly synthesized RNA (Adams and MacIntosh 1985).

10.5 Released Nucleic Acids Can Enter and Express in Other Cells

10.5.1 DNA

10.5.1.1 Released Prokaryote DNA Enters Eukaryote Cells

The spontaneous release of DNA by bacteria has been well-demonstrated (Ottolenghi and Hotchkiss 1960, 1962; Borenstein and Ephrati-Elizur 1969) as has the entry of DNA from *Micrcoccus lysodekticus* into rabbit spleen cells and lymphocytes (Olsen and Harris 1974). Anker and Stroun (1972) examined the possibility that bacteria could release DNA into eukaryotes where it could be expressed. The experiment was based on the fact that bacteria do not normally pass the blood-brain

barrier. Frogs were injected intraperitoneally with either *Escherichia coli* or *Bacillus subtilis* or *Agrobacterium tumefaciens* and, subsequently, were further injected intraperitoneally with antibiotics (2,000 μg colimycin for *E. coli* and *B. subtilis* and 2,000 μg ampicilin + 2,000 μg cloxacilin for *A. tumefaciens*) to destroy the bacteria prior to injecting ^{3}H-uridine intraperitoneally.

Total RNA was extracted from the isolated brains after they had been tested for sterility. The presence of bacterial RNA in the frog brains was demonstrated by hybridizing the RNA against each of the bacterial DNAs held on filters. This implied that the DNA released by the bacteria had travelled to the brain where it was incorporated and expressed (Anker and Stroun 1972). The passage of the released bacterial DNA to the brain across the blood-brain barrier was further demonstrated by the intraperitoneal injection of bacterial ^{3}H-DNA that was subsequently found to be present in the nuclei of the brain cells (Anker and Stroun 1972). Frog auricles were also shown to take up and express the DNA released from bacteria (Anker and Stroun 1972). Similar results were obtained if the spontaneously released complex from bacteria was incubated together with the frog auricles (Stroun and Anker 1972b, 1973). This phenomenon can also occur in plant cells. When DNA is naturally released from bacteria into plant cells, a bacterial RNA can be found in these cells (Stroun et al. 1971). Other autoradiographic and biochemical experiments showed that bacterial DNA was also able to enter higher plant cells and be expressed (Stroun et al. 1970; Gahan et al. 1973, 2003). Bacterial DNA can enter plant cells, be integrated into the plant cell genome, and be expressed. Sterile cut shoots of *Solanum aviculare* were fed for 6 h with *E. coli* DNA carrying three marker genes, namely: GUS gene (ß-glucuronidase), BAR gene (phosphinotricin, an inhibitor of glutamate synthase to yield resistance to bialophos), and NPT II gene (neomycin phosphotransferase II that blocks the antibiotic interaction with ribosomes so leading to resistance to, e.g., kanamycin) followed by chasing with water for 24 h. The cut shoots were rerooted and grown under sterile conditions for 2 months (Gahan et al. 2003). All three genes were expressed in all tissues of the established plants, their stable insertion into the host genome being confirmed by Southern blotting. The F1 generation was found to carry all three genes after the sterile, transformed plants were allowed to flower and seed (Gahan et al. 2003).

10.5.1.2 Release and Uptake of DNA by Eukaryote Cells

The uptake of released DNA by recipient cells and the subsequent expression of its biological activity has been demonstrated in in vitro studies on mammalian cells.

Immune response. An allogenic T-B lymphocyte co-operation involving lymphocyte subsets from human donors with different allotypes showed that B lymphocytes cultured in the presence of the supernatant from the culture medium of T cells, previously exposed to inactivated herpes simplex virus, synthesized an antiherpetic antibody carrying some allotypic markers of the T cell donor. DNA purified from the supernatant of the T cell culture medium had the same effect on B lymphocytes as did the nonultra-centrifuged supernatant (Anker et al. 1980).

In other experiments (Anker et al. 1984), nude mice were injected with DNA extracted from the complex released by human T lymphocytes previously exposed to inactivated Herpes or Polio viruses. The serum from these mice, tested for its neutralizing activity, showed that mice synthesized antiherpetic or antipolio antibodies depending on the antigen used to sensitize the T cells. The antibodies carried human allotypes as shown through their neutralization by human antiallotype sera. Furthermore, when concentrated, the newly synthesized complex transformed much more efficiently than did either the DNA purified from the supernatant or the crude supernatant itself.

Effect on cell division. The DNA released from mouse tumor cell lines J774 cells (leukaemia), P497 cells (glial tumor), and nonstimulated lymphocytes was isolated from the culture medium by agarose gel chromatography (Adams et al. 1997). After concentration, the released DNAs were added to the culture media of each of the different cell types. Thus, the tumor cell lines were each incubated in the presence of either of the two tumor cell line DNAs or the lymphocyte DNA, i.e., each cell type was incubated in the presence of either a self DNA or each of two foreign DNAs. ^{3}H-thymidine was added to the cultures of each cell type together with the particular donor DNA and the amount of nuclear incorporation of the ^{3}H-thymidine into DNA of the recipient cells was measured (the index of DNA synthesis). The levels of DNA synthesis in the tumor cell lines was the same in the presence of either of the DNAs released by the two tumor cell lines, but it was reduced by about 60% in the presence of the lymphocyte DNA (Adams et al. 1997).

Conversely, the incubation of the nonstimulated lymphocytes in the presence of either of the tumor cell line DNAs showed an initiation of DNA synthesis in the presence of the tumor DNAs but not in the presence of the lymphocyte DNA (Adams et al. 1997).

DNA effects in irradiated cells. On exposure to X-rays at an adaptation dose of 10 cGy, Chinese hamster ovarian cells showed a transposition of the chromosomal pericentromeric loci of homologous chromosomes from the perimembrane sites to approach each other and an accompanying activation of the chromosomal nucleolar-forming regions (Ermakov et al. 2008). The movement of the pericentric loci appears to be associated with repair of the DNA double-strand breaks during the development of an adaptive response to radiation. When untreated cells were grown in a medium containing DNA fragments isolated from the medium of the treated cells, they showed similar changes to those seen in the treated cells. Incubation of the untreated cells in medium containing the DNA fragments from medium of untreated cells had no such effect (Ermakov et al. 2008).

DNA effects on myocardiocyte contraction rates. AT-rich fragments of the human satellite 3 tandem repeat (1q12 region) and GC-rich fragments of the rDNA were isolated from the circulating DNA of patients with myocardial infarction (Bulicheva et al. 2008). When fed in vitro to neonatal rat ventricular myocytes in culture, AT-rich fragments at 1 ng/ml increased the frequency of cardiomyocyte contractions and GC-rich fragments at 0.5 ng/ml decreased the contraction frequency. The serum circulating DNA fraction from patients with acute myocardial infarction decreased the contraction frequency in proportion to the amount of rDNA fragments contained

thus implying that the GC-rich fragments circulating in the blood of myocardial infarct patients could affect contractile function of the myocardial cells (Bulicheva et al. 2008).

Hence, in these experiments, the DNAs that have been taken up by the cells have shown a biological activity.

Tumor induction. The SW 480 cell line, originating from a human colon carcinoma, contains a point mutation of the K-ras gene on both alleles. These cells in culture released the DNA/RNA-lipoprotein complex containing the mutated K-ras gene. When NIH/3T3 cells were cultured in the presence of nonpurified SW 480 cell supernatant, without the addition of any other compound, transformed foci appeared as numerously as those occurring after a transfection resulting from a cloned ras gene administered as a calcium precipitate. The presence of a mutated ras gene in the transfected foci of the 3T3 cells was confirmed by hybridization after PCR. This result was confirmed by sequencing the PCR product (Anker et al. 1994).

The effects on cultured cells of plasma from healthy individuals and patients with colon cancer. Cultures of NIH-3T3 cells and human adipose-derived stem cells (hASCs) were supplemented with samples of plasma from patients with either K-ras-mutated colorectal tumors or from healthy subjects by either direct addition of plasma to cultures in standard plates or avoiding plasma–cell contact by placing membranes with 0.4 μm pores between the plasma and the cells thus acting as a filter. K-ras mutated sequences were never detected by real-time PCR in hASCs. However, human gene transfer occurred in most cultures of NIH-3T3 cells, as verified by the presence of human K-ras sequences, p53 sequences, and ß-globin encoding sequences. Furthermore, NIH-3T3 cells were oncogenically transformed after being cultured with plasma from colon cancer patients as demonstrated by the development of carcinomas in NOD-SCID mice after they had been injected with the transformed NIH-3T3 cells. The presence of an artificial membrane containing 4μm diameter pores placed between the NIH-3T3 cells and the plasma gave similar results showing that the transforming factor had a diameter of less than 4 μm. A TEM study of the membranes from the plasma experiments confirmed the complete absence of cells though many structures of < 0.4 μm diameter were seen to pass through the pores of membrane (García-Olmo et al. 2010).

Methylation. The uptake of methylated fragments of RAR2 gene into HeLa and human umbilical vein endothelial cells was twice as efficient as that of unmethylated fragments. One of the most common alterations of tumor-related DNA found in CNAPS DNA from cancer patients is its hypermethylation. Since the methylated RAR 2 gene fragments are more prevalent than the unmethylated fragments in intracellular traffic, they would appear to pose a higher transformation potential (Skvortsova et al. 2008).

Clearly, both naked DNA and the newly synthesized, spontaneously released DNA complex released into the blood can move to other parts of the organism and into host cells. On entering cells of a similar type, there appears to be no expression of a changed biological activity. However, if the uptake is into cells of a different type, it can express a changed biological activity in the host cell. The complex was found to be more transformant than the DNA purified from the complex.

The results above have implications for (a) the formation of metastases by DNA released from tumor cells into the circulation termed "genometastasis" (García-Olmo et al. 1999, 2000) and (b) the ability of the DNA to move to cells in other parts of the body, including the brain, in spite of the blood-brain barrier, where it could be taken up and expressed (Anker and Stroun 1972). The uptake of CNAPS DNA by stem cells raises interesting possibilities (Garcia-Olmo et al. 2004).

10.5.2 RNA

Little work has occurred on the entry of CNAPS RNA into other cells. However, double-stranded RNA polyriboinosinic polyribocytidylic acid (polyI:C) has been found to activate murine glomerular endothelial cells via RIG-1 in the cytosol to produce inflammatory cytokines, chemokines, and type I interferons (Hägele et al. 2009).

The experimental delivery of siRNA causes many difficulties and so it may be considered that the natural uptake of siRNA from CNAPS will be minimal. However some experiments have shown that entry of siRNA has been shown to produce an apparent dose-dependent siRNA-mediated suppression of lamin A/C in primary human umbilical vein endothelial cells (Overhoff and Sczakiel 2005; Detzer et al. 2008). Perhaps more importantly, Skog et al (2008) have shown mRNA and miRNa to be taken up by normal host cells including brain and microvascular endothelial cells. In addition, glioblastoma-derived vesicles were shown to stimulate proliferation of a human glioblastoma cell line.

10.6 Mechanisms of CNAPS Entry into CELLS

The early studies on the uptake of DNA and RNA into cells were reviewed by Stroun et al (1977a). More recently, other mechanisms of nucleic acid entry have been demonstrated including the entry of various sources of DNA through the Toll receptor system (Chuang et al. 2000; Hemmi et al. 2002; Cornélie et al. 2004; Barton et al. 2006; Dalpke et al. 2006; El Kebir et al. 2008).

Nevertheless, there is every reason to believe that CNAPS can enter cells by other methods and, in some cases, change the biology of the recipient cells. The question arises as to the mechanisms involved in this cell entry process and the mechanism by which the nucleic acids avoid the digestive processes of the lysosomal system.

10.6.1 DNA Entry into Mouse Skeletal Muscle

Preliminary studies showed that either plasmid DNA or artificial mRNA injected intramuscularly resulted in the expression of the transgenes in myofibres (Wolff et al. 1990). It was thought that the plasmid DNA expression vectors had entered

the nuclei in order to be transcribed since they contained type II RNA polymerase promoters. The artificial mRNAs must have entered the sarcoplasm in order to have had access to the ribosomes for translation. Additional study of this system (Wolff et al. 1990) showed that the results were not due to damage by injection. Furthermore, TEM examination of the material demonstrated colloidal gold conjugated plasmid DNA to cross the external lamina and enter T tubules and caveoli, but none was seen in the endocytic vesicles. Interestingly, similarly labeled polylysine, polyethylene glycol, and polyglutamate primarily remained outside the myofibres.

