microRNA-based cancer cell reprogramming technology (Review)

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Abstract. Epigenetic modifications play crucial roles in cancer initiation and development. Complete reprogramming can be achieved through the introduction of defined biological factors such as Oct4, Sox2, Klf4, and cMyc into mouse and human fibroblasts. Introduction of these transcription factors resulted in the modification of malignant phenotype behavior. Recent studies have shown that human and mouse somatic cells can be reprogrammed to become induced pluripotent stem cells using forced expression of microRNAs, which completely eliminates the need for ectopic protein expression. Considering the usefulness of RNA molecules, microRNA-based reprogramming technology may have future applications in regenerative and cancer medicine.

Contents

1. Introduction

MicroRNAs (miRNAs) modulate mRNA expression through base pairing between seed sequences in miRNA and complementary sequences within the open reading frame or an untranslated region of the target mRNA, thereby destabilizing mRNA and/or inhibiting protein synthesis (1,2). miRNAs play crucial roles in developmental biology (3,4), stress response (5,6), stem cell physiology (3,4,7), and diseases such as cancer (8-12).

2. Complete reprogramming

Introduction of the transcription factor MyoD into a muscle lineage caused differentiated cells to become myoblasts (13), indicating that single defined biological factors can successfully execute reprogramming of differentiated cells. Three cDNAs, including MyoD, were identified by screening a myocyte cDNA library with proliferating myoblast-specific subtracted cDNA probes. The MyoD cDNA contained a short protein segment similar to an amino acid sequence present in the myc protein family. MyoD cDNA was then transfected into fibroblasts, where it is not normally expressed (mouse C3H10T1/2, NIH3T3, Swiss 3T3, and L cells), and this was sufficient to convert these cells into stable myoblasts. Furthermore, myogenesis occurs to a lesser extent when MyoD is expressed in other lineage-committed adipoblasts.

The hypothesis that one or few transcription factors are sufficient for lineage conversion of terminally differentiated cells is supported by a study showing that C/EBP overexpression can lead to a stepwise reprogramming of B cells, thereby causing them to become macrophages (14). Initial C/EBP expression in B cells inhibits the B-cell commitment transcription factor Pax5, leading to downregulation of its target CD19. This increases the activity of PU.1, an ETS family transcription factor, which results in the upregulation of its target gene Mac-1 and other myeloid markers. This ultimately leads to the conversion of B cells to macrophages (15). Furthermore, three transcription factors, *Ngn3* (also known as *Neurog3*), *Pdx1*, and *Mafa*, are capable of reprogramming differentiated pancreatic exocrine cells in adult mice into cells that closely resemble β-cells. These and several other studies (16,17) clearly indicate that defined biological factors are sufficient for complete lineage conversion of differentiated cells. Lineage conversion, i.e., direct cell reprogramming without reversion to a pluripotent stem cell (PSC) state, is indeed a major step, but the main aim of reprogramming studies is the successful generation of PSCs from differentiated cells. Understanding the mechanism responsible for the successful conversion of adult cells into other cell types would be beneficial for tissue repair, regeneration, and cancer therapy.

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Takahashi and Yamanaka reported that complete reprogramming can be achieved by the introduction of defined biological factors such as Oct4 (also known as Pou5f1), Sox2, Klf4, and cMyc into mouse (18) and human fibroblasts (19,20). This is a major breakthrough considering the ethical issues related to the use of fertilized oocytes for the establishment and production of embryonic stem (ES) cells and the immunological incompatibility that occurs when unrelated individuals act as donor sources.

3. Reprogramming-like phenomenon in cancer cells

Genetic and epigenetic modifications are the hallmarks of cancer (21), but their roles in the determination of biological behavior have not been completely elucidated (22,23). Considering that cancer initiation and development is based on genetic information, the uncontrolled behavior of cancer cells is probably due to the combined deregulation of genetic and epigenetic programs (23). In tumor cells, genes can be controlled by epigenetic modifications (24) and underlying genomic mutations that lead to reversible and irreversible changes, thereby resulting in activation of oncogenes and inactivation of tumor-suppressor genes (21,23,25-27). In other words, modifications in genetic information, such as double-strand breaks and fusions, can cause irreversible and stable changes in a cell (28), whereas epigenetic controls exert reversible and transient effects on cell behavior (23,29). Tumor development is a complex process driven by active (driver) and passive (passenger) mutations (30). The resultant tumors generally comprise heterogeneous tissues with tumor cell characteristics. It is generally considered that molecular mutations can lead to growth advantages during heterogeneous tumor formations, which ultimately increases tumor size. Furthermore, anti-apoptotic survival signals also support tumor development (31). The understanding of the involvement of changes in differentiation during tumor development remains somewhat elusive (23).

