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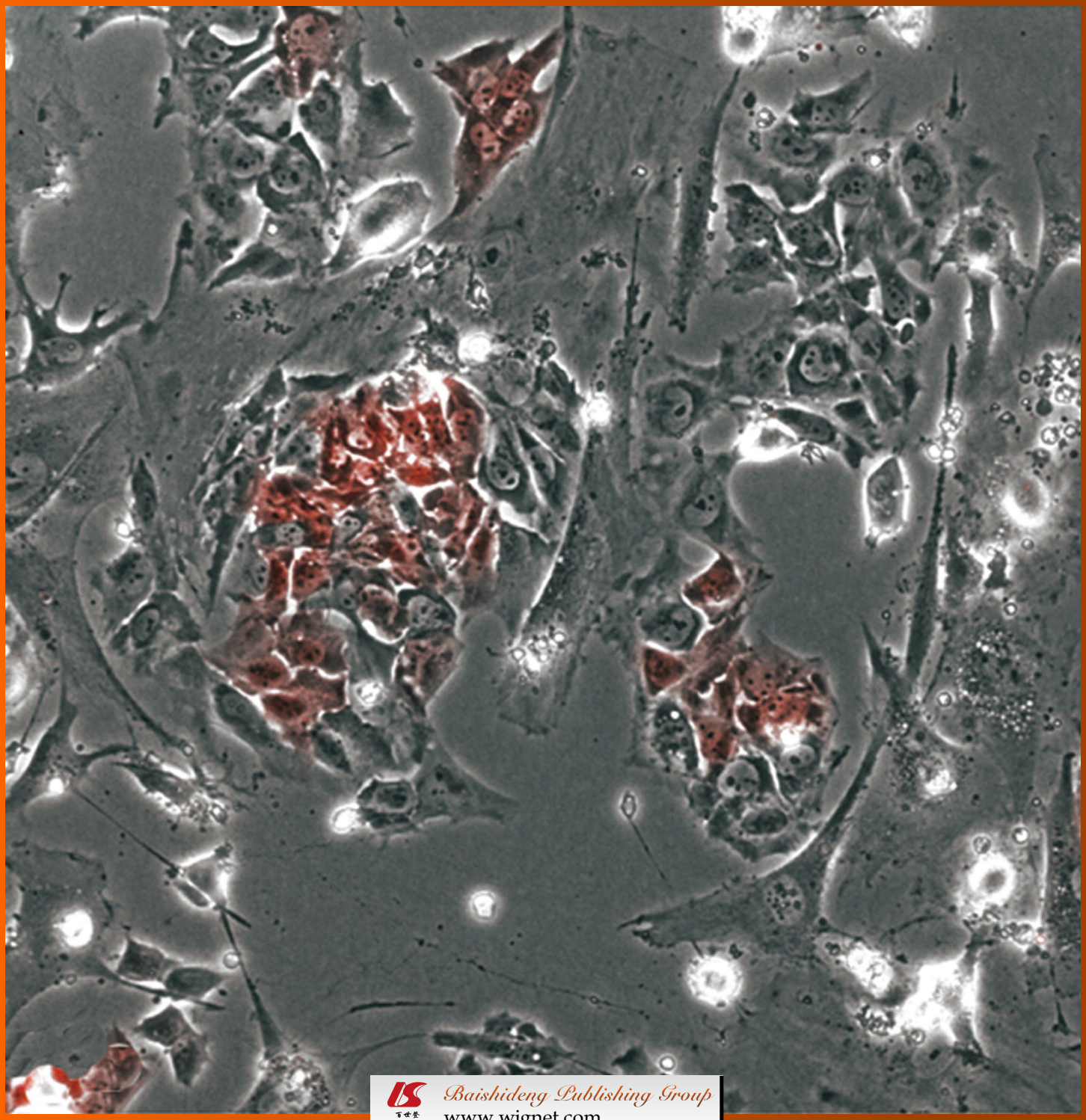

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EDITORIAL

MicroRNAs, stem cells and cancer stem cells

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Abstract

This review discusses the various regulatory characteristics of microRNAs that are capable of generating widespread changes in gene expression *via* post translational repression of many mRNA targets and control self-renewal, differentiation and division of cells. It controls the stem cell functions by controlling a wide range of pathological and physiological processes, including development, differentiation, cellular proliferation, programmed cell death, oncogenesis and metastasis. Through either mRNA cleavage or translational repression, miRNAs alter the expression of their cognate target genes; thereby modulating cellular pathways that affect the normal functions of stem cells, turning them into cancer stem cells, a likely cause of relapse in cancer patients. This present review further emphasizes the recent discoveries on the functional analysis of miRNAs in cancer metastasis and implications on miRNA based therapy using miRNA replacement or anti-miRNA technologies in specific cancer stem cells that are required to establish their efficacy in controlling tumorigenic potential and safe therapeutics.