10.6.2 DNA Entry into Human Keratinocytes

Visualization and measurement of the uptake of FITC-labeled plasmid by keratinocytes by FACS analyses detected up to 15% internalization in a dose- and time-dependent fashion. This uptake was 90% inhibited by cyclohexamide thus implying a protein-mediated uptake. The macropinocytosis pathway was blocked with amiloride and *N*, *N*-dimethyl amilorid inhibiting plasmid uptake by $> 85\%$, while the use of nystatin and filipin III to block coated pits and caveoli made no difference. Co-localization studies using confocal microscopy showed the presence of DNA in the endosomes and lysosomes. Further analysis of the membrane proteins showed the presence of two binding proteins, ezrin, and moesin that may be linked to the internalizing of the DNA via the macropinocytosis pathway (Basner-Tschakarjan et al. 2004).

10.6.3 DNA Entry into Mouse (Leukaemia) J774 Cells

The incubation of J774 cells with pcDNA3-Hsp65-Alexa 488 in the presence of LysoTracker red showed no DNA fluorescence in the endosomes and lysosomes. However, when the experiment was repeated using Texas Red dextran, significant co-localization was seen after 24 h. This implies that the plasmid DNA was present in the endosomes and lysosomes but has interfered with the acidification of the endosomes in a way not yet understood. A similar result was obtained if unlabeled plasmid DNA was employed. Replacement of the J774 cells by spleen-derived dendritic cells gave a similar result. The data were confirmed by incubating J744 cells with the mammalian expression vector-heat shock protein gene hpHsp65-fluophore Alexa 488 (pcDNA3-Hsp65-Alexa 488) and DQ Ovalbumin that gives a green color on degredation in the lysosomes. This did not occur, thus confirming that acidification of the lysosomes had not occurred (Trombone et al. 2007).

Such a mechanism would permit the DNA to enter endosomes without being digested and to travel safely through the cytoplasm toward the nucleus prior to either release from the endosomes or entry into the nucleus. It has been suggested that since the naked plasmid DNA carries a negative charge similar to the endosomal membrane, the charge similarity could lower the association between the membrane

and the DNA, thus impeding the escape of the DNA and hence aiding its transport through the cytosol (Trombone et al. 2007).

Nevertheless, the mechanism by which the DNA leaves the endosomes is not clear.

10.6.4 dsRNA Entry into Murine GEnC Cells

The uptake of poly I:C RNA into murine GEnC cells resulted in the production of IL-6, CCL2, CCL5, CXCL10, IFN-, and IFN-ß. This required complex formation with cationic lipids to be taken up into GEnC via clathrin-dependent endocytosis but was independent of endosomal acidification (Hägele et al. 2009).

10.6.5 RNA Entry into ECV304 Cells

On examining the co-incubation of Rh110-labeled siRNA phosphorothioate (PTO)-modified ON (TM6-6) into ECV304 cells, it was found that the siRNA was internalized by caveosomal vesicles and transported to the perinuclear region of the cytoplasm where it was released to become an active agent in the cell, though similar experiments performed on human and mouse lymphoid cells did not to support a caveolin-mediated pathway (Fra et al. 1994). Neither the human T-lymphoma cell line Jurkat (Schneider et al. 1977) nor the mouse B-lymphoid cell line BJA-B (Menezes et al. 1975) supported the PTO-stimulated uptake of siRNA under similar conditions. It was assumed that the caveosomes form non-acidic compartments. However, in the absence of PTOs, the uptake of the siRNA is reduced and the molecules are distributed throughout the cytoplasm (Overhoff and Sczakiel 2005).

10.6.6 Viral RNA Entry into 2B2318 Lymphocytes

Although they were unable to detect caveolae in earlier studies, Fra et al (1994) were able to detect SFV-VIP21 virus (dog/simian) entry into 2B2318 lymphocytes via the production of caveoli.

Thus, it is possible that CNAPS DNA can enter primarily via either macropinocytosis into cells in vitro or via caveoli into muscle in vivo and block the acidification of the endosomes, thus preventing their development into lysosomes and hence degradation of the DNA. Moreover, the DNA can exploit the endosomes' movements toward the nucleus as a transport mechanism prior to being released and possibly entering the nucleus as in the case of the complex (Adams and MacIntosh 1985). The entry of RNA could well occur by a similar mechanism to that of DNA

with a lack of either endosomal or caveosomal acidification allowing the RNA to remain undigested. It is not clear how the RNA escapes from either the endosomes or the caveosomes, but it is able to exert a biological effect upon the recipient cells (Skog et al. 2008; Hägele et al. 2009).

10.7 Conclusions

CNAPS nucleic acids are present in a variety of forms, which appear to be capable of entering cells with which they come into contact. The mechanism of entry, and in some cases exit, is still not clear for either RNA or DNA, although caveoli and pinocytosis seem to be implicated in their entry into cells. As yet, there is no information as the possible rôles that connexins, innexins, and pannexins might play in the intercellular movement of nucleic acids (Scemes et al. 2007; Nguyen and Taffet 2009).

Both naked DNA and the newly synthesized, spontaneously released DNA complex released into the blood can move to other parts of the organism and into host cells. On entering cells of a similar type, no effects appear to occur. However, if the uptake is into cells of a different type, the biological activity of the host cell may be modified. The uptake of CNAPS DNA by stem cells raises interesting possibilities (Garcia-Olmo et al. 2004). However, the modification of cellular biology on the uptake of DNA released from tumor cells has particular implications for both the formation of metastases by the DNA released from tumor cells into the circulation and the ability of the DNA to move to cells in other parts of the body. In particular, and in spite of the blood-brain barrier, DNA can move to the brain, where it can be taken up and expressed so possibly leading to the formation of metastases.

Similarly, RNA in its various forms can leave cells and enter others where, in a few cases, it has been shown to modify the biology of the host cell though fewer studies have been made on this aspect of CNAPS RNA. In view of the fact that both CNAPS DNA and RNA have been implicated in the transmission of information leading to tumor induction, it is pertinent to ask a question concerning blood collected for blood transfusions. Should such blood be screened for specific forms of CNAPS prior to use?

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Chapter 11
Modulation and Regulation of Gene Expression by CpG Oligonucleotides

Dennis Klinman and Sven Klaschik

Contents

Abstract Immune cells are triggered when their Toll-like receptors (TLR) interact with pathogen-associated molecular patterns present on infectious agents. For example, TLR9 recognizes unmethylated CpG motifs present in bacterial DNA, whereas TLR3 recognizes single-stranded RNA present in viruses. Microarrays were used to examine the pattern of gene activation elicited following engagement of TLR9 and TLR3. Two distinct waves of activation were induced by CpG DNA. Each wave was regulated by a small group of major and minor inducers. Included in these waves of gene activation were suppressive networks that served to then downregulate

D. Klinman (✉) and S. Klaschik
Cancer and Inflammation Program, National Cancer Institute, NCI in Frederick, Frederick, MD 21702
e-mail: klinmand@mail.nih.gov

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TLR-dependent cellular stimulation. Triggering via TLR3 activated a larger and more complex regulatory network than did TLR9. Synergy was observed when cells were stimulated with both TLRs. This was characterized by the early and persistent activation of multiple regulatory pathways, resulting in a prolonged signaling cascade that increased, magnified, and diversified gene expression.

11.1 Introduction

Unmethylated CpG motifs present in bacterial DNA interact with Toll-like receptor 9 (TLR9) and trigger an innate immune response that promotes the production of Th1 and proinflammatory cytokines, and stimulates the functional maturation of immune cells, including B lymphocytes and dendritic cells (Klinman et al. 1996; Ballas et al. 1996; Broide et al. 1998; Sparwasser et al. 1997, 1998; Sun et al. 1998; Roman et al. 1997; Stacey et al. 1996). Synthetic oligodeoxynucleotides (ODN) expressing CpG motifs mimic the immunostimulatory activity of bacterial DNA. The interaction of CpG DNA with TLR9 triggers a signaling pathway that proceeds via myeloid differentiation factor 88 (MyD88), IL-1R-associated kinase (IRAK), and tumor necrosis factor receptor-associated factor 6 (TRAF6), and subsequently involves the activation of several mitogen-activated kinases (MAPK) and transcription factors (such as NF-kB, AP-1) (Akira et al. 2001). This results in a complex pattern of gene upregulation and the transcription of several proinflammatory chemokines and cytokines.

Unmethylated CpG motifs are present in bacterial DNA at a frequency nearly 20 times that of mammalian DNA (Krieg et al. 1995). Consistent with evolutionary divergence in CpG recognition by different species (Kanellos et al. 1999), the precise sequence motif that optimally stimulates cells from one species may be inactive in a different species (Rankin et al. 2001). For example, the sequences of murine and human TLR9 differ by 24% at the amino acid level (Hemmi et al. 2000), such that CpG ODN that are highly effective in mice are poorly stimulatory in Man (Hartmann and Krieg 2000). Moreover, TRL9 expressing cell populations differ among species. Immune cells of the myeloid lineage in mice (including monocytes, macrophages, and myeloid DC) express TLR9 and respond to CpG stimulation, whereas TLR9 is not expressed in these cell types in humans and therefore are not directly activated by CpG ODN (Kadowaki et al. 2001; Krug et al. 2001b; Bauer et al. 2001a).

Several structurally distinct classes of synthetic CpG ODN are capable of stimulating cells that express human TLR9 (Klinman et al. 1999; Krug et al. 2001a; Hartmann et al. 2003; Marshall et al. 2003). K-type ODN (also referred to as B-type) typically express multiple CpG motifs on a phosphorothioate backbone. K-ODN trigger pDC to differentiate and produce TNFa, and B cells to proliferate and secrete Ig (Klinman et al. 1999; Krug et al. 2001a; Hartmann and Krieg 2000). Binding of K-Typ ODN to TLR9 in lysosomal vesicles initiates stimulation of the corresponding cells (Honda et al. 2005) and proceeds through an IRF5 mediated

pathway. D-type ODN (also referred to as A-type) consist of a mixed phosphodiester/phosphorothioate backbone and contain a single palidromic CpG motif linked to a poly-G tail at the 3′ end (Klinman et al. 1999). CXCL16 expressed on the surface of pDC interacts with this poly-G tail, which improves the uptake of D-ODN and directs them into early endosomes (Honda et al. 2005; Gursel et al. 2006). TLR9-mediated CpG stimulation proceeds through IRF7 in these lysosomes, culminating in the production of IFNa (Gursel et al. 2006). C-type ODN resemble K-type in being composed entirely of phosphorothioate nucleotides. This class of ODN combines some of the stimulatory effects of K and D-ODN, since they stimulate B cells to secrete IL-6 but also pDCs to produce IFNa (Hartmann et al. 2003; Marshall et al. 2003).

Microarray technology provides a powerful technique for analyzing global mRNA expression and can be used to examine both gene expression and gene regulation (Miller et al. 2002). This technique provides information on changes in mRNA levels of thousands of genes simultaneously from cells, tissues, or organisms treated under a variety of different conditions. Analyzing the output of microarray studies via bioinformatics can provide novel insights into biological systems and the underlying regulatory mechanisms responsible for mediating diverse changes in gene expression. Microarray technology has been harnessed to provide new insights into the nature, breath, magnitude, and regulation of the cellular changes triggered by the interaction of CpG DNA with TLR9. Initial works identified those changes in gene expression triggered from 1–72 h after CpG treatment *in vitro* or *in vivo* (Gao et al. 2002; Kato et al. 2006; Schmitz et al. 2004; Klaschik et al. 2007; Klaschik et al. 2009; Tross et al. 2009).