Recent studies indicate that aggressive tumors are associated with the preferential expression of ES-expressing genes, which suggests that common mechanisms are involved in the regulation of cancer stem cells (CSCs) and ES cells. Epigenetic regulating agents or events (including cell fusion and several epigenetic agents for DNA demethylation and histone acetylation) may reverse epigenetic information to normal biological characteristics in malignant tumors. A recent study that investigated the overexpression of the ES-expressing transcription factor indicated a modification in the epigenetic status and cancer cell behavior, after which the cells mimicked induced PSC (iPSC) behavior (32). Taken together, it is suggested that the viral-mediated transfection of transcription factors resulted in marked modification of epigenetic alterations in cancer cells due to its high magnitude of exogenous gene expression, whereas somewhat distinct alterations may develop in *de novo* cancer in terms of epigenetic net works, i.e., the activation of growth-promoting oncogenes and inactivation of tumor-suppressor genes. This suggests a possible experimental approach for using a reprogramming-like event in cancer research.

Cell reprogramming events observed in normal cells should be distinguished from reprogramming-like events observed in cancer cells. i) Numerous studies have suggested that the genetic and epigenetic pathways in cancer cells differ from those in normal cells. Although some pathways may appear to be common, recent studies have indicated that only aggressive tumors are associated with the preferential expression of ES-expressing genes. ii) Cancer cells involve irreversible genetic changes that can be controlled by a one-way irreversible program; however, normal cells largely exert their differentiation program via reversible epigenetic modifications. For example, pancreatic PDX-1 was expressed transiently in pancreatic somatic stem or progenitor cells, whereas it was not expressed in differentiated endocrine or pancreatic cancer cells (33). iii) The introduction of four defined transcription factors, Oct4, Sox2, Klf4, and cMyc, resulted in complete reprogramming of mouse (18) and human fibroblasts (19). The introduction of the same four transcription factors into cancer cells induced the expression of genes related to an undifferentiated status and showed multipotentiality by differentiating into three germ layers, although they did not form a teratoma, a hallmark of pluripotency. This suggests that the induced pluripotency program may be partially altered by genetic mutations in cancer cells or that the cancer program initiated in normal multipotent cells may be different from the pluripotency program in normal cells.

4. Investigation of other factors

Reprogramming factors such as Oct3, Sox2, Klf4, and c-Myc were identified on the basis of their differential expression in fibroblasts and ES cells (18). However, like previous studies (34,35), future studies may elucidate the role of other new important factors in cellular reprogramming that may contribute considerably to the development of safe and effective clinical therapeutic options. Recent studies using an expression library of transcription factors expressed by unfertilized oocytes and fertilized one-cell zygotes have identified a novel maternal transcription factor, Gli-like transcription factor (Glis1; Glis family zinc finger 1). Glis1 considerably enhances the generation of iPSCs from mouse and human fibroblasts, when it is expressed together with Oct4/Sox2/Klf4 (36). Glis1 effectively promotes multiple reprogramming pathways, including Myc, Nanog, Lin28, Wnt, Essrb, and the EMT related-pathways, thereby indicating the usefulness of Glis1 in effectively promoting the direct reprogramming of somatic cells during the generation of iPSC (36).

Small non-coding RNAs such as miRNAs, endogenous small interfering RNAs (endo-siRNAs), and Piwi-interacting RNAs (piRNAs) (37-39), have different but essential roles in mammalian development (40). Differences among the small non-coding RNA types can be determined using numerous properties, such as the size of endo-siRNAs (21 nucleotides, nt), which is distinct from that of piRNAs (31 nt). The sequence of an endosiRNA is completely complementary to its target transcripts, whereas that of an miRNA is usually only partially complementary to its target (37-39). Mature miRNAs are derived from precursor miRNAs that possess short stem-loop structures, whereas endo-siRNAs are processed from long dsRNAs without short stem-loop structures (37-39). It is

Table I. Summary of published studies concerning normal somatic cell reprogramming.