Key words: Stem cell functions; Cancer stem cells; Cellular pathways; miRNA; oncomiR; Tumor suppressor miRNAs; miRNA based therapeutics

INTRODUCTION

Stem cells, a pool of precursor cells, exist in an undifferentiated state and have exclusive capability to self-renew over an extended period of time and undergo asymmetrical division which promotes healthy growth in normal cells due to polarity involved in cell division. One of the daughter cells retains stem cell properties while another becomes the committed progenitor called a transit amplifying cell and differentiates into a variety of cells that contribute to organ formation and function[1].

Stem cells are classified into two major classes: embryonic stem cells (ESCs) and adult stem cells. ESCs can be isolated at the blastocyst stage from the embryo, are pluripotent and induce lineage specific differentiation in cell culture. Adult stem cells are multipotent, have a tissue specific role in growth and maintenance in adult tissues and can produce only a limited number of differentiated cell types *in vivo*. The role of stem cells in tissue growth, homeostasis and repair in many organ systems make it an important therapeutic tool in the treatment of many human diseases[2].

The stem cell properties, including proliferation, self-renewal and differentiation, are controlled by a complex network of extrinsic and intrinsic signaling pathways. Dysfunction of these regulators can adversely affect the normal functions of stem cells and may either result in the loss of tissue homeostasis or cancer. Following ge-

nomic stress, appropriate DNA repair pathways, including mismatch repair, O^6-alkylguanine DNA alkyltransferase repair, nucleotide excision repair, base excision repair, non-homologous DNA end-joining repair, and homologous recombination repair, are activated in order to maintain the genomic integrity. However, in the absence of DNA repair, cellular responses are activated to induce apoptosis and remove damaged cells from the organ as a part of a defense mechanism.

This review briefly focuses on the critical functions of microRNAs as regulators of post transcriptional gene expression that play a vital role, not only in maintaining the normal stem cell functions, but they also may modulate various signaling pathways that may turn stem cells into cancer stem cells with extensive self-renewal potential and aberrant differentiation. Recently, culture as well as *in vivo* studies in animal models with human cancers have shown the significance of miRNAs in modulating the expression level of responsive proteins by target mRNA cleavage and translational repression *via* the RNA interference (RNAi) pathway in the potential elimination of cancer stem cells.

MicroRNAs

MicroRNAs are the regulators of gene expression in many biological processes, including development, proliferation, apoptosis, stress response and fat metabolism. These newly discovered classes of molecules are 21-23 nucleotide short non coding RNA sequences, many of them are evolutionary conserved among distantly related organisms and may be expressed in a tissue-specific or developmental stage-specific manner. They are normally expressed as polycistronic transcripts and play an important role in various fundamental biological processes, such as cell cycle, cell growth and differentiation, apoptosis and embryo development, and cardiac and immune system function *via* regulating mRNA functions at post transcriptional as well as post translational level[3].

MicroRNAs were discovered in 1993 during a study of the gene *lin-14* in *Caenorhabditis elegans* (*C. elegans*) development, where partial binding of 61 nucleotide precursor from lin-4 gene matured to a 22 nucleotide to complementary sequences in the 3' UTR of the lin-14 and mRNA inhibited the translation of lin-14 mRNA[4]. This is followed by the characterization of second miRNA, lethal-7 (let-7), which repressed lin-41, lin-14, lin-28, lin-42 and daf-12 expression during developmental stage transitions in *C. elegans* in 2000[5]. Computational and experimental evidence provide a recent estimate of around 700 miRNAs hairpin sequences which are currently known to be contained in the publicly accessible miRNA database, miRBase (http://microrna.sanger.ac.uk/)[6]. More than 5300 human genes are supposed to be regulated by miRNA, which accounts for 30% of all the genes and around 60% of protein non coding genes. Many of the miRNAs are conserved between distantly related organisms, suggestive of their important roles in the biological system.