11.2 Modulation and Regulation of Gene Expression by CpG ODN *In vitro*

11.2.1 Gene Expression Studies Involving a Single Cell Type In vitro

Gao et al. (2002) and Schmitz et al. (2004) examined the response of a murine macrophage cell line to CpG and several additional TLR ligands by means of microarray technology (Gao et al. 2002; Schmitz et al. 2004). Gao et al. found that LPS treatment upregulated the expression of a significantly greater number of genes than did CpG DNA, and that all of the genes induced or repressed by CpG DNA were also induced or repressed by LPS. They concluded that CpG DNA signaling via TLR9 activated a subset of the same genes induced by LPS-TLR4 signaling (Gao et al. 2002). Similarly, Schmitz et al. found that the TLR4 ligand LPS had a stronger effect on gene expression than ligands for TLRs 2, 3, 7, or 9. Taking advantage of the known dependency of TLR on specific adaptor molecules, they concluded with a model for TLR signaling that defines MyD88 and TRIF "private" genes required for transcriptional activation (Schmitz et al. 2004).

11.2.2 *Gene Expression Studies with Mixed Cell Populations* In vitro

CpG ODN have broad and well-documented effects on multiple types of immunologically relevant cells. These include the ability to directly trigger B cells and dendritic cells to produce factors that subsequently activate additional cell types, including natural killer cells and T cells (Klinman et al. 1996; Ballas et al. 1996; Broide et al. 1998; Sparwasser et al. 1997, 1998; Sun et al. 1998; Stacey et al. 1996; Roman et al. 1997). To gain a clearer understanding of the breadth of these CpG-induced interactions, it is important to monitor changes in gene expression using mixed cell populations. Although using organs, e.g., the spleen (contain multiple TRL9 expressing cells), cannot identify the effect of CpG treatment on changes in mRNA levels among individual cell types, it provides the critical advantage that changes in gene expression associated with complex cell–cell interactions can be detected over time. In contrast, studies examining CpG-mediated activation of cloned cell lines are not designed to detect interactions between different cell types (see above; (Gao et al. 2002; Schmitz et al. 2004)).

Kato et al. (2003) analyzed gene expression in CpG-stimulated human peripheral blood mononuclear cells (PBMC) (Kato et al. 2003). They detected an "early" and "late" gene response cluster. Inflammatory cytokines such as IL-6 and GM-CSF were upregulated predominantly 3–6 h after stimulation with CpG ODN, presumably through activation of the transcription factor NF-kappaB. Interferon (IFN)-inducible antiviral proteins, including IFIT1, OAS1 and MX1, and Th1 chemoattractant CXCL10, were upregulated predominantly 6–24 h after stimulation. Blocking with mAb against IFN-alpha/beta receptor strongly inhibited the induction of these IFN-inducible genes by CpG ODN. They concluded that CpG effects were mediated via an interferon (IFN)-alpha/beta receptor paracrine pathway (Kato et al. 2003)).

Klaschik et al. (2007) stimulated murine spleen cells with CpG ODN (Klaschik et al. 2007). Spleen cells were selected on the basis of earlier studies establishing that the response of this population reflected the breadth of immunity induced by CpG ODN *in vivo* (Takeshita et al. 2000; Klinman et al. 1996; Ishii et al. 2002; Zelenay et al. 2003; Datta et al. 2003; Ito et al. 2005). The study of Klaschik et al. (2007) analyzed changes in gene expression at 2, 4, and 8 h after *in vitro* CpG stimulation (Klaschik et al. 2007). Results are shown in more detail below.

11.2.3 *Temporal Activation of Genes in the TLR9 Signaling Pathway*

The genetic components of various TLR pathways have been defined over the past few years (Hemmi et al. 2000; Takeshita et al. 2001; Bauer et al. 2001b; Yamamoto et al. 2002; Hacker et al. 2000; Aderem and Ulevitch 2000). Microarray technology

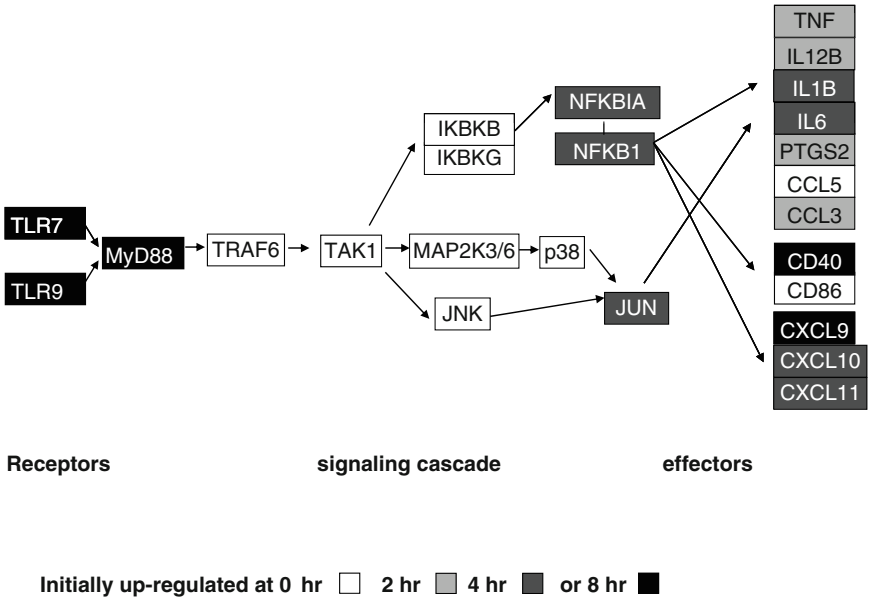

Fig. 11.1 CpG DNA stimulation induces a time-dependent increase in the expression of elements of the TLR signaling cascade. BALB/c spleen cells were stimulated *in vitro* with 1 μM of CpG ODN for 2, 4, or 8 h. Gene upregulation was determined by comparison to identical cultures stimulated with control (non-CpG) ODN in 3-4 independent experiments. Upregulation of those genes associated with the TLR9 signaling cascade (based on KEGG pathway database, Kanehisa Laboratory of Kyoto University Bioinformatics Center) that were present on the microarray was determined using a stringency cutoff of $p < 0.001$. Results reflect the first time point when each gene was significantly upregulated

enabled analysis of the kinetics with which mRNA for each element of this signaling cascade was upregulated following stimulation with CpG ODN (Klaschik et al. 2007). As seen in Fig. 11.1, mRNA levels of genes known to mediate the effector functions of CpG DNA (e.g., TNF, IL12B, CCL3) were significantly upregulated within 2 h ($p < 0.001$). After 4 h, additional effector genes were upregulated (e.g., IL6, IL1B, CXCL10) as were genes known to be directly proximal in the TLR pathway (e.g., NFKB1, NFKBIA, JUN; Fig. 11.1). By 8 h, the genes encoding TRL9 and MyD88 were themselves upregulated. This pattern of gene expression indicates that mRNA for effector cytokines/chemokines are upregulated first, followed by intermediate transcription factors and finally by ligand recognition genes. These findings further document that the receptors and transcription factors responsible for initiation of the signaling cascade are present at sufficient concentration that further mRNA-dependent production is unnecessary. This allows cellular activation to proceed in the absence of increased receptor expression. Subsequently, the genes encoding these molecules are upregulated, presumably to replace the proteins used up during the initial activation. By comparison, secreted effector molecules that protect the host from infection are not stored

at high concentration and thus must be rapidly upregulated following cellullar activation.

Also of interest, several hundred genes not directly linked to the TLR signaling pathway were reproducibly upregulated following CpG stimualtion. Data were evaluated and genes categorized using the GeneOntology (GO) database and Ingenuity Pathway Analysis (IPA). Genes were mapped to specific functional categories: at 2 h, genes fell mainly into the category of innate and adaptive immunity (80%). Many of the genes upregulated at both 4 and 8 h were primarily associated with general immune activation (74%), induction of innate immunity (29%), adaptive immunity (44%), inflammation (15%), and antiviral immunity (15%). These findings confirm that stimulation of the TLR9 pathway boosts host resistance to infection, and suggest that CpG ODN may be of particular benefit for the prevention/treatment of viral infections.

11.2.4 Regulatory Networks Triggered by CpG DNA Stimulation In Vitro

The impact of CpG stimulation on TLR9 has been widely studied (Hemmi et al. 2000; Takeshita et al. 2001; Bauer et al. 2001b; Yamamoto et al. 2002; Hacker et al. 2000; Aderem and Ulevitch 2000). While important elements of the signaling pathway have been defined, the broader results of this interaction require considerably greater definition. Klaschik et al. therefore analyzed the regulatory networks modulating gene expression after CpG stimulation (Klaschik et al. 2007). IPA was used to map each gene within a global molecular network developed from microarray data. The IPA gene networks were generated algorithmically based on their connectivity in terms of expression, activation, transcription, and/or inhibition, and reflect a graphical representation of the molecular relationship between genes (which are represented as nodes) and the biological relationship between nodes (shown as a connecting line). All connections are supported by published data stored in the IPA database. Results indicate that TNF and NFKB1 are key regulators of gene expression at 2 h after CpG ODN stimulation and continue to play a dominant regulatory role at 4 h (Fig. 11.2) together with additional regulators, most notably IL1B. These data are consistent with previous works showing that NFKB1 and TNF are rapidly upregulated following CpG stimulation and that TNF is rapidly produced by CpG-stimulated cells (Ida et al. 2006). Of interest, TNF is a potent inducer of IL1B, NKFB1, and itself (Nawroth et al. 1986; Balkwill 2000), and therefore, these data suggest that CpG stimulation induces a self-perpetuating autocrine loop.

8 h after CpG stimulation, TNF, IFNG and MYC become major regulators of gene activation, accompanied by other regulators (STAT1, IL6, IL12B) that had detectable but lesser effects (Fig. 11.2). Most of the genes associated with gene function at 8 h were regulated by IFNG and/or TNF, whereas MYC contributed to

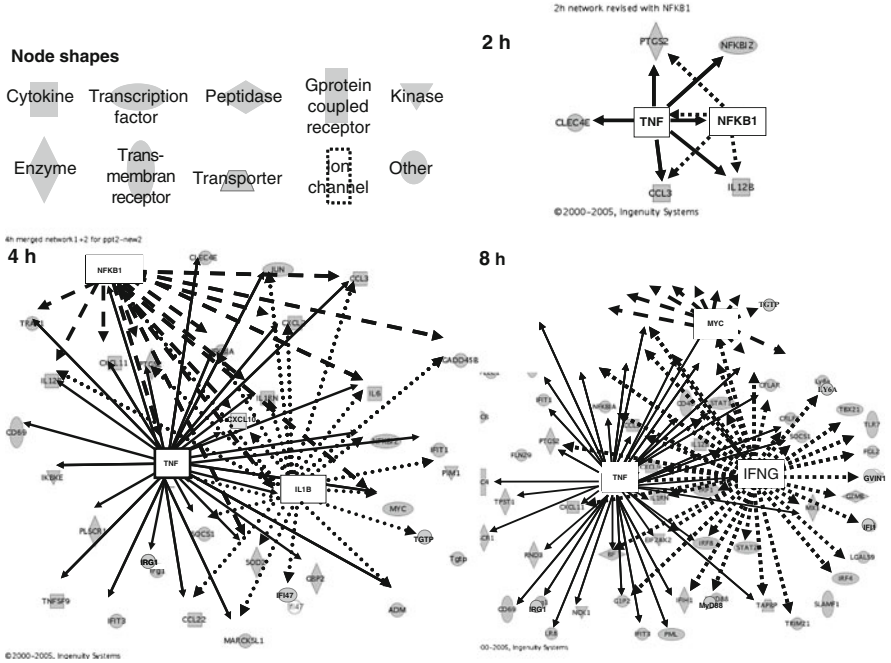

Fig. 11.2 Network analysis of CpG-induced gene activation. BALB/c spleen cells were stimulated *in vitro* with 1 μM of CpG ODN for 2, 4, or 8 h. The regulators identified by Ingenuity Pathway Analysis as controlling the expression of those genes stimulated at each time point are shown (*open labels*) as is the functional class of each gene product

the regulation of multiple nonimmune genes involved in cell proliferation, particularly the G1 to S phase transition (King and Cidlowski 1998; Oster et al. 2002). Thus, MYC might play an important role in orchestrating the division of immune cells induced by CpG stimulation (Krieg et al. 1995; Stacey et al. 1996; Ballas et al. 1996).

