How the techniques of molecular biology are developed from natural systems

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Abstract

A striking characteristic of the highly successful techniques in molecular biology is that they are derived from natural occurring systems. RNA interference (RNAi), for example, utilises a mechanism that evolved in eukaryotes to destroy foreign nucleic acid. Other examples include restriction enzymes, the polymerase chain reaction, fluorescent proteins and CRISPR-Cas9. I propose that natural molecular mechanisms are exploited by biologists for their effectors' (protein or nucleic acid) activity and biological specificity (protein or nucleic acid can cause precise reactions). I also show that the developmental trajectory of novel techniques in molecular biology, such as RNAi, is four characteristic phases. The first phase is discovery of a biological phenomenon. The second is identification of the mechanism's trigger(s), the effector and biological specificity. The third is the application of the technique. The final phase is the maturation and refinement of the molecular biology technique. The development of new molecular biology techniques from nature is crucial for both biological and biomedical research.

Keywords: mechanism; experiment; specificity; scientific practice; PCR; GFP.

Introduction

Philosophy of science, with its emphasis on the structure of scientific theories, struggles to account for molecular biology (Darden 2006; Schaffner 1996). This area of biology is principally concerned with explaining the complex molecular phenomena underlying living processes by identifying the mechanisms that produce such processes (Tabery et al. 2015). In order to access the causal structure of molecular mechanisms it is necessary to manipulate the components of the mechanism and to observe the resulting effects with sophisticated molecular techniques. These techniques generate knowledge that cannot be obtained by any other means. Scientific knowledge in molecular biology is therefore acquired in a distinctive way compared to other areas of biology, progress is driven by the introduction and use of novel techniques. However, what drives the development of molecular biology techniques?

In this paper, I firstly provide evidence that molecular biology techniques are derived by biologists from natural systems. In the second section, I identify that the natural systems' strategy for technique development means biologists utilise the activity of a mechanism's effector (protein or RNA) and exploit biological specificity (protein or nucleic acid can cause precise reactions). In the third section, I show that molecular biology technique development from nature can be separated into four phases and I present RNA interference (RNAi) as an exemplar case study. I conclude by discussing the implications of deriving techniques from nature for molecular biology.

From natural systems to techniques

A striking feature of the development of molecular biology techniques, which biologists themselves often highlight (for example, Lander 2016; Mello and Conte 2004), is that they are derived from natural occurring systems. These techniques are not developed through 'rational design', such as using engineering principles (discussed in O'Malley 2009) nor do they merely mimic nature (Ahn et al. 2015). In this paper I examine eight contemporary techniques that are derived from natural systems and are the most scientifically successful. These eight techniques have been patented, produced landmark scientific articles and been the subject of a Nobel prize (Ronai and Griffiths in press). The scientific community sees these techniques as significant advances. In chronological order these techniques are: restriction enzymes; DNA sequencing, polymerase chain reaction (PCR); gene targeting; fluorescent proteins (such as, green fluorescent protein); RNAi; induced pluripotent stem cells (iPS); and clustered regularly interspaced short palindromic repeats-CRISPR associated 9 (CRISPR-Cas9) (see Table 1). Throughout this paper I use RNAi as my detailed case study. This technique was chosen due to its contemporary history (see Fire et al. 1998), which means that it has not yet been examined to a great extent by philosophers and historians of biology. RNAi is a technique that introduces molecules of RNA into an organism in order to reduce the expression of a gene of interest (reviewed in, Mello and Conte 2004) (Figure 1). The eight molecular biology techniques are so ubiquitous that they are regarded as common knowledge by biologists. So when these techniques are mentioned in the Methods section of a scientific article, a citation to the technique is often not necessary.

The eight molecular biology techniques discussed are derived from mechanisms that each evolved for a particular biological function in a natural system (see Table 1). The biological