BIOGENESIS OF MicroRNAs

MiRNAs are endogenous and naturally generated within animal cells. They can inhibit the translation of mRNAs bearing the partially complementary target sequences, thus is one of the key components of RNAi within the cells. MiRNAs control various cellular, physiological and developmental processes and their aberrant expression link them with various diseases, including cancer; cardiovascular disease; schizophrenia; renal function disorders; Tourette's syndrome; psoriasis; primary muscular disorders; Fragile-X mental retardation syndrome; chronic hepatitis; polycythemia vera; AIDS; and obesity[7-18]. To better understand the potential role of miRNA as important regulatory molecules in various cellular pathways by negatively controlling the gene and protein expression and their links with cancer, it is important to discuss the miRNA biogenesis pathway (Figure 1).

The biogenesis of miRNA involves multiple processing steps, including transcription, processing, maturation and degradation. MiRNAs are randomly placed in a mammalian genome and found as isolated transcriptional units, co-transcribed as part of other transcriptional units, or clustered together and transcribed as polycistronic primary transcripts. They are either produced from their own genes or from introns. The process begins with the transcription of primary (pri) miRNA transcript, generally by RNA polymerase II, while those with upstream Alu sequences, transfer RNAs, and mammalian wide interspersed repeat promoter units by RNA polymerase Ⅲ[19,20]. Primary miRNA having hundreds or thousands of nucleotides and one or more miRNA stem loops are then capped at 5' and polyadenylated at 3' end[21]. This is followed by the cleavage of pri-miRNA with the enzyme Drosha, RNA Ⅲ endonuclease and a double stranded RNA binding protein, DiGeorge syndrome critical region gene 8 (DGCR8), together form a microprocessor complex or Pasha in invertebrates to form a resulting hairpin of around 70 nucleotides in length, known as a precursor-miRNA (pre-miRNA) which has 5' phosphate and 2 nucleotide 3' overhang[22]. Pre-miRNAs that are spliced directly out of introns are known as miRtrons.

Nucleocytoplasmic shuttle Exportin-5 exports processed pre-miRNA from the nucleus, by a RAs-related Nuclear protein-GTP dependent process[23]. This follows the subsequent cleavage of Pre-miRNA by another RNA Ⅲ endonuclease known as Dicer in cytoplasm in partnership with its TRBP (human immunodeficiency virus transactivating response RNA binding protein), a RNA binding protein to form a final product of 21-23 nucleotide miRNA with 5'phosphates and a 2-nucleotide 3' overhangs and generate two complementary RNA fragments. General inhibition of Drosha-mediated processing of many nuclear pri-miRNAs and Dicer-mediated processing of cytoplasmic pre-miRNA can regulate many important biological mechanisms[24,25]. One of either the strands of the duplex mature miRNAs are incorporated into the members of the argonaute (Ago) protein family,