Activation of these multiple gene upregulatory networks suggests that CpG stimulation may induce a positive feedback loop of continued immune stimulation. However, it is well established that the immune response triggered by CpG DNA is rapid but of short duration (Takeshita et al. 2000; Klinman et al. 1996). This suggests that the duration of gene activation is limited by one or more downregulatory processes. Coherent with this conclusion, genes capable of downregulating the CpG-induced stimulatory cascade were themselves upregulated by 4 h (IL1RN, MYC, NFKBIA, SOCS1). Many of the genes targeted by these inhibitors manifest declining expression levels from 4–8 h, despite a nearly threefold increase in general gene activation over the same period of time. This strongly indicates that a well-defined counter regulatory process is embedded in the gene expression profile stimulated by CpG ODN. Due to the limitations of *in vitro* cultures (artifacts in gene expression become extreme by 12 h), the existence of such inhibitory networks could not be confirmed *in vitro* (Kato et al. 2003; Klaschik et al. 2007).

11.3 Modulation and Regulation of Gene Expression by CpG ODN *In vivo*

To resolve these issues, Klaschik et al. (2009) treated mice *in vivo* with immunostimulatory CpG ODN and splenic mRNA levels monitored over the next 3 days by microarray (Klaschik et al. 2009). The spleen was selected for analysis on the basis of previous studies establishing that this lymphoid organ accurately reflected the breadth of immunity induced by CpG ODN *in vivo* (Takeshita et al. 2000; Klinman et al. 1996; Ishii et al. 2002; Zelenay et al. 2003; Datta et al. 2003; Ito et al. 2005).

11.3.1 Regulatory Networks Underlying CpG-Dependent Gene Expression In vivo

Changes in gene expression were present within 30 min, peaked at 3 h, and declined progressively thereafter (Fig. 11.3). IPA (see above) was used to analyze the regulatory patterns underlying CpG-dependent gene activation *in vivo*. Initially, analysis focused on the 3 h time point when gene activation was maximal (Fig. 11.3). 90% of all evaluable genes were under the regulatory control of TNF

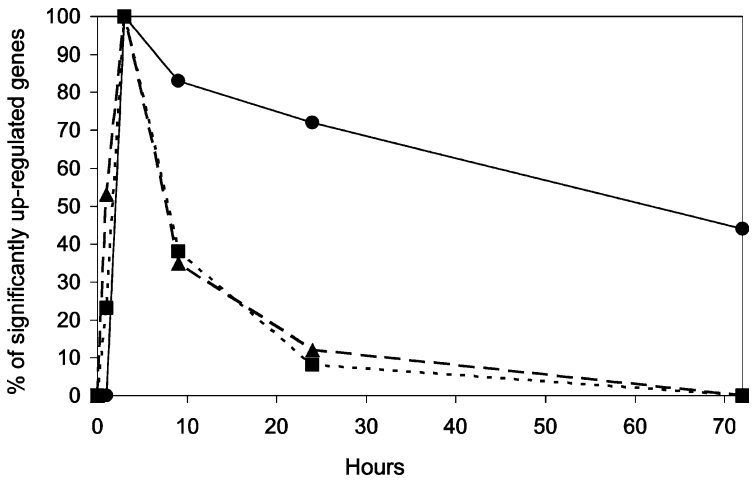

Fig. 11.3 Changes in gene expression over time following CpG ODN stimulation *in vivo*. BALB/c mice were injected i.p. with 400 μg of CpG ODN. Changes in splenic gene expression were monitored over time by microarray. Four biological replicates were independently analyzed at each time point. Upregulated genes were identified by comparison to untreated mice ($N = 6$) using a stringency cutoff of $p < 0.00001$ and are displayed as % of maximum gene expression. The behavior of genes upregulated by *IFNG* (*filled circle*), *TNF* (*open triangle*), or minor inducers (*filled square*) is shown. Note that the expression of genes regulated by *IFNG* persists significantly longer than those under the control of other inducers ($p < 0.001$)

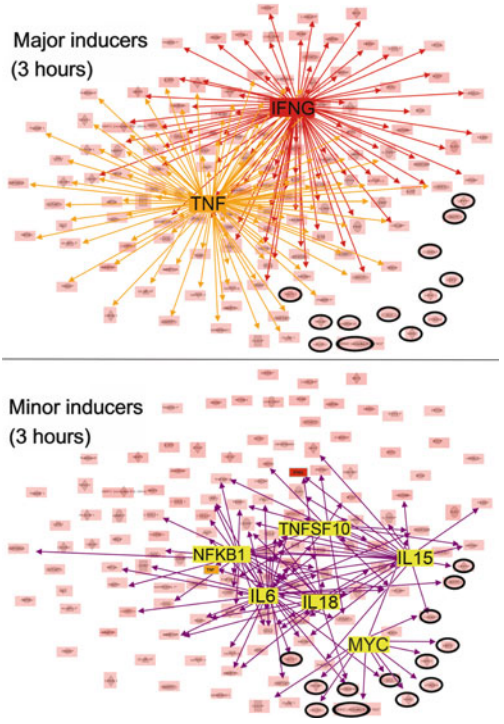

Fig. 11.4 Key regulatory networks activated by CpG ODN treatment (3 h time point). All genes activated at 3 h ($p < 0.00001$) whose regulatory interactions could be mapped by IPA are shown. (*Top panel*) Network analysis identifies two major inducers: *IFNG* (*red network*) and *TNF* (*orange network*). Those genes not regulated by *IFNG* or *TNF* are circled. (*Bottom panel*) Network analysis also identifies minor inducers (*IL6, NFKB1, IL15, TNFSF10*, *MYC*, and *IL18*, purple network) that regulate 10–50% of all genes, including those not regulated by *IFNG* or *TNF*

Table 11.1 Kinetics of CpG-mediated gene upregulation

Time (h)	Major inducers	Minor inducers
0.5	*TNF*, *IL1B*, *IL1A*	None
1	*TNF*, *IL1B*	*IFNG*, *IL6*, *IL1A*
3	*TNF*, *IFNG*	*IL6*, *NFKB1*, *IL15*, *TNFSF10*, *IL18*, *MYC*
9	*TNF, IFNG*	*IL15*, *IL18*
24	*IFNG*	*PARP9*, *STAT1*, *STAT2*
72	*IFNG*	*STAT1*, *STAT2*

IPA was used to identify the major and minor inducers of gene activation ($p < 0.00001$) from 0.5–72 h after CpG ODN treatment of BALB/c mice. Results reflect the analysis of four biological replicates independently analyzed at each time point. Major inducers are defined as activating >50% of all genes upregulated at a given time point, whereas minor inducers regulate 10–50% of the corresponding network

and/or IFNG (Fig. 11.4, top panel). To clarify the regulatory importance of the individual genes, they were defined as "major" or "minor." Major inducers individually mediated the expression of >50% of all genes at a given time-point, while minor inducers impacted less than half but greater than 10% of these genes. Major inducers also triggered higher levels of gene expression than did minor inducers.

Among the minor inducers identified by network analysis were IL6, NFKB1, IL15, TNFSF10, IL18, and MYC (Table 11.1 and Fig. 11.4, bottom panel). These

Table 11.2 Effect of multiple inducers on the level of gene expression

Inducer	N	Avg fold increase in gene expression	p-value
TNF alone	18	4.0 ± 0.47	
TNF plus minor inducer	19	4.7 ± 0.71	0.4
TNF plus major inducer	19	6.2 ± 1.10	0.08
TNF plus major and minor inducer	35	6.7 ± 0.99	0.016

The network of genes significantly upregulated at 3 h ($p < 0.00001$) was analyzed by IPA. The fold increase in mRNA expression of all genes regulated by *TNF* alone was compared to genes regulated by *TNF* plus various major and/or minor inducers (mean ± SEM). Genes targeted by multiple major and/or minor inducers showed higher levels of mRNA expression (p values vs. *TNF* alone)

both influenced the magnitude of expression of genes regulated by the major inducers and controlled the expression of a smaller group of genes not stimulated by any of the major inducers. Indeed, the level of expression of genes targeted by both a major and minor inducer was significantly greater than that of genes targeted by a single inducer alone (Table 11.2).

11.3.2 Temporal Pattern of Gene Upregulation Mediated by TLR9 Engagement

Bioinformatic analysis showed that TNF, IL1B, and IL1A were major inducers of gene expression at 0.5 and/or 1 h post CpG stimulation (Fig. 11.5 and Table 11.1). The genes targeted by these major inducers were upregulated rapidly, but for only a short period of time (typically <1 day). Moreover, they worked in tandem to control early gene expression, with 85% of the genes upregulated at 30 min and 75% at 1 h being targeted by two or more major inducers.

This is contrasted by the pattern of gene activation attributable to IFNG. IFNG became a major inducer 3 h after CpG ODN stimulation and remained a dominant source of stimulation through at least day three (Figs. 11.4, 11.6 and Table 11.1). Genes whose expression was regulated by IFNG generally remained active long-term. Thus, the persistent immune activation observed following CpG ODN administration *in vivo* most likely reflects the ongoing effects of IFNG.

These findings provide evidence that multiple waves of activation occur following CpG stimulation, regulated over time by a small number of major and minor inducers. To establish the biological relevance of the results generated by this bioinformatic analysis, the effect of eliminating the major inducer TNF was evaluated in knockout (KO) mice. Results established that the genes observed to be under the unique regulation of TNF in wild-type mice were not stimulated following CpG treatment of KO mice (Klaschik et al. 2009).

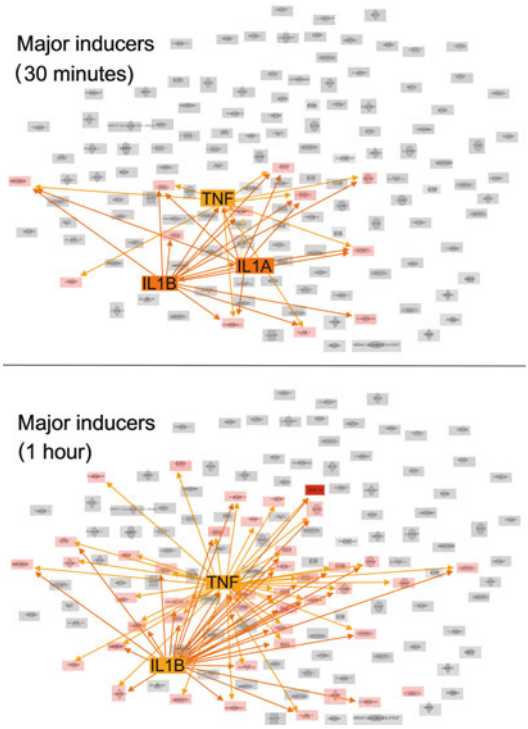

Fig. 11.5 Key regulatory networks activated by CpG ODN treatment (30 and 60 min time points). All genes upregulated 3 h after CpG treatment are shown (modified from Fig. 11.2). Those upregulated ($p < 0.00001$) within (*top panel*) 30 min and (*bottom panel*) 1 h are highlighted in *pink*. *Arrows* identify the network involving major inducers (*IL1A, IL1B,* and *TNF*) responsible for gene upregulation at these early time points

11.3.3 Mechanism of Downregulation of CpG Induced Gene Expression

As noted above, the immune stimulation mediated by CpG DNA treatment is relatively short-lived (Takeshita et al. 2000; Klinman et al. 1996). Consistent with those results, the number of genes upregulated following CpG treatment *in vivo* peaked at 3 h and declined thereafter (Fig. 11.3). The mechanisms responsible for this decline in gene expression were defined by bioinformatic analysis. Results showed that a small group of "suppressors" (genes capable of downregulating the expression of other genes) were induced by CpG treatment. These suppressors included IL10, MYC, NFKBIA, SOCS1, SOCS3, IL1RN, and FOS (Fig 11.6A, blue boxes).

Nearly 40% of all genes whose expression levels declined after 3 h were targeted directly by these suppressors, including major regulators like TNF, IL1A, and IL1B. Moreover, by suppressing these major inducers, the suppressors extended their effects (albeit indirectly) on gene expression by eroding the foundation upon which continued network stimulation relied (Fig. 11.6B). These findings demonstrate that active suppression is key to the temporal decline in gene expression and that by targeting "inducers," a small cohort of "suppressors" can extinguish entire networks. (Fig. 11.6).