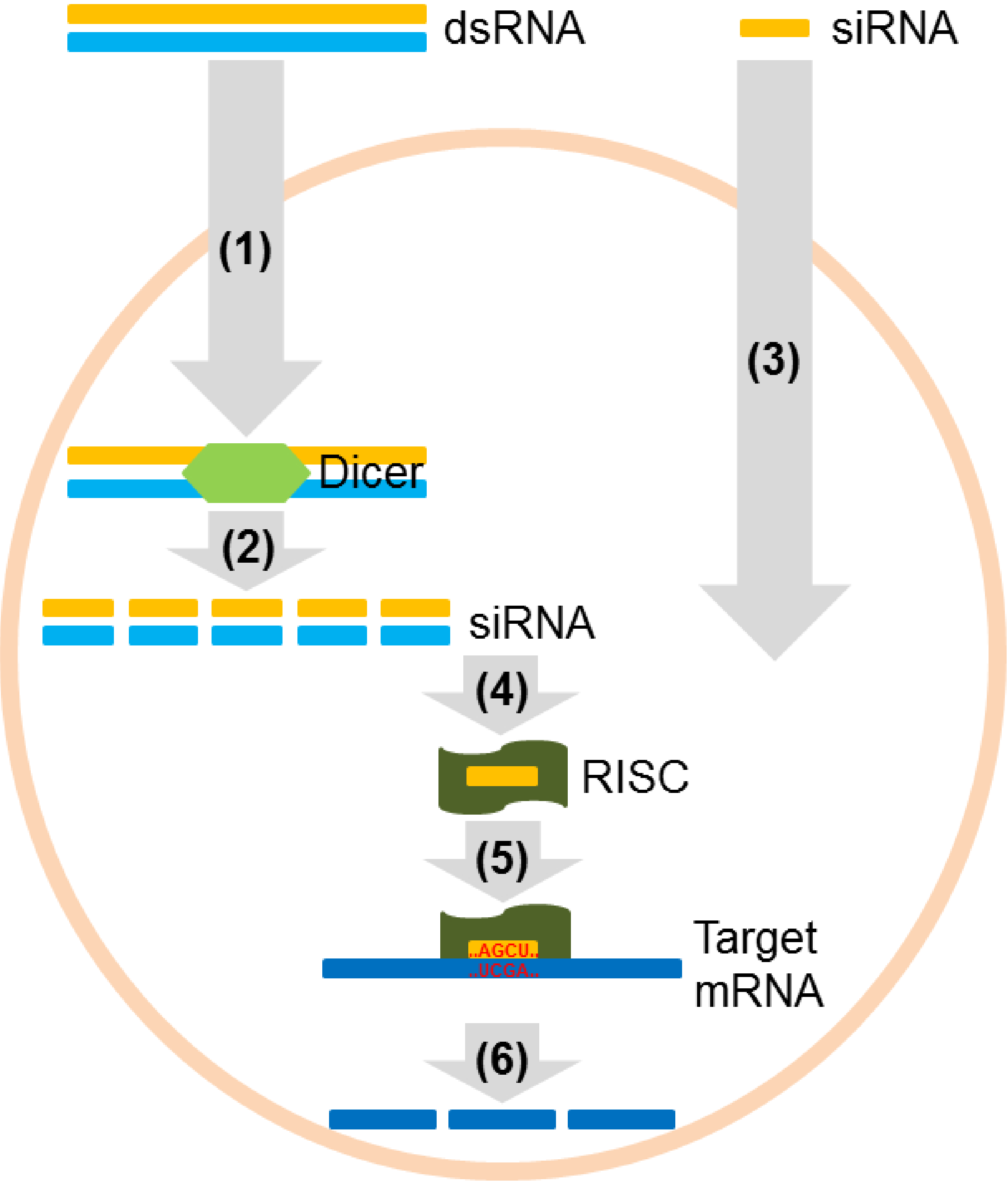

Fig. 1: A model of the molecular biology technique of RNA interference (RNAi). (1) Double-stranded RNA (dsRNA) is introduced into the experimental system. (2) The endogenous endonuclease Dicer cleaves the dsRNA into small fragments known as small interfering RNA (siRNA). (3) Or the siRNA is added directly into the experimental system. (4) The siRNA antisense strand attaches to the endogenous RNA-induced silencing complex (RISC), (5) which binds sequence specifically to the target mRNA. (6) RISC cuts the target mRNA which causes it to be degraded, therefore no gene function is executed.

Table 1: Summary and characterisation of eight highly successful molecular biology techniques. These techniques are all derived from natural systems and are now utilised as methodologies. For key references see Table 2.

Technique (in chronological order of development)	**Originating natural system**	**Biological function of mechanism**	**Experimental context**	**Type of effector**	**Type of biological specificity**
1. Restriction enzymes	Bacteria	Destroy foreign nucleic acid from bacteriophages	In vitro	Restriction endonuclease	Stereochemical: DNA recognition sequence
2. DNA sequencing	Bacteria	DNA replication	In vitro	DNA polymerase I	Informational: dideoxynucleotides (also DNA primer) has sequence match to DNA template
3. PCR	Bacteria	DNA replication	In vitro	DNA polymerase I	Informational: DNA primers has sequence match to DNA template
4. Gene targeting	Organism or cell culture	Homologous recombination	Organisms & cell culture	Endogenous endonuclease (for example, SPO11)	Informational: exogenous DNA has sequence match to target DNA/gene
5. Fluorescent proteins	Jellyfish	Unknown - emitted when jellyfish is agitated[1]	Organisms & cell culture	Fluorescent protein, in particular the flurophore	Engineered informational specificity: fluorescent protein DNA placed in specific location
6. RNAi	Eukaryote or cell culture	Destroy foreign nucleic acid or gene regulation	Eukaryote organisms & cell culture	Endogenous RNA-induced silencing complex (RISC), in particular the Argonaute endonuclease	Informational: dsRNA (& siRNA) with sequence match to target mRNA
7. iPS	Embryonic stem cells	Stem cell function (unlimited self-renewal & pluripotency)	Cell culture	Transcription factors (*Oct4*, *Sox2*, *cMyc* & *Klf4*)	Stereochemical: DNA binding site
8. CRISPR-Cas9	Bacteria	Destroy foreign nucleic acid from bacteriophages	Organisms & cell culture	RNA-guided DNA endonuclease (cas9)	Informational: guide RNA (crRNA + tracrRNA) with sequence match to target DNA

[1] (Davenport and Nicol 1955)

function of the RNAi mechanism, for example, is an eukaryotic defence system[1] for the destruction of foreign nucleic acid and mobile elements (van Rij and Andino 2006; Waterhouse et al. 1998; Waterhouse et al. 2001). The same biological function, to destroy foreign nucleic acid in the organism, underlies the techniques of RNAi (derived from eukaryotes) and CRISPR-Cas9 (derived from prokaryotes) (Bhaya et al. 2011; Wright et al. 2016), but the two techniques involve different molecular mechanisms (Table 1). The 'arms race' that occurs between viruses and their organismal hosts has provided biologists with the basis of two techniques. In contrast, green fluorescent protein was derived from a relatively unique biological phenomenon in jellyfish and is therefore taxonomically restricted.