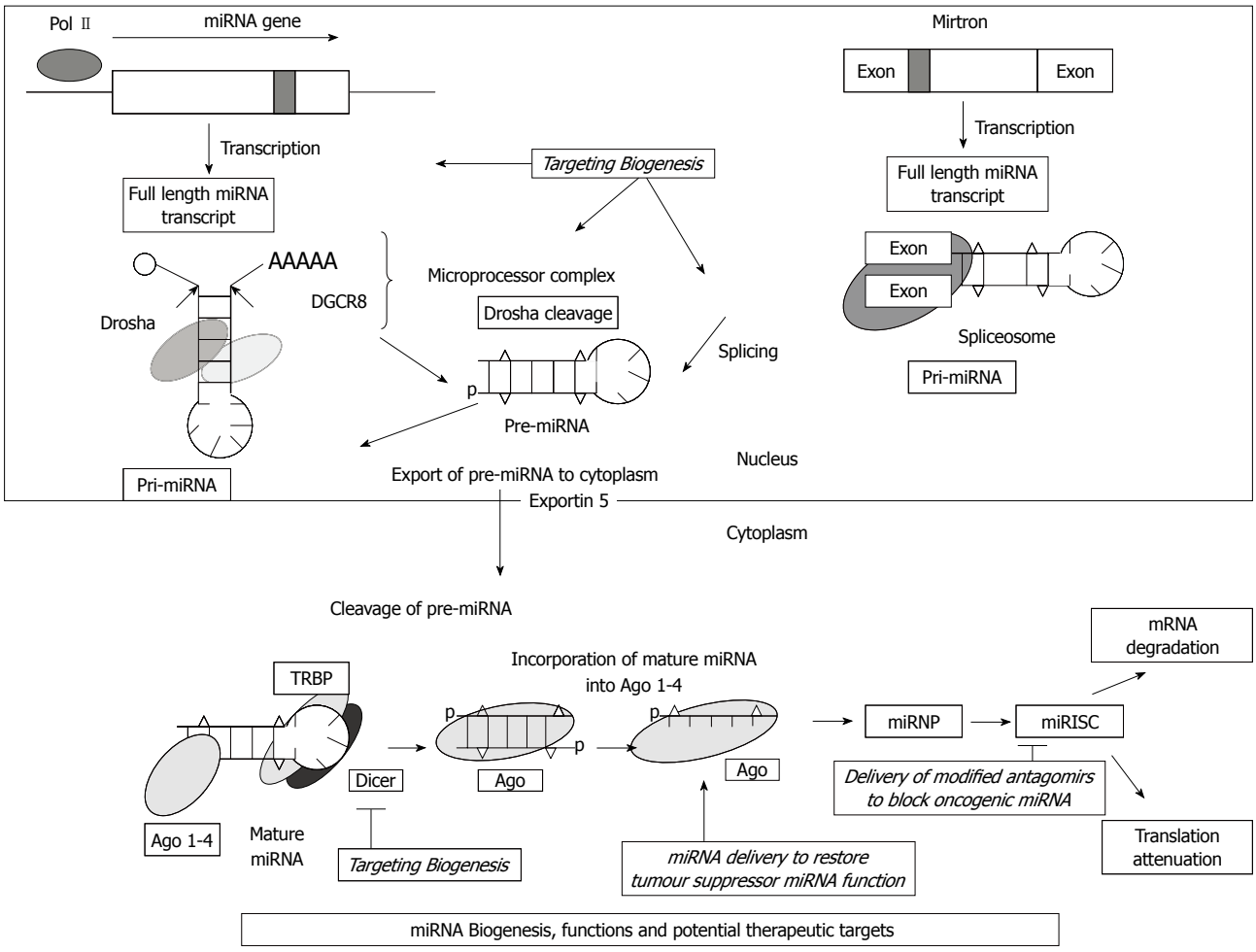

Figure 1 MiRNA biogenesis, functions and potential therapeutic targets. miRNA transcript excised to form pri-miRNA, gets cleaved by Drosha and exported from nucleus to cytoplasm by Exportin-5. 70 n hairpin-loop precursor-miRNA (pre-miRNA) then processed by Dicer into mature RNA. The figure also explains the various potential miRNA therapeutic targets including biogenic pathways, restoring the tumor suppressor functions of miRNAs and blocking the oncogenic properties of miRNAs. miRNA mediated silencing involves either inhibition of translation or degradation of their target mRNA transcripts depending on the degree of complementarity. TRBP: Transactivating response RNA binding protein; miRISC: miRNA-induced silencing complex; miRNP: MicroRNA ribonucleoprotein complex; DGCR8: DiGeorge syndrome critical region gene 8.

Ago 1-4, forming miRNPs (microRNA ribonucleoprotein complex) along with other proteins such as GW182 and known as miRNA-induced silencing complex. Mature miRNAs direct miRNPs to target mRNAs which share complementation with the seed region consisting of nucleotides at positions 2-8 of 5' end of mature miRNA which result in either translational repression or more commonly mRNA degradation[26]. Targeting the regulators involved in the alternative splicing of mRNAs has been shown to upregulate the expression of mRNAs[27,28].