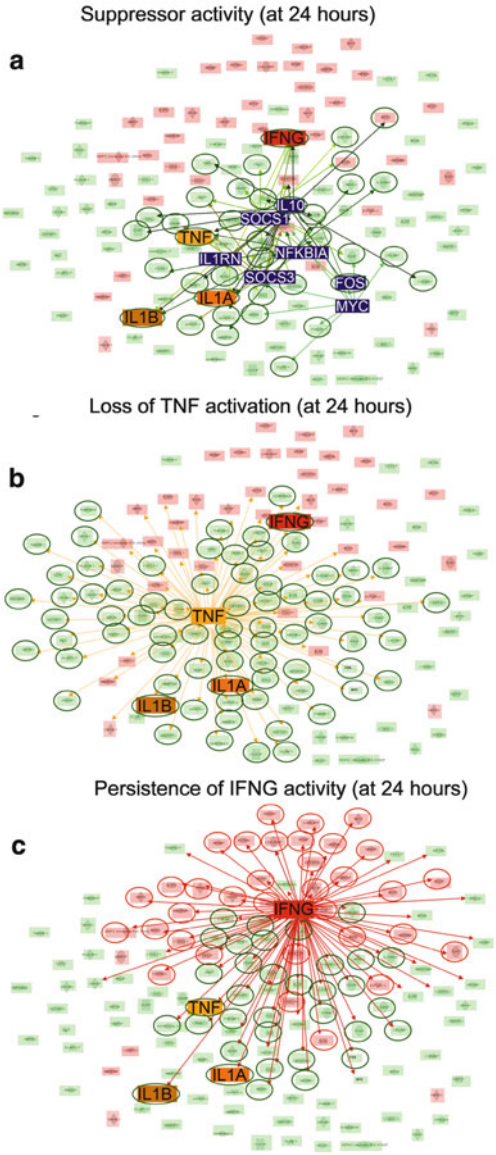

Fig. 11.6 Mechanisms underlying the downregulation of genes 24 h after CpG ODN administration. All genes upregulated 3 h after CpG treatment are shown (modified from Fig. 11.2). Genes that remain active through 24 h are highlighted in pink ($p < 0.00001$), while those whose expression fell to background are in *green*. (**a**) *Green arrows* identify genes downregulated by suppressors. Note that 33/35 of the genes targeted by suppressors (*circled in green*) are significantly downregulated. Also note that five suppressors (*SOCS1*, *SOCS3*, *IL1RN*, *NFKBIA*, and *IL10*) target the major inducer *TNF*. (**b**) As *TNF* levels decline, the expression of most genes regulated by *TNF* (*yellow arrows*) also falls (*green circles*). (**c**) *IFNG* upregulates 92% of all genes that remain active at 24 h (*red arrows* leading to *red circles*). Nevertheless, 84% of the genes regulated by *IFNG* but targeted by a suppressor declined to background levels by 24 h (*red arrows* leading to *green circles*)

11.4 Synergistic Modulation and Regulation of Gene Expression by TLR3 and TLR9 Ligands

TLR 3 is stimulated by double-stranded viral RNA (Alexopoulou et al. 2001). Signal transduction after stimulation through TLR3 is dependent on the adaptor protein TRIF, which initiates the activation of NF-kB and the production of type I

interferons, which then mediate antiviral immunity (Kulka et al. 2004). Multiple pathogen-associated molecular patterns (PAMPS) are typically expressed by individual pathogens and thus can activate the innate immune system via several discrete TLR pathways, which has the potential to trigger a synergistically enhanced immune response (Gautier et al. 2005; Trinchieri and Sher 2007; Zhu et al. 2008). Data suggest that such synergy is enhanced when TLR ligands that signal through different adapter proteins, e.g., stimulation with CpG ODN (TLR9 ->adapter MyD88)) and poly (I:C) (TLR3 -> adapter TRIF) (Gautier et al. 2005; Napolitani et al. 2005; Trinchieri and Sher 2007; Zhu et al. 2008) are involved. Yet the regulatory mechanisms responsible for the breath of this synergistic activation has not been fully explained (Whitmore et al. 2007).

11.4.1 Genome-Wide Changes in Gene Expression After CpG ODN and Poly (I:C) Treatment

Tross et al. (2009) were the first to compare global changes in gene regulation triggered by co-stimulation with CpG ODN and poly (I:C) and to delineate the synergy resulting from the co-administration of both TLR agonists (Tross et al. 2009). RAW264.7 macrophages were stimulated with CpG and/or poly (I:C) for 4 and 12 h and genes whose level of expression was significantly increased ($p <$ E−6) indentified. The number of genes stimulated by poly (I:C) exceeded the number of genes activated by CpG ODN at 4 h by nearly fivefold and at 12 h by tenfold (Table 11.3).

Ninety-seven percent of the genes most strongly upregulated by CpG ODN were also stimulated by poly (I:C), whereas only 66% of the genes most strongly activated by poly (I:C) were also triggered by CpG ODN. The magnitude of gene induction was also significantly different: 37-fold for poly (I:C) vs 15-fold for the CpG-treated group ($p < 0.001$). These findings indicate that poly (I:C) induces broader, more complex, and more persistent gene upregulation than CpG ODN, and that CpG ODN stimulates only a subset of the genes triggered by poly (I:C).

Table 11.3 Number of genes significantly upregulated following TLR stimulation

TLR ligand	Time point	
	4 h	12 h
CpG	190[a]	105[a]
Poly (I:C)	574	1,051
CpG + Poly (I:C)	773[a]	943[a]

RAW 264.1 cells were stimulated with 3.2 ug/ml of CpG ODN and/or 32 ug/ml of poly (I:C) in three independent experiments. The numbers of genes significantly upregulated after 4 and 12 h is shown ($p < 0.000001$)

[a]Significantly different form the number of genes upregulated by poly (I:C) alone at the same time point, $p < 0.001$

Co-stimulation with both ligands resulted in a significant increase in the overall magnitude of gene activation ($p < 0.0001$). Unexpectedly, certain regulatory genes (such as IL6) were upregulated rapidly following stimulation with both ligands but not until considerably later when each ligand was administered independently. This suggests that co-stimulation with CpG ODN and poly (I:C) magnifies and accelerates the gene expression profile induced by each ligand independently.

11.4.2 Regulatory Networks Triggered by CpG ODN Vs. Poly (I:C)

As noted above, TNF, IFNG, and IL1B were important regulators of CpG-driven macrophage activation, impacting ~95% of all genes upregulated at 4 and 12 h (Klaschik et al. 2007). Poly (I:C) stimulation resulted also in the upregulation of TNF and IFNG, but these key regulators accounted only for the upregulation of 84% of the genes, but additionally many type I interferons were induced. Most notably, IFNbeta1 controlled about 50% of the genes strongly stimulated by poly (I:C) but not CpG ODN (Tross et al. 2009). This suggests that IFN_{beta1} plays an important role in modifying the magnitude and breath of the poly (I:C)-induced immune response.

The functional groups upregulated by CpG and poly (I:C) were analyzed using IPA. Despite the differences in regulatory pattern noted above, both stimuli activated groups of genes with similar functions. These included genes associated with cell death, immune response, and immune system development (all groups $p < E{-}4$; Table 11.4). This finding is consistent with considerable evidence showing that both ligands contribute to the induction of an innate immune response.

Table 11.4 Top functional categories of genes up-regulated by CpG ODN and poly (I:C)

Functional category	CpG ODN	Poly (I:C)
	p-value	p-value
Cell death	$<4.7^{-06}$	$<1.1^{-05}$
Cellular growth and proliferation	$<5.0^{-06}$	$<8.7^{-06}$
Immune response	$<4.9^{-06}$	$<1.3^{-05}$
Cell-to-cell signaling and interaction	$<5.0^{-06}$	$<1.2^{-05}$
Hematological system development and function	$<4.9^{-06}$	$<1.3^{-05}$
Immune and lymphatic system development and function	$<4.6^{-06}$	$<1.2^{-05}$
Gene expression	$<4.0^{-06}$	$<1.1^{-05}$
Cellular development	$<4.0^{-06}$	$<1.0^{-05}$
Tissue morphology	$<4.6^{-06}$	$<3.5^{-06}$
Tissue development	$<4.2^{-06}$	$<9.8^{-06}$

IPA was used to assess the functional categories associated with CpG ODN or poly (I:C) upregulated genes

11.4.3 Genes Synergistically Upregulated by Co-administration of CpG ODN and Poly (I:C)

CpG ODN plus poly (I:C) synergistically enhance the production of certain cytokines, such as IL1A, IL6, IL12B, and IL10. Microarray studies of RAW264. Seven cells were performed to determine whether this synergistic interaction was mirrored on the mRNA level. Synergistic interactions were defined by that subset of genes whose level of expression exceeded the mean + 3 SD of the sum induced by each ligand alone. By this definition, 60 genes were synergistically upregulated by the combination of CpG ODN plus poly (I:C). 80% of these belonged to the functional group "immune response." This included primarily chemokines/cytokines (e.g., IL1A, several typ I interferons, IL12A, IL12B, IL10, and IL6).

In overview, poly (I:C) stimulation via TLR3 activated a larger and broader regulatory network than CpG stimulation of TLR9. Gene activation was accelerated when poly (I:C) was co-administered with CpG ODN and triggered the synergistic upregulation of many immune-related genes including a subset that was not activated by either ligand alone. Synergy was characterized by the early and persistent activation of multiple regulatory pathways, resulting in a prolonged signaling cascade that increased, magnified, and diversified gene expression.

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Chapter 12
Immune Recognition of Nucleic Acids and Their Metabolites

Shohei Koyama, Shizuo Akira, and Ken J. Ishii

Contents

S. Koyama
Department of Respiratory Medicine, Tohoku University Graduate School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai-city, Miyagi 980-8575, Japan

S. Akira
Laboratory of Vaccine Science, WPI Immunology Frontier Research Center, Osaka University, 3-1 Yamada-oka, Suita, Osaka 565-0871, Japan

K.J. Ishii (✉)
Laboratory of Vaccine Science, WPI Immunology Frontier Research Center, Osaka University, 3-1 Yamadaka, Suita, Osaka 565-0871, Japan
Laboratory of Adjuvant Innovation, National Institute of Biomedical Innovation, 7-6-8 Asagi Saito, Ibaraki, Osaka 567-0085, Japan
e-mail: kenishii@biken.osaka-u.ac.jp

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Abstract Recent research suggests that nucleic acids are active modulators of the immune system. RNA and DNA can be detected by specific receptors – the so-called Toll-like receptors, RIG-I-like receptors, and NOD-like receptors. Resultant intra- and intercellular activations of the innate immune system are pivotal in both protective and pathological immune responses during infection and other immunological disorders. Moreover, our immune system is substantially modulated by metabolic intermediates of nucleic acids. Elucidation of such manifold mechanisms involved in immune recognition of nucleic acids and their metabolites offers the possibility of novel nucleic acid-based immunotherapies as well as interventions for nucleic acid-related immune disorders.

12.1 Introduction

Nucleic acids of mammalian cells have long been thought to be immunologically inert as they are normally tightly packed and sequestered within the nucleus. However, recent progress in immunology has shown that nucleic acids and their metabolites are specifically detected by the innate immune receptors, also called Pattern Recognition Receptors (PRRs); these include Toll-like receptors (TLRs), Retinoic Acid Inducible Gene-I (RIG-I)-like Receptors (RLRs), and Nod-like Receptors (NLRs). These receptors are able to distinguish "non-self," such as infectious organisms or damaged host cells, from "self" moieties of host or environmental entities, activating intra- and intercellular signaling pathways of the immune system through receptor-mediated recognition of dangerous "non-self" signatures.

Here, we show how the host immune system recognizes and responds to "non-self" nucleic acids in abnormal conditions, such as microbial infections as well as to "self" nucleic acids in certain immunological disorders, such as autoimmune diseases. We also illustrate some clinical applications of nucleic acids that utilize their immunogenic potential, such as vaccine adjuvants.