Natural systems show biologists what is mechanistically possible. Natural mechanisms have been selected by evolution and are likely to have a high level of effectiveness. However, the components of these mechanisms are contingent on historical, iterative events rather than being at an optimal state. Biologists can alter these components to reach an optimal state but are constrained by their possibility space.

Biologists use molecular biology techniques developed from pre-existing, natural mechanisms because they can be 'biologically normal interventions' (Weber forthcoming). These techniques are compatible with living processes and do not create artificial phenomena. Furthermore, the use of a natural mechanism may allow the continuing function of the biological process (for example, fluorescent proteins) and cellular based techniques can be stably inherited in designed constructs with transgenerational effects. These techniques can be used to observe or intervene in active, complex biological processes even when no comprehensive understanding of these processes exists.

The importance of natural systems for the development of techniques in molecular biology

In this section I analyse why natural systems are used for the development of techniques in molecular biology. The techniques exploit two key components of natural mechanisms: an effector's activity and the use of biological specificity. It is important to note that biologists implicitly recognise the importance of the effector's activity and the use of biological specificity for molecular biology techniques[2].

Effector activity

Living systems use effector molecules (such as, proteins or RNAs) to generate a particular activity within a mechanism. I have identified the protein effector, all from a natural system, for each of my eight molecular biology techniques (Table 1). The majority of the techniques utilise proteins that are catalytic enzymes (note, enzyme names normally end with '-ase') and the

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[1] The RNAi mechanism is thought to have been repurposed (Cerutti and Casas-Mollano 2006) for the precise regulation of endogenous gene expression, in particular for the regulation of developmental genes (Carrington and Ambros 2003). Therefore, the RNAi mechanism is a deeply entrenched process in eukaryotic organisms.

[2] For example, many studies on RNAi discuss the technique's effector's activity (Fellmann and Lowe 2014; Filipowicz et al. 2005; Li et al. 2006; Rana 2007; Siomi and Siomi 2009; Vaucheret et al. 1998) and specificity (Bartel 2004; Elbashir et al. 2001b; Fellmann and Lowe 2014; Fire et al. 1998; Hamilton and Baulcombe 1999; Hammond et al. 2000; Hammond et al. 2001; Kennerdell and Carthew 1998; Parrish et al. 2000; Rana 2007; Siomi and Siomi 2009; Waterhouse et al. 1998; Waterhouse et al. 2001).

techniques leverage the efficiency of the enzymatic activity (Table 1). The two exceptions are the techniques of fluorescent proteins and iPS which utilise a protein's stereochemistry, a flurophore or structural motif, respectively (Table 1).

A technique's effector is either endogenous or exogenous to the experimental system (Table 1). Endogenous based techniques use the effector for its original purpose but they appropriate the overall mechanism. For example, the effector of RNAi is the RNA-induced silencing complex (RISC), which is an endogenous component of a molecular mechanism present in all eukaryotes (Cerutti and Casas-Mollano 2006) (Figure 1). Whereas, exogenous based techniques use the effector for its original purpose but in another biological context. Therefore, the exogenous effector needs to be introduced into the experimental system and is more tractable than an endogenous effector.

Biological specificity

Living systems need biological specificity to achieve precise control over their molecular mechanisms (Griffiths et al. 2015; Waters 2007; Woodward 2010). In the eight molecular biology techniques studied here biologists introduce biological specificity into their experimental systems to precisely access the target mechanism with fine-grained control[3]. I have identified that the majority of the eight molecular biology techniques use nucleic acid sequence informational specificity (Griffiths and Stotz 2013), nucleic acid is the substrate of the mechanism (Table 1). For example, RNAi provides fine-grained control of gene expression because it uses nucleic acid sequence informational specificity (Figure 1). Before RNAi only a non-specific, permanent disruption in gene expression via mutagenesis was possible (Bellés 2010). One molecular biology technique, fluorescent proteins, uses what I term 'engineered informational specificity', where the biologist creates the specificity by placing the effector in a highly specific location. The last two molecular biology techniques, iPS and restriction enzymes, use protein stereochemical specificity (Griffiths and Stotz 2013) (Table 1).

Informational and stereochemical specificity differ in an important aspect. For nucleic acid guided techniques the informational specificity is artificially designed whereas stereochemical specificity uses naturally derived specificity. Therefore, stereochemical specificity is fixed before the start of the experiment which means it is less programmable than informational specificity and not the preferred choice by biologists.