Method for factor delivery	Starting material	Efficiency	Reference
Retrovirus vector, OSKM	Mouse embryonic and adult fibroblasts	n.d.	(18)
Retrovirus vector, OSKM	Human fibroblasts	10 colonies/5x10^4	(19)
Lentivirus, OSNL	Human fetal fibroblasts	198 colonies/0.9x10^6	(73)
Plasmid transfection, OSKM	MEF	Lower than viral delivery method	(74)
Adenovirus (non-integrating vector), OSKM	MEF and hepatocytes	n.d.	(75)
Retrovirus, OSKM	Adult mouse liver and stomach cells	n.d.	(76)
Retrovirus, O(SKM)	Mouse neural stem cells	3.6-0.11%	(77)
Retrovirus, OSK(M)+VPA/5'Aza	MEF	0.5-11.8%, 100x higher than OSKM method	(78)
Dox-inducible lentiviruses, OSKM	Secondary somatic cells containing Dox-inducible OSKM expression (MEF, intestinal epithelium)	20-50x higher than direct infection method	(79)
Retrovirus, OSKM	Adult human adipose stem cells	0.2%	(80)
Repeated protein transduction, OSK(M)+VPA	MEF	Slower kinetics than viral delivery method	(81)
Retrovirus, OK+BIX/ BayK compounds	Neural progenitor cells	12 colonies/3.5x10^4	(82)
Doxycycline-inducible, OSKM transcription factors delivered by piggyBac transposition	Murine and human embryonic fibroblasts	n.d.	(83)
Retrovirus, OSK, miR-291-3p, miR-294 and miR-295	MEF	0.1-0.3%	(84)
Nucleofection, 2A-peptide linked cassette, OSKM	MEF	2.5%	(85)
Sendai virus, OSKM	Human terminally differentiated circulating T cells	0.1%	(86)
Lentivirus, Oct4 + compounds	Neonatal human epidermal keratinocytes, small human umbilical vein endothelial cell and amniotic fluid-derived cell	4-6 colonies/1x10^6	(87)
Repeated transfection of synthetic modified mRNAs (OSKM)	Primary human neonatal epidermal keratinocytes, BJ human neonatal foreskin fibroblasts, human fetal lung fibroblasts and human fetal skin fibroblasts	1.4%, 36-fold higher than retrovirus	(88)
Lentivirus, miRNA 302s and 367, VPA	MEF, human fibroblasts	Faster kinetic efficiency, 2x higher than OSKM, efficiency 10000x higher	(46)
Repeated transfection & miR-302s, -369-3p, -369-5p, and -200c	Human and mouse adipose stromal cells, dermal fibroblasts	5 colonies/5x10^4	(47)
Retrovirus, OSKM, miR-106b, -93, -106a and -17	MEF	Addition of miRNA enhanced efficiency 4- to 6-fold (miR-106b, 93) and 3 to 4-fold (miR-106a,17)	(89)
Retrovirus, OSK(M), miR-302b, -372 and -294	Human fibroblasts	Addition of miRNA enhanced efficiency by promoting MET	(90)

O, Oct4; S, Sox2; K, Klf4; M, c-myc; L, Lin28; N, Nanog; n.d., not determined.

Table II. Summary of published studies concerning cancer cell reprogramming.

Method	Type of cancer	Characterization	Reference
Nuclear transfer	Medulloblastoma (Primary culture, mouse, Ptc1 heterozygous)	Cloned blastocyst can support postimplantation development, as the embryo appeared normal and showed extensive differentiation, although not viable after E8.5.	(91)
Nuclear transfer (2-step cloning)	Melanoma RAS^{+}/Ink4a/$Arf^{-/-}$	NT ES cells were able to form a teratoma and generate a chimera. Injection into tetraploid blastocyst resulted in normal embryo, viable until E9.5.	(92)
Nuclear transfer	Embryonal carcinoma	Nuclei from embryonal carcinoma can direct preimplantation development, resulting in normal blastocyst appearance. Higher efficiency when producing an ESC line compared to differentiated cells, although the degree of differentiation depended on the cell line character.	(93)
Embryonic micro-environment	Metastatic melanoma, breast cancer	n.d.	(94)
microRNA (miR 302a, b, c, and d)	Melanoma (Colo), prostate	Expression of pluripotency markers: Nanog, Oct4, Sox2, SSEA3, and SSEA4, demethylation of Oct4, cancer cell line (PC3) teratoma (+)	(48)
Defined transcription factor (OKM)	Melanoma (R545)	Teratoma (+), chimera (+), expression of ESC marker, demethylation of Nanog and Oct4	(95)
Defined transcription factor (OSKM)	Gastrointestinal cancer (colon, liver, pancreatic) DLD-1 completely characterized	Expression of pluripotency marker, demethylation of Nanog, *in vitro* differentiation into adipocyte, epithelial, mesenchymal, and neural lineage, teratoma (-)	(32)
Defined transcription factor (OSKM)	KBM7 cells derived from blast crisis stage chronic myeloid leukemia	Expression of ESC marker (+), demethylation of Oct4 and nanog, teratoma (+)	(96)
Defined transcription factor (OSLN)	A549 lung cancer	Demethylation of Oct4 promoter, expressed endogenous Nanog and Oct4 although lower than HESC, ALP (+), teratoma (-) Reprogramming efficiency was higher than that of normal primary lung fibroblast	(97)
Oocyte extract	Breast cancer (cell line, MCF7, HCC1945)	n.d.	(98)

O, Oct4; S, Sox2; K, Klf4; M, c-myc; L, Lin28; N, Nanog; n.d., not determined.

generally accepted that piRNAs are expressed during male gametogenesis where they play a crucial role, whereas endo-siRNAs are essential during oocyte meiosis (37-40). In contrast, miRNAs are ubiquitously expressed in somatic tissues and function throughout postimplantation development (37-40).