12.2 The Fundamental Mechanism to Avoid False Recognition of Harmless Nucleic Acids: The Case of Food Metabolism

Metabolizing food enables us to generate energy through ATP and acetyl CoA production. It also allows us to synthesize nucleic acids for cell proliferation (de novo pathway) and to recycle nucleic acids within food metabolites (salvage pathway). Nucleic acids in foods are metabolized from polynucleotides to nucleosides to free bases, using several kinds of digestive enzymes, including endonucleases, phosphodiesterases, and nucleoside phosphorylases. For instance, in the case of purine nucleotide metabolism, phosphate and ribose are successively separated from purine bodies in food by the above enzymes in the small intestine,

yielding free bases like guanine and hypoxanthine (Ishii and Akira 2008). Free bases can be absorbed for recycling through special transporters on cell surface such as Nucleobase-Cation-Symport-1 (NCS1), the structure of which was recently published (Weyand et al. 2008). Unnecessary bases are degraded to uric acids and eliminated to urine, via xanthine. Neither polynucleotides nor nucleosides can enter cytoplasm unless they are degraded to free bases. While this mechanism seems inefficient, it is a fundamental means of avoiding misidentification of harmless or self nucleic acids inside host cells (Ishii and Akira 2008).

12.3 The Mechanism to Distinguish Healthy "Self" Nucleic Acids

Recent research has shown several unique receptors to recognize nucleic acids and their metabolites (Table 12.1; Figs. 12.1 and 12.2). Some of these receptors are expressed on cell surfaces and some are in cytosol or on endosomal membranes. Some of these receptors are specialized to recognize Pathogen-Associated Molecular Patterns (PAMPs); others detect Danger-Associated Molecular Patterns (DAMPs), which are endogenous products released from damaged host cells (Matzinger 2002; Kono and Rock 2008).

Here, we review how the immune system senses and responds to exogenous detrimental nucleic acids adequately in the case of microbial infections (3.1), controls reactions to endogenous nucleic acids in the case of host cell damage (3.2; 3.3), and misidentifies endogenous nucleic acids in the case of autoimmune diseases (3.4).

Table 12.1 Pattern recognition receptors for nucleic acids and their metabolites

Receptor family	Location	Major ligands	Receptor adaptor
Toll-like receptors (TLRs)	Endosome	ssRNA	TLR7/8 MyD88
		dsRNA	TLR3 TRIF
		Unmethylated CpG in ssDNA, abnormal DNA	TLR9 MyD88
RIG-I-like receptors (RLRs)	Cytosol	5′-triphosphate ssRNA	RIG-I IPS-1
		dsRNA	MDA5 IPS-1
		dsRNA	LGP2 IPS-1
NOD-like receptors (NLRs)	Cytosol	Microbial and synthetic RNA	NLRP3 ASC
Purinergic receptors	Cell surface	Adenosine	P1 None
		Nucleotides (ATP, UTP, ADP, UDP)	P2X None
		ATP	P2Y None
Others	Cytosol	dsDNA	AIM2 ASC
		dsDNA	DAI Unknown

MyD88 myeloid differentiation primary response gene 88, *TRIF* TLR-domain-containing adapter-inducing interferon-β, *RIG-I* retinoic acid-induced gene I, *IPS-1* interferon-β promoter stimulator-1, *MDA5* melanoma differentiation-associated gene-5, *ASC* apoptosis-associated speck-like protein containing a CARD, *AIM2* absent in melanoma 2

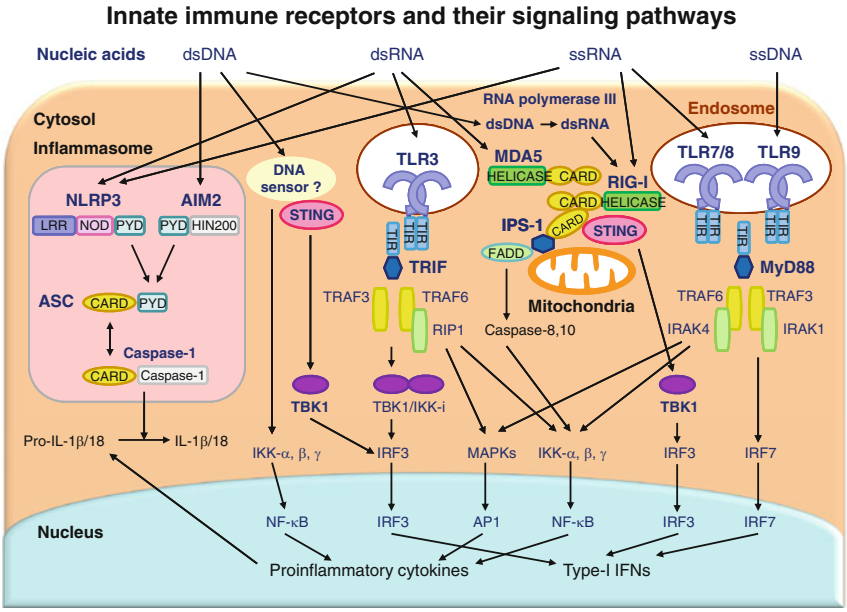

Fig. 12.1 ssRNA and dsRNA are detected by TLR7/8, RIG-I, NLRP3, and TLR3, MDA5, NLRP3, respectively. ssDNA and dsDNA are detected by TLR9 and TBK-dependent unknown DNA sensors, AIM2, RNA polymerase III, respectively. TLR3, TLR7/8, and TLR9 are located in endosomes. TLR3 signals through TRIF. TRIF associates with TRAF3, TRAF6, and RIP1. TRAF3 activates IRF3 via TBK1/ IKK-i, whereas TRAF6 and RIP1 activate MAPKs and NF-κB. TLR7/8 and TLR9 signal through MyD88. MyD88 also associates with TRAF3 and TRAF6. They form a complex with IRAK-4, IRAK-1, and activate IRF7, NF-κB, and MAPKs. RIG-I, MDA5, NLRP3, and AIM2 are located in the cytoplasm. Both RIG-I and MDA5 signal through IPS-1, which interacts with STING and FADD. STING activates IRF3 via TBK1 and FADD activates NF-κB through cleavage of caspase-8/10. NLRP3 and AIM2 associate with ASC. ASC-dependent inflammasome activation induces IL-1β /18 production via caspase-1. RNA polymerase III converts dsDNA into dsRNA and such dsRNA detected by RIG-I. *TBK1* TANK-binding kinase 1, *TRAF* TNF receptor-associated factor, *RIP* receptor-interacting protein, *IRF* Interferon regulatory factor, *IKK* IκB kinase, *NFκB* nuclear factor-kappa B, *MAPKs* mitogen-activated protein kinases, *IRAK* IL-1R-associated kinase; *NFκB* nuclear factor-kappa B; *MAPKs* mitogen-activated protein kinases; primary response gene 88, *STING* stimulator of interferon genes, *FADD* Fas-associated death domain

12.3.1 The Mechanism to Detect Exogenous Nucleic Acids Breaking into Host Cells: The Case of Microbial Infections

The immune response is the host's first-line protection from pathogens. In the case of microbial infections, nucleic acids can be released from microorganisms and infected host cells simultaneously. Therefore, the host has evolved a specialized mechanism to discriminate whether these nucleic acids should be eliminated or ignored, based on their sequences, structures, or modifications (Akira et al. 2006;

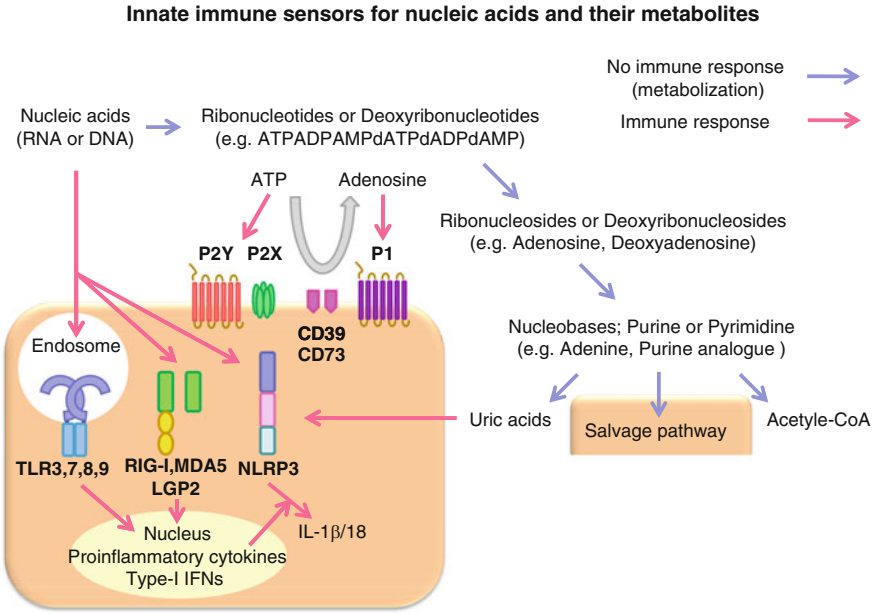

Fig. 12.2 Metabolization (*blue arrow*): Nucleic acids in foods are metabolized from polynucleotides to nucleosides to free bases, using several kinds of digestive enzyme. Free bases can be used for acetyl CoA production (energy) or recycling (salvage pathway) or degraded to uric acids and eliminated to urine. Immune response (red arrow): Nucleic acids and their metabolites can be released from damaged or dying host cells in the case of infection or tissue injury. For example, viral RNA or DNA can be directly detected by endosomal TLRs and cytoplasimic RLRs and NLRs. In addition, aberrant high concentration of extracellular nucleotides incuding ATP, adenosine, and their metabolic end products, uric acids, can be detected by innate immune sensors containing NLRs, ATP-gated P2 receptor, and G protein-coupled P1 receptors as danger signals. These responses mainly culminate in IL-1β/18 production via inflammasome activation. Adenosine is degraded from ATP through a cascade of ectonucleotidases, including nucleoside triphosphate diphosphorylase (NTPDase, also called CD39) and 5′-ectonucleotidase (Ecto50NTase, also called CD73)

Ishii et al. 2008b). Here, we illustrate several systems involved in the recognition of exogenous nucleic acids through immune receptors, including TLRs, RLRs, and NLRs.

12.3.1.1 Endosomal Recognition for Exogenous Nucleic Acids via TLRs

Currently, 11 members of the TLR family have been identified as functional receptors in mammals. Among them, TLR-3, -7, -8, and -9 have been shown to recognize nucleic acids within early endosomes (Takeda and Akira 2005). TLRs have ectodomains that contain variable numbers of Leucine-Rich-Repeat (LRR) motifs and a cytoplasmic signaling domain termed the Toll/IL-1R homology (TIR) domain. LRR motifs are responsible for sensing nucleic acids; the crystal structures

of TLR-3 (but not TLR-7, -8 or -9) have been shown to have horseshoe-like solenoid shapes (Choe et al. 2005; Leonard et al. 2008). TLR-3 recognizes double-stranded (ds) RNAs, which can be intermediates for single-stranded (ss) RNA viral replication, symmetrical transcription byproducts of DNA viruses, or synthetic compounds (e.g., poly IC) (Alexopoulou et al. 2001). TLR-3 is expressed in immune cells, including Dendritic Cells (DCs) and macrophages; it is also expressed in nonimmune cells such as epithelial cells, and it is inducible through type-I IFN response (Alexopoulou et al. 2001; Tissari et al. 2005). Double-stranded RNA recognition via TLR-3 can occur when virus-infected cells are phagocytosed by immune cells (Schulz et al. 2005). TLR-3 signals through the adaptor molecule TIR domain containing adaptor inducing IFN-β (TRIF, also known as TICAM1) (Yamamoto et al. 2003; Oshiumi et al. 2003). TRIF associates with tumor necrosis factor-receptor associated factor (TRAF)-3, TRAF-6, and receptor-interacting protein (RIP)-1. This complex activates NF-κB via IκK-complex, AP-1 via MAPK, and Interferon Response Factor (IRF)-3 via a complex of the TRAF family member-associated NK-κB activator-Binding Kinase 1 (TBK1) with Inducible IκB Kinase (IκKi), initiating pathways that culminate in proinflammatory cytokines and IFN-β production (Akira and Takeda 2004) (Fig. 12.1).