The importance of an effector's activity and biological specificity

The effector activity and specificity of a technique are critical to its success. If there are multiple techniques available to achieve the same experimental purpose, then the one with the greatest efficiency or superior type of specificity is preferred by the scientific community. For example, transcription activator-like effector nucleases (TALENs)[4] and zinc finger nucleases (ZFNs)[5] are techniques used for the same experimental purpose as the CRISPR-Cas9 technique, DNA editing. However, a TALENs' and ZFNs' specificity is stereochemical so it needs to be reengineered for every experiment and are not as easily programmable for a wide range of targets

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[3] Biologists need interventions with minimal off-target events. Also, high specificity means that the technique can be 'multiplexed' as multiple nucleic acid sites can be targeted at the same time.

[4] TALENs are a technique derived from the bacteria *Xanthomonas* (Boch et al. 2009; Moscou and Bogdanove 2009).

[5] ZFNs are a technique that uses two protein domains coupled together (Kim et al. 1996).

compared to CRISPR-Cas9[6]. For this reason CRISPR-Cas9 became commercially viable and has replaced TALENs and ZFNs as the premier gene editing technique (Corbyn 2015; Doudna and Charpentier 2014). Therefore, the effector's activity and specificity are likely to be critical for the commercialisation of the technique for widespread usage.

Molecular biology technique development has four phases

I propose that molecular biology techniques derived from natural systems have a specific pattern of development with four critical phases. These phases are: the discovery of a phenomenon; identification of the mechanism's trigger(s); application of the technique; and maturation of the technique. Each of the eight molecular biology techniques show the four phases of technique development (see Table 2) and in this section I use the development of RNAi as a detailed case study.

The first phase: discovery

Biologists identify and describe an unusual phenomenon in a natural system. At this stage the underlying mechanism is not well characterised and the biological function of the mechanism is typically unknown. This phase can be identified by examining the studies that the application of the technique phase built upon.

For example, in the early 1990s the RNAi[7] phenomenon was first identified in plants (Table 2). Napoli et al. (1990); van der Krol et al. (1990) wanted to increase colour intensity in the *Petunia hybrida* flower. They introduced synthetic sense RNA into the plant in order to overexpress a gene in the pathway that controls formation of the flower pigment. Contrary to expectation, these flowers had less, rather than more, pigment. Therefore, the sense RNA had reduced the mRNA of the endogenous gene. During the 1990s multiple studies were conducted on how different organisms actively respond to the introduction of RNA (Fire et al. 1991; Guedes and Priess 1997; Guo and Kemphues 1995; Lin et al. 1995; Mello et al. 1996; Powell-Coffman et al. 1996; Romano and Macino 1992). These early studies produced knowledge that was critical to the development of RNAi.

The second phase: identification of the trigger(s)

Biologists identify the specificity and effector[8] component of the mechanism (see Table 3A&B). I term the specificity and effector 'the trigger(s)' because they are the key causative agents and are 'the causally specific actual difference maker' under typical conditions (Carrier 2004; Waters 2007; Woodward 2010). Once biologists identify the trigger(s) they can use it to precisely access

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[6] CRISPR-Cas is limited by the protospacer adjacent motif (PAM) but this sequence is dependent on the Cas being used (Doudna and Charpentier 2014).

[7] During the 1990s the RNAi phenomenon was described using many different terms. The initial study by Napoli et al. (1990) termed this phenomenon 'co-suppression' but a follow up study by Blokland et al. (1994) demonstrated that silencing occurred post-transcriptionally so it was referred to as 'post transcriptional gene silencing'. Another study by Romano and Macino (1992) identified the RNAi phenomenon in a fungus, *Neurospora crassa*, and termed it 'quelling'. An early *Caenorhabditis elegans* study by Rocheleau et al. (1997) coined the term 'RNA-mediated interference'.

[8] For techniques that have stereochemical specificity (Table 1) the effector is the specificity.

Table 2: The four phases of development for the eight highly successful molecular biology techniques. For each technique I identify the first paper that: discovered the phenomenon; identified the mechanism's effector; identified the mechanism's specificity applied the trigger(s); and any highly cited papers that demonstrate the maturation of the technique.