Recent studies indicate that small non-coding RNAs undergo a complex, but finely tuned regulation in the early stages of cell reprogramming, i.e., during transition from a differentiated oocyte to a pluripotent blastomere. piRNAs and endo-siRNAs correspond to mRNAs or retrotransposons in growing oocytes (41). In oocytes, piRNAs are bound to Mili and regulate retrotransposons, while siRNAs are exclusively mapped to retrotransposons or other genomic regions that form dsRNA structures (41). The components of the piRNA pathway are also required for *de novo* methylation of the differentially methylated region (DMR) of the imprinted mouse Rasgrf1 locus, but not other paternally imprinted loci. A retrotransposon sequence within a non-coding RNA region that spans DMR was targeted by piRNAs generated from a different locus (42). Male germ cells express abundant endogenous siRNAs that can potentially target hundreds of transcripts and/or thousands of DNA regions in the genome, including retrotransposon sequences. This suggests the possibility of complex uncharacterized regulation imposed by small non-coding RNAs during male germ cell development (43). Interestingly, miRNAs were shown to be non-essential during preimplantation embryonic development and their function was suppressed during oocyte meiosis. Furthermore, endo-siRNAs, rather than miRNAs, play a role during oocyte maturation and preimplantation development (40,44,45). The introduction of miRNAs is sufficient to induce a pluripotent phenotype in differentiated somatic cells (46,47) without the addition of transcription factors. miRNA-induced reprogramming is probably related to the alteration of aggressive cancer phenotypes (32,48,49). Thus, these studies suggest that a fraction of endogenous siRNAs are involved in oocyte reprogramming. Future studies are required to search for endogenous siRNAs in meiotic oocytes, mitotic zygotes, and early embryos. A detailed understanding of these processes may lead to small non-coding RNAs proving beneficial in altering cancerous phenotypes.

Retrotransposon-type repetitive sequences are involved in cancer chromosome translocation. These repetitive sequences are characteristic genomic sequences in fragile sites commonly found in various chromosomes, including *FRA3B* and *FRA16D* (50-54). Rare fragile sites are often associated with particular sequences (55). Fragile sites often coincide with genes that are frequently rearranged or deleted in human cancers. More than half of cancer-specific translocations contain breakpoints within fragile sites (56,57). Replication stress is usually caused by carcinogen exposure or other cancer-induction events, and the ataxia-telangiectasia-related (Atr) DNA damage checkpoint pathway plays a crucial role in maintaining genomic stability at fragile chromosome sites (58). Recent findings have confirmed that Atr protein binds to three regions of FRA3B during mild replication stress (57). During dysplasia, loss of Fhit expression leads to a direct and significant influence on changes in checkpoint proteins, suggesting a connection between Fhit loss and modulation of checkpoint activity (57,59). In cancer, changes in miRNA expression are frequently associated with changes at fragile chromosome sites (60). Interestingly, the DNA damage-susceptible *FRA3B*/*FHIT* chromosome fragile site encodes a protein required for protecting cells from accumulated DNA damage through its ability to modulate the checkpoint proteins Atr and Chk1, whereas inactivation of Fhit contributes to the accumulation of abnormal checkpoint phenotypes during cancer development (61-63). The absence of Fhit protein in stem cells, a reduction in oxidative stress, and efficient but not error-free DNA damage repair, facilitate the unscheduled long-term survival of genotoxin-exposed Fhit-deficient hematopoietic stem cells that carry deleterious mutations (64). Thus, the small noncoding RNAs are nucleotides that can affect cancer risk. The catalog of known miRNAs and a large fraction of genomic ultraconserved regions (UCRs) that encode a particular set of non-coding RNAs located at fragile sites and/or cancer susceptibility loci, the expression of which is altered in human cancers, was recently updated (65). These types of studies are the first step toward discovering novel approaches for cancer therapies (65). The next logical research step would be to study the relationship between UCRs, the stemness-regulating region of CSCs, and the therapeutic control of CSCs and EMT (66,67). The regulation of cancer phenotypes by small noncoding RNAs has emerged as an important component of pharmacogenomics, and one day it may be used as an efficient therapeutic approach for cancer (68-70).

5. Perspectives

The regulatory mechanism of cell differentiation in cancer is unclear, and it is debatable whether cancer cells can actually be reprogrammed. The cell of origin for cancer remains unknown, and hence, the underlying mechanism controlling initial programming during cancer remains elusive (32,48,71,72). Epigenetic modifications are involved in the aggressive behavior of cancer cells; therefore, utilizing these modifications for therapeutic purposes may be challenging.

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