TLR-7 and -8 recognize uridine-rich or uridine/guanosine-rich ssRNA of virus (e.g., influenza virus, human immunodeficiency virus) and synthetic compounds (e.g., imidazoquinolines) (Diebold et al. 2004; Jurk et al. 2002). The expression of TLR-7 is limited to B cells and DCs in human and mice, while TLR-8 functions primarily in human monocytes and myeloid DCs (Hornung et al. 2008). Both genes are located on the X chromosome. TLR-9 preferentially recognizes unmethylated CpG motifs (lack of cytocine methylation) in microbial DNA, which is thus discernable from highly methylated CpG motifs in mammalian DNA (Hemmi et al. 2000; Krieg 2002; Wagner 2004; Klinman 2004); TLR-9 binding to its cognate ligand induces a conformational change that activates the downstream intracellular signaling pathway (Latz et al. 2007). Sequence restriction of TLR-9 recognition varies as unmethylated CpG motifs are needed to activate TLR-9 only in ssDNA with phosphorothionate backbones, whereas natural phosphodiester DNA can activate TLR-9 independently of CpG motifs (Haas et al. 2008). In addition, TLR-9 activation by synthetic oligonucleotides (ODNs) is dependent on cell types (Ishii et al. 2004) and secondary structures of ODNs, such as aggregated forms (Kerkmann et al. 2005; Hou et al. 2008). The expression of TLR-9 is restricted to Plasmacytoid DCs (pDCs) and B cells in humans, while expression of mouse TLR-9 is broader, as TLR-9 is highly expressed in murine myeloid DCs and macrophages (Kadowaki et al. 2001; Bauer et al. 2001; Hornung et al. 2002).

The proteins TLR-7, -8, and -9 share a common signaling pathway through a TIR domain – containing adaptor molecule, Myeloid Differentiation Factor 88 (MyD88). MyD88 associates with TRAF3/6 and interleukin-1-receptor-associated kinase (IRAK)-1/4. This complex activates NF-κB, AP-1, and IRF7 via IκK-complex, MAPK and IκKα, respectively, and culminates in proinflammatory cytokines and IFN-α/β production (Akira and Takeda 2004). IRF7 is constitutively expressed in pDCs and is especially involved in inducing massive type-I IFNs response via

the TLR-7/9-MyD88-dependent signaling pathway (Kawai and Akira 2006) (Fig. 12.1).

Notably, TLR activation upon recognition by nucleic acids occurs mainly in the endosome. Conceivably, TLR localization in the endosome is necessary to prevent contact with "self" nucleic acids, which are not taken into the endosome without additional components, as described below.

12.3.1.2 Cytoplasmic Sensors for Exogenous Nucleic Acids

Although endosomal TLRs that recognize nucleic acids are expressed mainly in the specialized immune cells, such as B cells and DCs, there are receptors in the cytoplasm that are also capable of sensing nucleic acids, namely RLRs and NLRs. In addition, several molecules have been proposed as capable of recognizing double-stranded B-form DNA (Takeshita and Ishii 2008; Vilaysane and Muruve 2009).

RIG-I and Melanoma Differentiation-Associated gene 5 (MDA5) belong to RLRs (Yoneyama et al. 2004; Takeuchi and Akira. 2008). Both of these cytoplasmic proteins contain helicase domains, including ATP-binding domains, C-terminal regulatory domains capable of binding tRNA, and Caspase Activation and Recruitment Domains (CARDs) to interact with adaptor molecule Interferon-β Promoter Stimulator 1 (IPS-1, also known as MAVS, Cardif and VISA). RIG-I discerns viral RNA by detecting the 5′-triphosphates of ssRNA and its short double-stranded form, while MDA5 recognizes long double-stranded RNA (Takeuchi and Akira 2008; Saito and Gale 2008). However, the exact element in viral RNA identified by RIG-I and MDA5 is currently unknown. It has been shown so far that genomic RNA of influenza viruses, paramyxoviruses, and HCV and short (≈1 kb) dsRNAs are detected by RIG-I, while picornaviruses such as Encephalomyocarditis Virus (EMCV) and longer (> 2 kb) dsRNA such as poly I:C are detected by MDA5 (Kato et al. 2008; Saito et al. 2008). Both RIG-I and MDA5 signal through IPS-1, which lies on the outer membrane of mitochondria and is associated with Stimulator of IFN Genes (STING; also known as MITA) (Ishikawa and Barber 2008; Zhong et al. 2008) and Fas-Associated Death Domain (FADD) (Balachandran et al. 2004). This complex activates NF-κB and IRF3 via IκK complex and TBK1/IκKi, respectively, initiating pathways that culminate in proinflammatory cytokines and IFN-β production (Fig. 12.1). LGP2 is also an RLR and shares homology with RIG-I and MDA5 in the helicase domain but lacks a CARD domain. It was shown in vitro to be a negative regulator of RIG-I and MDA5; however, results derived from knockout mice suggest that it is actually a positive regulator (Venkataraman et al. 2007; Satoh et al. 2010).

Cytoplasmic DNA recognition is quite distinct from RLR-mediated cytoplasmic RNA recognition. As initially shown by Isaacs et al. (1963) and rediscovered by Suzuki et al. (1999), DNA, especially double-stranded DNA, has been shown to be immunomodulatory. Ishii et al. refined their findings that transfection by natural DNA, or by synthetic polynucleotides that form double-stranded structures,

stimulates cells to produce type-I IFNs and induces cell-autonomous protection from viral replication, independently of TLR9. Unlike the CpG motifs needed for TLR9 activation, methylation of such dsDNA has no effect on activity. Rather, poly (dA-dT) • poly(dT-dA) induces higher levels of type-I IFNs compared with poly (dG-dC) • poly (dC-dG), suggesting that the right-handed helical structure of B-form DNA (B-DNA) is essential for cellular activation of type-I IFNs production; this process is mediated through a TLR-independent, TBK1-dependent means (Ishii et al. 2006). Therefore, TLR-independent, TBK1-dependent cytoplasmic DNA recognition plays an important role in immune responses during viral and bacterial infections (Yasuda et al. 2005; Ishii et al. 2006; Stetson and Medzhitov 2006; Cortez-Gonzalez et al 2006; Martin and Elkon 2006), and in controlling the ensuing adaptive immune responses (Ishii et al. 2008a; Baccala et al. 2007; Babiuk et al. 2004; Spies et al. 2003).

DNA derived from dying host cells can reportedly accumulate when nuclease functions are obstructed, including DNase-I, II, and -III (also known as TREX) (Okabe et al. 2005; Yoshida et al. 2005; Yasutomo et al. 2001; Morita et al. 2004; Napirei et al. 2000). The resultant activation of immune responses through TLR-independent DNA recognition by as-yet undefined receptors can lead to immunological disorders, such as autoimmune diseases (Kawane et al. 2006; Stetson et al. 2008).

Many receptors have been proposed for this TLR-independent, TBK1-dependent type-I IFN production by ds B-form DNA. The first candidate DNA sensor was reported to be DAI (DNA-dependent activator of IFN-regulatory factors), previously called DLM-1 and Z-DNA binding protein 1 (ZBP1) (Takaoka et al. 2007). However, mice lacking DAI (ZBP-1) did not show any expected phenotypes, in vitro or in vivo, suggesting that DAI is not essential for DNA-induced, TBK1-dependent type-I IFN production, or for DNA vaccine immunogenicity (Ishii et al. 2008a). More recent reports suggest that RNA polymerase-III can recognize AT-rich dsDNA, to generate 5′-triphosphate RNA, activating RIG-I in human cells (Ablasser et al. 2009), or in transformed cells (Chiu et al. 2009). Moreover, RIG-I (Choi et al. 2009), HMGB proteins (Yanai et al. 2009), and histone H2B (Kobiyama et al. 2010) were shown to recognize ds B-form DNA and respond by promoting TBK1-dependent type-I IFN production. Although it may take time to resolve how these distinct proteins recognize ds B-form DNA, it will be an exciting field of research.

Recent reports indicate that both type-I IFN (through RLRs) and IL-1β/18 (through inflammasome activation) are engaged in immune response to cytoplasmic nucleic acids (Muruve et al. 2008; Kanneganti et al. 2006a, b). Initially, microbial recognition by innate immune receptors like TLRs induces pro-IL-1β/18 accumulation in the cytosol through MAPK or NF-κB activation. Pro-IL-1β/18 is then cleaved with caspase-1, a major component of all types of inflammasomes, and released as mature form, IL-1β/18. Currently, four types of inflammasome complexes, NLRP1, NLRC4, NLRP3, and AIM2 inflammasome, have been partially characterized. Among them, only NLRP3 and AIM2 appear to be involved in nucleic acid sensing (Vilaysane and Muruve 2009; Franchi et al. 2009).

The NLRP3 inflammasome consists of NLRP3 (also known as NALP3 or cryopyrin), which is an NLR – an Apoptosis-Associated Speck-Like Protein Containing a CARD (ASC) and caspase-1. NLRs are a large family of cytoplasmic sensors, containing 23 members in human and 34 members in mice; their ligands have been only partially elucidated. NLRP3 consists of three domains: C-terminal LRR, central Nucleotide-Binding Oligomerization (NOD) domain and N-terminal ligand-sensing domain, and Pyrin Domain (PYD). A variety of ligands, including nucleic acids and their metabolites, such as RNA, RNA analogs, uric acids crystals, and ATP, are known to trigger NLRP3 inflammasomes (Martinon et al. 2006; Mariathasan et al. 2006; Kanneganti et al. 2006a), inducing formation of inflammasome complexes. These complexes include NLRP3 multimers, the adaptor molecule ASC, and pro-caspase-1 recruited via the ASC CARD domain, leading to autocleavage of caspase-1 (Fig. 12.1). However, it is unclear how NLRP3 detects its nucleic acid ligands.

In contrast to the NLRP3 inflammasome, which is mainly engaged in the recognition of RNAs, such as bacterial and viral RNA, synthetic dsRNA (poly I:C), and ssRNA (imidazoquinoline), the AIM2 inflammasome is activated by ds B-form DNA derived from bacteria, viruses, and host. AIM2 was recently identified as a member of HIN200 protein family; it consists of two domains: HIN200 domain, which binds to cytoplasmic dsDNA , and PYD, which recruits ASC (Roberts et al. 2009; Burckstummer et al. 2009; Fernandes-Alnemri et al. 2009; Hornung et al. 2009). The AIM2 inflammasome leads to activation of caspase-1 in the same manner as the NLRP3 inflammasome (Fig. 12.1).

12.3.2 The Mechanism to Avoid the False Recognition of the Endogenous Nucleic Acids: The Case of Tissue Damage

The innate immune system protects the host against invading infectious agents; however, the same system can also respond to endogenous stimuli (Gallucci et al. 1999; Ishii et al. 2001; Tsan and Gao 2004; Kono and Rock 2008). These stimuli include molecules released from damaged or dying host cells; some are endogenous cytokines and chemokines, including HMGB proteins, IL-1α, and IL-33; the others are heat-shock proteins, hyaluronan degradation fragments, oxidized lipids, nucleic acids, etc. RNA and DNA are normally sequestered tightly in the cells but can be released from host cells in the case of tissue damage, such as necrosis and apoptosis (Matzinger 2002; Ishii and Akira 2005). Nevertheless, nucleic acids are barely recognized by host immune system as there are safety mechanisms. One of these is the limited accessibility to the endosomal compartments where nucleic acid-sensing TLRs are expressed. Another is the immediate elimination of nucleic acids by RNases or DNases within the phagosome, extracellular matrix, or in the serum. A third is the sequential or molecular modification of nucleic acids (Kariko et al 2005). The presence of so many safety mechanisms suggests that any nucleic acid

species can be immunostimulatory if not in the right place in the cells or tissues. In fact, endogenous RNA and DNA are quite immunostimulatory if these safety mechanisms are broken – for example, if immune complexes of RNA or DNA with anti-RNA/DNA antibodies or RNPs are exogenously introduced into cells by transfection reagents, or if nucleotides are subjected to nuclease-resistant modification , or host nucleotides are removed or naturally modified. Otherwise, host RNA and DNA are normally inert to our immune system.