Technique (in chronological order of development)	**Phase**	**Reference**	**Description**
1. Restriction enzymes	**Discovery**	Luria and Human (1952)	Discovered that bacteriophage (T1, T2, T3, T4, T5, T6 and T7) vary in their ability to grow in different bacterial (*Escherichia coli* and *Shigella dysenteriae*) strains.
		Dussoix and Arber (1962)	Discovered that bacteriophage λ DNA degrades in *Escherichia coli* strains.
	Identification of specificity/effector	Kelly Jr and Smith (1970); Smith and Welcox (1970)[1]	Identified the nucleotide recognition sequence that causes restriction enzymes (in particular, a type II which recognises DNA and cuts sites at the same place, endonuclease R from *Hemophilus influenza*) to cut DNA.
	Application of trigger	Danna and Nathans (1971)	Applied restriction enzyme (endonuclease R from *Hemophilus influenza*) to cut up DNA.
	Maturation	Feinberg and Vogelstein (1983)	Developed restriction enzymes using radiolabelling to efficiently recover DNA fragments.
2. DNA sequencing	**Discovery**	Watson and Crick (1953)	Discovered the complementary DNA structure in calf thymus (possibly) and proposed a mechanism for DNA replication. Also, predicted the existence of DNA polymerase.
		Matthaei et al. (1962)	Discovered that three nucleotides code for a specific amino acid in a cell-free system of *Escherichia coli*. Also, predicted the code was universal.
	Identification of effector	Kornberg et al. (1956b)	Identified DNA polymerase in *Escherichia coli*.
	Identification of specificity	Atkinson et al. (1969)	Identified that dideoxynucleotides cause DNA polymerase to terminate synthesis of DNA.
	Application of triggers	Sanger et al. (1977)	Applied dideoxynucleotides with DNA polymerase from *Escherichia coli* to determine the DNA sequence of bacteriophage φX174.
	Maturation	The *C. elegans* Sequencing Consortium (1998)	Developed DNA (Sanger) sequencing to sequence the first multicellular organism (*Caenorhabditis elegans*) genome.
		International Human Genome Sequencing Consortium (2001)	Developed DNA (Sanger) sequencing to sequence the human genome.
3. PCR	**Discovery**	Watson and Crick (1953)	Discovered the complementary DNA structure in calf thymus (possibly) and proposed a mechanism for DNA replication. Also, predicted the existence of DNA polymerase.
		Meselson and Stahl (1958)	Discovered that DNA replicates semi-conservatively in *Escherichia coli*.

	Identification of effector	Kornberg et al. (1956b)	Identified DNA polymerase in *Escherichia coli.*
	Identification of specificity	Kornberg et al. (1956a)	Identified that a primer causes DNA polymerase to initiate synthesis of DNA.
	Application of triggers[2]	Saiki et al. (1985)	Applied primers with DNA polymerase from *Escherichia coli* to amplify DNA region.
	Maturation	Saiki et al. (1988)	Developed PCR to be thermostable using DNA polymerase from *Thermus aquaticus*.
4. Gene targeting	**Discovery**	Gluzman et al. (1977); Vogel et al. (1977)[1]	Discovered that a mutant phenotype can be rescued in a simian virus 40 (SV40) temperature-sensitive mutant (tsD202) when added to monkey CV1 cells (containing endogenous integrated SV40). Also, discovered that the rescue is due to recombination.
	Identification of specificity	Hinnen et al. (1978)	Identified that exogenous DNA of *LEU2* causes site specific recombination with homologous chromosomal DNA in *Saccharomyces cerevisiae.*
	Application of trigger	Smithies et al. (1985)	Applied exogenous DNA to modify only the target gene (β-globin) in human cells.
	Maturation	Thomas and Capecchi (1987)	Developed gene targeting to inactivate an endogenous gene (*hptr*) in mouse embryonic stem cells.
		Doetschman et al. (1987)	Developed gene targeting to correct mutant *hptr* in mouse embryonic stem cells.
		Mansour et al. (1988)	Developed gene targeting selection (positive for cells that have incorporated exogenous DNA and negative for cells that have randomly incorporated exogenous DNA) in mouse embryonic stem cells.
	Identification of effector	N/A[3]	Endogenous endonucleases create a double-stranded break and this initiates repair pathway. For example, SPO11.
5. Fluorescent proteins	**Discovery**	Davenport and Nicol (1955)	Discovered the green fluorescence in *Aequorea victoria.*
	Identification of effector	Shimomura et al. (1962)	Identified the green fluorescent protein (GFP) in *Aequorea victoria.*
	Identification of specificity	Prasher et al. (1992)	Identified the genomic DNA and cDNA sequence of GFP that causes fluorescence in *Aequorea victoria.*
	Application of trigger	Chalfie et al. (1994)	Applied GFP cDNA to generate fluorescence in *E. coli* and *Caenorhabditis elegans* cells.
	Maturation	Heim et al. (1995)	Developed GFP spectral characteristics using a point mutation in *Escherichia coli.*
		Cormack et al. (1996)	Developed GFP variants that fluoresce more intensely in *Escherichia coli.*
6. RNAi	**Discovery**	Napoli et al. (1990)	Discovered the knockdown of *chalcone synthase* in *Petunia hybrida.*
	Identification of specificity (component) & application of trigger	Fire et al. (1998)	Identified that dsRNA causes sequence specific regulation of mRNA in *Caenorhabditis elegans.* Applied dsRNA to knockdown gene expression in *Caenorhabditis elegans.*
	Identification of specificity (processed component)	Hamilton and Baulcombe (1999)	Identified that siRNA (processed product of dsRNA) causes sequence specific regulation of mRNA in plants.