12.3.3 The Mechanism to Recognize Endogenous Nucleic Acids and Their Metabolites as Danger Signal: Another Case of Tissue Damage

Normally, extracellular concentrations of nucleic acids are very low. However, several kinds of molecules, including nucleic acids and their metabolites, which are mainly stored inside the host cell, are released from damaged or dying host cells in the case of tissue injury. Aberrant high concentration of extracellular nucleotides, such as ATP and its metabolic end products, uric acids, can be detected by innate immune sensors as danger signals (Mariathasan et al. 2006; Martinon et al. 2006) (Fig. 12.2). These responses culminate in IL-1β/18 production via NLRP3 inflammasome activation. Although several kinds of mechanisms that activate inflammasomes appear to be involved, the ATP-gated P2X7 receptor contained in the P2 receptor, a class of ubiquitous plasma membrane receptors, plays an important role in the upstream signals that trigger inflammasome activation (Kahlenberg et al. 2005; Ferrari et al. 2006; Di Virgilio 2007). Extracellular high concentration of ATP, a predominantly intracellular molecule, stimulates the P2X7 receptor and induces the activation of a cation channel that mediates potassium efflux. It also induces high concentrations of end-product uric acids and generates uric acids crystals, both of which can activate NLRP3 inflammasomes in gout (Martinon et al. 2006). Extracellular ATP is also engaged in the pathogenesis of bronchial asthma (Idzko et al. 2007). Uric acids, ATP, and adenosine – which is degraded from ATP through a cascade of ectonucleotidases, including nucleoside triphosphate diphosphorylase (NTPDase, also called CD39) and 5′-ectonucleotidase (Ecto50NTase, also called CD73) – induce immune responses (Hasko and Cronstein 2004). Extracellular adenosine binds to and activates four G protein-coupled cell surface receptors, A1, A2A, A2B, and A3, contained in P1 receptors, which signal through alterations in intracellular cyclic AMP and Ca^{2+} concentrations.

Nucleotides and their metabolites can both activate immune responses as danger signals and control or suppress immune reactions (Di et al. 2009). Adenosine's interaction with A2A receptors, which are the predominant subtype in immune cells, may inhibit inflammation by cAMP induction. For instance, hypoxia-inducible factor-1α (HIF-1α) mediates 5′-nucleotidase induction following accumulation of extracellular adenosine; therefore, hypoxic conditions can also

induce immunosuppressive effects via the A2A receptor (Ohta and Sitkovsky 2009). Thus, the recovery process from tissue damage is built upon a strict immunomodulatory mechanism.

12.3.4 *Endogenous Nucleic Acids Recognition: The Case of Autoimmune Diseases*

Despite the mechanisms to prevent false recognition of "self" nucleic acids and their metabolites described above, innate immune recognition of endogenous nucleic acids can induce detrimental adaptive immune response to "self" antigen – the so-called autoimmune diseases (Marshak-Rothstein and Rifkin 2007). Two major mechanisms of "self" nucleic acids have been identified to trigger autoimmune diseases: delayed clearance of damaged host cells, and unfavorable reactions to immune complexes (Fig. 12.3).

Damaged host cells generated from microbial infections and tissue injuries are immediately eliminated by phagocytes in normal conditions; however, necrotic cells always lose membrane integrity and unavoidably release intracellular contents,

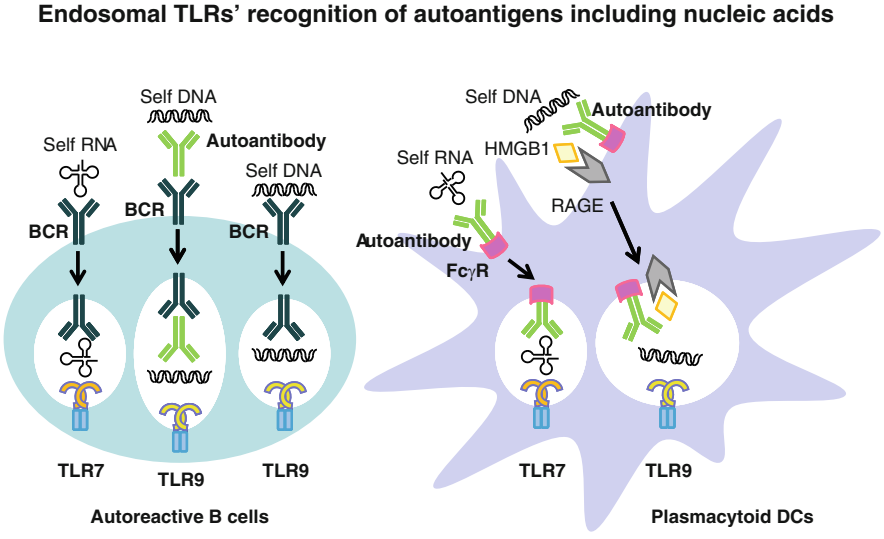

Fig. 12.3 B-cell receptors (BCRs) expressed on the surface of autoreactive B cells can bind autoantigen (DNA or RNA) directly or immune complexes including DNA or RNA and transport both BCRs and autoantigen or immune complexes to the cytoplasmic endosomal compartment containing TLR7/9. pDCs express receptors for the Fc portion of IgG (FcγRs). Immune complexes including DNA or RNA bind FcγRs on pDCs and are transported to cytoplasm. The interaction between HMGB1 (High-Mobility Group Box 1) and RAGE (receptor for advanced glycation end products) also contributes to the recognition of self-DNA-containing immune complexes

inducing immune responses via danger signals (Matzinger 2002). In contrast to necrotic cells, apoptotic cells are strictly cleared through interactions between opsonins and their receptors, such as scavenger receptors, complement receptors on phagocyte cell surfaces (Savill et al. 2002; Erwig and Henson 2008), and the recently identified peroxisome proliferator-activated receptor-δ (PPAR-δ) sensing system (Mukundan et al. 2009). Recent studies also suggest that impaired clearance of nucleic acids in apoptotic cells plays an important role in autoimmunity pathogenesis (Kawane et al. 2006; Napirei et al. 2000; Stetson et al. 2008).

Autoreactive B cells can bind autoantigens released from dying host cells via specific B-Cell Receptors (BCR), which are shown to exist in 5–20% of healthy individuals (Wardemann et al. 2003), and in a greater percentage of autoimmune-disease patients, due to defective early B-cell tolerance (Yurasov et al. 2005). Once these autoantigens are endocytosed by BCR, nucleic acids contained in the cells can stimulate endosomal TLR-7 and -9. These immune system activations, especially type-I IFNs responses via TLRs, play a key role in autoantibody production from proliferated and differentiated autoreactive B cells (Le Bon and Tough 2002; Theofilopoulos et al. 2005; Marshak-Rothstein and Rifkin 2007). B cells also express TLR-9 and the antigen receptor for self-immunoglobulin-gamma (IgG). Both are engaged in the recognition of IgG2a-chromatin immune complexes and induce production of a class of autoantibodies known as Rheumatoid Factors (RF) (Leadbetter et al. 2002). Both B cells and pDCs are involved in recognition of immune complexes – including self-RNA and DNA. PDCs express FcγRs (also known as CD32) to detect the Fc portions of autoantibodies, and Receptor for Advanced Glycation End Products (RAGE), to detect extracellular High-Mobility Group Box-1 (HMGB1) derived from necrotic cells (Tian et al. 2007). These interactions induce engulfment of immune complexes and stimulate TLR-7/8 and -9 in endosomal components, as with B cells.

Microbial infections can also trigger or accelerate a detrimental cascade mediated through increased proinflammatory cytokines (Munz et al. 2009) and damaged host cell products; interestingly, the gene dosage of TLR-7 directly contributes to the risk of autoimmune diseases (Pisitkun et al. 2006; Subramanian et al. 2006).

12.4 Therapeutic Applications of Nucleic Acids as Innate Immune Activators: Vaccine and Vaccine Adjuvants (Table 12.2)

As described above, extracellular nucleic acids and their metabolites can induce both immune activation and suppression via immune receptors, suggesting that nucleic acids and their analogs are candidates for therapeutic agents against infectious diseases, such as vaccines and vaccine adjuvants (Table 12.2) as well as therapies for autoimmune diseases and allergies. Here, we discuss vaccines and vaccine adjuvants that utilize the immunostimulatory effect of nucleic acids: DNA

Table 12.2 Nucleic acids based vaccines and vaccine adjuvants

Vaccine/Vaccine adjuvants (Formation of nucleic acids)	Receptors and adaptors involved in the recognition
Inactivated influenza whole virus vaccine (ssRNA)	TLR7/8-MyD88, RIG-I-IPS-1, NLRP3-ASC
Imidazoquinolines (ssRNA)	TLR7/8-MyD88, NLRP3-ASC
Poly I:C, Poly I:C[12]U (dsRNA)	TLR3-TRIF, MDA5-IPS-1, NLRP3-ASC
CpG oligonucleotides (ssDNA)	TLR9-MyD88
Poly dA:dT (dsDNA)	Unknown-STING-TBK1
DNA vaccine (dsDNA)	Unknown-STING-TBK1

Underlined innate immune signalings are essential for their immunogenicity
Poly I:C: polyinosinic:polycytidylic acid, STING: stimulator of interferon genes

vaccine (dsDNA), inactivated Whole Virus Influenza vaccine (influenza WV) (ssRNA) and poly I:C (dsRNA). CpG-ODNs (ssDNA), which are also very good candidates for therapeutic agents, are discussed in detail in the next section (Klinman 2004).

DNA vaccines are DNA plasmids encoding target antigen genes. Once these vaccines are administered, target antigens are expressed in host cells, inducing adaptive humoral and cellular immune responses. The immunogenicity of DNA vaccine was recently shown to depend on plasmid dsDNA (but not unmethylated CpG-motifs) and mediated through the DAI-independent, TBK1-dependent signaling pathway (the sensor for DNA vaccine has yet to be identified) (Ishii et al. 2008a). On the other hand, the immunogenicity including B cells and Th1-type CD4T cells activation of the influenza WV is dominantly controlled by a TLR7/MyD88-dependent signaling pathway, although TLR7/MyD88, RIG-I/IPS-1, and inflammasome activation are shown to be involved in live influenza virus infection (Allen et al. 2009; Koyama et al. 2007; Thomas et al. 2009; Ichinohe et al. 2009; Koyama et al. 2009). It suggested that genomic ssRNA remaining in influenza WV is essential for vaccine efficacy as an immune activator, detectable by only TLR but not RLR or NLR (Koyama et al. 2010).

A vaccine adjuvant is a compound that promotes and modulates vaccine immunogenicity. In theory, all PAMPs and DAMPs could be candidates for vaccine adjuvants (if safety were not an issue). Poly I:C, a synthetic dsRNA, is a vaccine adjuvant commonly used in animal models. Poly I:C can be detected by both endosomal TLR3 and cytoplasmic MDA5 (Miyake et al. 2009; McCartney et al. 2009); its adjuvanticity is mediated via both pathways (Kumar et al. 2008). The adjuvanticity of poly I:C (Longhi et al. 2009), DNA vaccines (Ishii et al. 2008a), and influenza WV (Koyama et al. 2010) requires dendritic cell activation and type-I IFNs production.

These studies demonstrate that specific signaling pathways in specific host cells play key roles in the efficacy of nucleic acids-based vaccines and vaccine adjuvants, though they can stimulate multiple immune sensors. Therefore, improved efficacy of vaccines and clinical applications require that target antigens and adjuvants be delivered to key immune cells, utilizing various manipulations of drug delivery systems, vaccine formation, routes of administration, and so on.

12.5 Conclusions

Nucleic acids and their metabolites are recognized by the specific host receptors such as TLRs, RIG-like receptors (RLRs), and NLRs, purinergic receptors such as P2X and P2Y receptors, and adenosine receptors such as A2A receptors. Resultant responses vary and may contribute to host defenses, aid homeostatic clearance of dying host cells, or even promote deleterious autoimmune diseases. As more questions emerge about nucleic acids and their relationships with the immune system, more efforts will be needed to elucidate the mechanism of immune recognition of, and regulation by, poly- and oligonucleotides such as RNA and DNA and their metabolites – including mononucleotides, nucleosides, bases, sugars, and uric acids – and the therapeutic potential of nucleic acids and their metabolites.

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