	Identification of effector	Hammond et al. (2000)	Identified the RNA-induced silencing complex (RISC) which contains an endonuclease that cleaves target mRNA in *Drosophila* cells.
	Maturation	Elbashir et al. (2001a)	Developed RNAi to knockdown gene expression in mammalian and *Drosophila* cells.
7. iPS	**Discovery**	Gurdon (1962)	Discovered that cell differentiation is reversible because the nucleus of a somatic cell can successfully replace the nucleus of an egg cell in *Xenopus laevis*.
	Identification of specificity/effector & application of trigger	Takahashi and Yamanaka (2006)	Identified the genome and transcriptome changes that cause four transcription factors (Oct3/4, Sox2, c-Myc and Klf4 in mice) to make somatic cells become pluripotent stem cells. Applied the four transcription factors cDNA to reprogram embryonic and adult fibroblast mice cells.
	Maturation	Takahashi et al. (2007)	Developed iPS in human cells.
8. CRISPR-Cas9	**Discovery**	Ishino et al. (1987)	Discovered the CRISPR motif (repeated sequence with spacers) in the DNA sequence of *Escherichia coli*.
	Identification of effector	Makarova et al. (2002)	Identified the CRISPR-associated (cas) genes in the genome sequences of bacteria and archaea. In particular, the class 2, Type II (recognises DNA and cleavage results in double-stranded break) Cas9 (COG3513) in *Streptococcus pyogenes*, *Campylobacter jejuni*, *Neisseria meningitidis* and *Pasteurella multocida*.
	Identification of specificity (component A)	Brouns et al. (2008)	Identified that CRISPR RNAs (crRNAs) cause Cas9 to sequence specifically cleave DNA in *Escherichia coli*.
	Identification of specificity (component B) & application of triggers	Jinek et al. (2012)	Identified that crRNA and trans-activating CRISPR RNA (tracrRNA) must complementary base pair to cause Cas9 to site-specifically cleave DNA. Applied a tracrRNA-crRNA complex (the 'single-guide RNA') with Cas9 from *Streptococcus pyogenes* to cleave DNA.
	Maturation	Cong et al. (2013)	Developed CRISPR-Cas9 to edit the genome of mammalian (human and mouse) cells.
		Mali et al. (2013)	

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[1] This paper was published in two parts.

[2] Kleppe et al. (1971) only applied primers with DNA polymerase to replicate short synthetic DNA rather than amplify a DNA region.

[3] A single study cannot be identified because the biological mechanism underlying gene targeting has multiple effectors.

Table 3: The key experiments for the RNAi technique conducted by Fire et al. (1998). Experiments that (A) identified the triggers in the RNAi mechanism; and (B) identified the target of the specificity in the RNAi mechanism.

(A)

Specificity	**Range tested**	**Result**
Non-purified single-stranded RNA (ssRNA)	Sense RNA or antisense RNA	When non-purified ssRNA was introduced into the experimental system caused RNAi.
Purified ssRNA	Sense RNA or antisense RNA	Purified ssRNA led to weaker RNAi compared to purified dsRNA, indicated that dsRNA causes RNAi.
Complementary sense and antisense strand RNA	Pre-annealed; injected sequentially; or injected sequentially but with long time interval between RNAs	Pre-annealing of RNA led to stronger RNAi, indicated that the formation of dsRNA was important for RNAi. Sequential injection of sense and antisense RNA led to RNAi, indicated that RNA strands could hybridise to form dsRNA in the experimental system. If there was a long time interval between sequential injection of RNAs no RNAi occurred, indicated that over time ssRNA are degraded or become inaccessible in the experimental system.
Time post-injection of RNA	6; 15; 27; 41; or 56 hours	When there was a long time interval after RNA was introduced into the experimental system RNAi decreased.
ssRNA and control gene dsRNA	ssRNA not attached to dsRNA; ssRNA attached at its 5' end to dsRNA; or ssRNA attached at its 3' end to dsRNA	For the gene that the ssRNA targeted no RNAi occurred, indicated that sequence specificity not double stranded structure was important for RNAi.
dsRNA length	299 to 1033 nucleotides	Nucleotide length of dsRNA did not affect RNAi.
RNA dosage	30,000 to 3,600,000 RNA molecules per organism	Very low dsRNA dosages triggered RNAi, indicated that RNAi is a catalytic process (i.e. enzymes involved) otherwise there would be not enough RNA molecules to bind to all the endogenous mRNA in the experimental system.
Site of injection of RNA in organism	Body cavity of head; body cavity of tail; or gonad	In tissues other than the ones injected RNAi occurred, indicated that RNAi is systemic. Also, injection of adults sometimes led to offspring with RNAi, indicated that trans-generational inheritance of RNAi occurred. These results suggested that the RNAi mechanism existed throughout the whole organism.

(B)

Target of specificity	**Range tested**	**Result**
Gene regions	One exon, multiple exons; intron; or promoter	RNAi occurred only when the coding sequence of the mRNA was targeted, indicated that RNAi works through post-transcriptional regulation.
Conserved gene segment		RNAi led to an unexpected phenotype, indicated that RNAi affects genes with a similar sequence to the gene of interest.
Gene of interest	*unc-22*; *unc-54*; *fem-1*; *hlh-1* ;*gfp;* or *mex-3*	The target of RNAi was genes that are non-essential and have previously been characterised with an easily identifiable visual phenotype. Also, the relationship between the gene's expression and phenotype was in the manipulable direction (i.e. reduced expression increased the severity of the phenotype).
Transgenic line expressing two GFP reporter proteins		RNAi occurred in individual cells of the organism.
mex-3 in an *in situ* hybridisation experiment		The target of RNAi was a gene that is abundant in early embryos (a useful developmental period for an *in situ* experiment). Endogenous mRNA disappeared suggesting it was destroyed, visually indicated that mRNA (not precursor mRNA nor protein) was the target of RNAi.

the causal structure of the mechanism. If the effector is endogenous to the experimental system (Table 1), then it does not need to be added to the experiment and its identification is not essential for the development of the technique.

For example, in the late 1990s dsRNA was found to be causally specific for the RNAi mechanism (Table 2). The dsRNA was investigated due to it being accidently produced in earlier experiments as it was found that:

> … polymerases, although highly specific, produce some random or ectopic transcripts. DNA transgene arrays also produce a fraction of aberrant RNA products[3]… we surmised that the interfering RNA populations might include some molecules with double-stranded character. (Fire et al. 1998, p. 807)

Fire et al. (1998) tested the specificity of RNA molecules to control the RNAi mechanism in *C. elegans* (Table 3A). The dsRNA was identified as the cause of sequence specific regulation of mRNA. Fire et al. (1998, p. 806):

> … investigate[d] the requirements for structure and delivery of the interfering RNA. To our surprise, we found that double-stranded RNA was substantially more effective at producing interference than was either strand individually.

Therefore, the study was a conclusive demonstration of how dsRNA can be used to control the RNAi mechanism.

Biologists then wondered how dsRNA could bind to the mRNA to sequence specifically cleave it. They found that dsRNA is processed into small RNA fragments (antisense and sense) in many different organisms and suggested that these were necessary for RNAi (Hamilton and Baulcombe 1999; Hammond et al. 2000; Parrish et al. 2000; Zamore et al. 2000). The small interfering RNAs (siRNA), 21-23 nucleotides in length, were shown to sequence specifically guide the cleavage of the mRNA (Elbashir et al. 2001b) (Table 2).

Two years after the RNAi technique was developed the endogenous effector component that degrades the target mRNA was identified as the RNA-induced silencing complex (RISC) (Figure 1, Table 2). The endonuclease that cuts the target mRNA sequence-specifically was identified in *Drosophila* cells as Argonaute, which is part of RISC (Hammond et al. 2000; Martinez et al. 2002). The effector that cleaves dsRNA into siRNAs was identified as a ribonuclease type III named Dicer (Bernstein et al. 2001). Biologists then pursued the mechanistic details such as the functions of different forms of Argonaute (Rana 2007).

The third phase: application of the trigger(s)

Biologists conclusively determine that when the trigger(s) is introduced into the experimental system it achieves some intended effect on the target of the specificity. The trigger(s) is exploited in three types of investigative strategies: to manipulate an effector's activity in a non-cellular experimental system (for example, restriction enzymes); to intervene on a cellular experimental system (for example, RNAi); or as a tracer to follow a biological process[9] (for example, fluorescent proteins) (Table 1). At this stage a deep understanding of the mechanism underlying the technique is not necessary for the technique to work.

For example, the RNAi technique was first applied in the Fire et al. (1998) paper 'Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*' published in the journal *Nature* (Table 2). The study was a conclusive demonstration of how dsRNA can be

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[9] For an in depth discussion see (Griesemer 2007).

applied as a molecular biology technique to manipulate gene expression in *C. elegans*. Fire et al. (1998, p. 810) concluded that RNAi:

> … adds to the tools available for studying gene function in *C. elegans*. In particular, it should now be possible functionally to analyse many interesting coding regions[21] for which no specific function has been defined.

Interestingly, Fire et al. (1998, p. 810) explicitly stated that they did not understand the biological function of the RNAi mechanism:

> Whatever their target, the mechanisms underlying RNA interference probably exist for a biological purpose.

It is important to note that when a molecular biology technique is developed for an organismal experimental context (Table 1) it is first tested in a 'model organism' system. For example, RNAi was first developed using the model organism *C. elegans* (Fire et al. 1998). Model organisms provide standardised experimental systems that are relatively well characterised at the molecular level act as a prototype for technique development (Ankeny 2000; Leonelli and Ankeny 2013). When a technique has been validated in a model organism there is the expectation that, due to the fundamental unity of living systems, it might be applied to other organisms. The use of model organisms is particularly important given the complexity and cost of molecular biology experiments.

The fourth phase: maturation

Once the technique is established biologists improve and expand its performance. The scientific community invests considerable research activity into characterising, both spatially and temporally, the mechanism in natural systems. Therefore, the technique generates further research on the mechanism that underlies it. The new knowledge acquired may improve access to the mechanism or allow the technique to be better controlled, enabling the technique to continue to be refined and standardised.

Immediately following Fire et al. (1998), RNAi was shown to work in multiple organisms[10] (Table 2). In mammals RNAi (using dsRNA) initially failed due to the immune response elicited, however, when siRNAs were used gene expression could be altered (Elbashir et al. 2001a). RNAi has become a highly selective molecular biology technique for reducing expression of a target gene and today it is widely used for both basic and applied research (Deng et al. 2014; Fellmann and Lowe 2014; Mello and Conte 2004). To this day the mechanism of RNAi is still being investigated.

Molecular biology technique development

The four phases are necessary features of technique development when derived from a natural system. I have shown that eight highly successful molecular biology techniques have these four phases of development (Table 2), additional techniques include: reverse transcription; molecular cloning; monoclonal antibodies; site directed mutagenesis; and immunotherapy. Future research

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[10] The RNAi technique was used in *C. elegans* (Fitzgerald and Schwarzbauer 1998; Montgomery et al. 1998; Ogg and Ruvkun 1998; Page and Winter 1998; Skop and White 1998; Tabuse et al. 1998; Timmons and Fire 1998); two species of plants, *Nicotiana tabaccum* and *Oryza sativa* (Waterhouse et al. 1998); and *D. melanogaster* (Kennerdell and Carthew 1998).

will be able to show that these techniques also have the four phases of development and identify these techniques' effector's activity and specificity.

The development of new molecular biology techniques accelerates research and generates new scientific knowledge that would otherwise not exist. A new technique can help uncover previously undetected phenomena and paradoxically, in turn lead to the development of yet another technique. For example, restriction enzymes were instrumental to the initial detection of the RNAi phenomena (Napoli et al. 1990; van der Krol et al. 1990) and during the application phase of development for RNAi green fluorescent protein was used to visualise that the RNAi mechanism occurs within the cell (Table 3B, Fire et al. 1998). Therefore, the techniques in molecular biology build upon one another and are cumulative.

Cognitive values and the success of the techniques

Cognitive values[11] play an important role in the assessment of theory change in the sciences (Darden 1991; Douglas 2013; Kuhn 1977). Here I identify three cognitive values that are important for the scientific community's adoption of a technique. First, the technique needs to be fruitful for further research. Techniques need to generate new knowledge and open up areas of research that were previously unimaginable. For example, RNAi has helped biologists manipulate RNA thus leading to a more sophisticated understanding of the function of RNA (Mello and Conte 2004) and this has allowed biologists to manipulate genes that are lethal in development in order to investigate their functions (for example, Fitzgerald and Schwarzbauer 1998). Second, the technique should allow expansion of its scope of application far beyond its original biological context. After the effector protein is identified it must either be endogenous to the experimental system (and also conserved in the taxa that will be the experimental system) or be exogenous and able to operate in a range of experimental systems. A technique that has applications in many contexts means a larger scientific community can use the technique. In addition, a technique that can be used in mammals is particularly desired due to the value placed on medical and therapeutic research. For example, the RNAi effector, RISC, is present in all eukaryotes (Cerutti and Casas-Mollano 2006) and RNAi can be used in human cell lines (Elbashir et al. 2001a). Third, the technique needs to have extendability. The technique should accommodate modifications so that it can be used for different or expanded capabilities. Therefore, a technique can become the progenitor for a family of related techniques. For example, a form of RNAi was developed that used RNA molecules targeted at promoters to increase rather than decrease gene expression (Li et al. 2006). It is important to note that whether a technique rates highly on these three cognitive values it can only be identified in hindsight as that judgment is based on the employment of the technique (Darden 1991; Douglas 2013). The three cognitive values I have identified do not compete with one another as similar theoretical values do (Darden 1991) - a technique can be fruitful, have broad scope and be extendable at the same time.

Conclusions

A deeper understanding of the characteristics of natural systems and the development of scientific practice is gained by examining how molecular biology techniques are developed by biologists. In this paper I have investigated eight highly successful techniques of contemporary molecular biology that are derived from natural systems. I have argued that the development of

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[11] Otherwise referred to as epistemic values (Douglas 2013).

these techniques falls into four phases. What are the implications of the fact that biologists develop molecular biology techniques from natural systems? Biologists' knowledge about natural systems limits what can be developed as a technique. Molecular biology techniques, and therefore molecular biology knowledge, are contingent. If biologists had discovered different phenomena in natural systems in the past then different techniques would have been developed. Molecular biology knowledge would have been altogether different, although we might speculate that deeply entrenched biological processes that are highly conserved across taxa (for example, the RNAi mechanism) will always be discovered.

It is an open question whether molecular biology will continue to progress through the development of molecular techniques derived from natural systems. Perhaps knowledge construction in molecular biology requires a natural systems strategy. Alternatively, as a relatively immature science that is still discovering its fundamental phenomena, adopting this strategy could be just an immature stage for molecular biology. There is some evidence that biologists working on synthetic biology have started to use rational design in organisms, for example, the high profile 'Human Genome Project–Write' (Boeke et al. 2016). However, biologists often find that rational design is laborious and that selection methods lead to improved technique development and outcomes (Silverman 2003). Furthermore, a rational design strategy cannot be used to access the causal structure of molecular mechanisms when no comprehensive understanding of these mechanisms exists.

What makes molecular biology such a unique area of the biological sciences is perhaps the fact that its scientific practice is based on a collection of research tools (Burian 1993). I therefore suggest that molecular biology is a historically accumulated set of techniques to manipulate, intervene on, and trace biological processes. Further, a biologist's explanation of a molecular mechanism is dependent upon the molecular biology techniques they use to investigate the mechanism (Trujillo et al. 2015). In molecular biology, even more than in other areas of science, the development of technological capabilities and scientific knowledge are inextricably linked.

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