STEM CELLS AND miRNA FUNCTIONS

Differential gene expression under epigenetic, transcriptional, translational and posttranslational control, as well as signaling from neighboring cells, regulates normal stem cell properties. The regulatory miRNA levels are lower in stem cells but their dynamic expression profile in these cells provide evidence of their significance in maintaining the self-renewal, pluripotency and regulating differentiation of their progeny cells (Table 1). miR-15b/miR-16r, miR17-92, miR-21 and the miR-290-295 clusters are the four prominently expressed miRNA clusters in ESCs and are an integral part of their control. Many transcription factors regulated by miRNAs control the pluripotency and differentiation that are the major functions of stem cells. MiRNAs facilitate differentiation in murine ESCs with conditional knockout of Dicer1 and DGCR8 by downregulating the pluripotency markers like Oct4 and Nanog homeobox (Nanog)[29,30]. Directly targeting the transcripts of self-renewing factors, like *Oct4, sex-determining region Y-box containing gene 2* (*Sox2*), *Kruppel-like factor 4* (*KLF4*) with miR-145 and *Nanog*, *liver receptor homologue 1*, *the positive regulators of Oct4 expression*, with miR-34 in human ESCs promote differentiation. Lin-28, marker for pluripotent stem cells, forms a negative feedback loop with the let-7 family miRNAs, whereas let-7 miRNAs in differentiated stem cells target the Lin-28 miRNA[31]. MiR-290 and two other related families, including miR-370 and miR-302 cluster, showed an altered

Table 1 miRNA mediated regulation in the maintenance and function of stem cells

miRNA	Functions in stem cells	Mechanism(s)	Ref.
Pluripotent miRNAs			
miR-290 cluster, miR-370, miR-302	Promotes self-renewal	Regulate embryonic stem cell cycle	[32]
miR-141, miR-200, miR-429	Maintenance of self-renewal in the absence of leukemia inhibitory factor	Regulated by cMyc proteins	[66]
miR-9	Proliferation and promote NSC migration Neurite outgrowth	Target Stmn1, which increases microtubule instability Inhibit Cdc42 expression and altering the localization of Rac1	[67]
miR-184	NSC proliferation	Represses the expression of Numb-like 1	[68]
miR-137	Promotes NSC proliferation but inhibits neuronal maturation, dendritic morphogenesis, and spine development	Target Mind bomb 1, an ubiquitin ligase	[69]
Pro-differentiation miRNAs			
miR-134, miR-145, miR-296, miR-470	Initiate differentiation	Suppress pluripotent markers including Nanog, Oct4, Sox, Klf4	[33]
Let-7	Stabilize differentiation	Target transcripts that are regulated by the pluripotency transcription factors Oct4, Sox2, Nanog and Tcf3	[34]
		Promote somatic cell cycle by targeting both directly and indirectly the multiple activators of the G1-S transition including cdc25a, cdk6, cyclinD1 and cyclinD2	[35-37]
miR-124	NSC differentiation	Suppress Sox9 expression in adult NSCs and exhibit mutual inhibition mechanism of Ephrin-B1	[70]

Let-7: Lethal-7; NSC: Neuronal stem cell; Sox: Sex-determining region Y-box containing gene.

cell cycle profile and disrupt ESC transition from a self-renewing to a differentiated state[32].

The two classes of pro-differentiation miRNAs play an important role in the differentiation process. MiRNAs, including miR-134, miR-145, miR-296 and miR-470, grouped under the first class of miRNAs and they directly suppress the self-renewal state by suppressing Nanog, Pou5f1 (also known as Oct4), KLF4 and Sox2, the markers of pluripotency[33]. The other class of miRNAs include the let-7 family of miRNAs that stabilizes the differentiated cell fate by targeting the transcripts that are regulated by the pluripotency transcription factors Oct4, Sox2, Nanog and Tcf3[34]. In addition, Let-7 also promotes the somatic cell cycle by targeting, both directly and indirectly, the multiple activators of the G1-S transition, including cdc25a, cdk6, cyclinD1 and cyclinD2, thereby making the G1 phase cells most susceptible to pro-differentiation signaling cascades, including MAPK signaling[35-37].

Studies have shown the potential role of miRNAs in different aspects of neuronal development, such as proliferation of neural stem cells (NSCs) and progenitors, neuronal differentiation, maturation and synaptogenesis[38]. Overexpression of miR-124 and miR-137 in undifferentiated NSCs result in morphological changes and expression of markers indicating neuronal differentiation[39]. Trim-NHL proteins, a new class of regulatory RNA binding proteins, act as an ESC expressed E3 ubiquitin ligase that function to degrade Ago2 protein, a component of the RISC complex, and modulate the activity of the entire miRNA pathway and are found to be associated with the differentiation of NSCs[40,41].

MiRNA expression profiles and functional studies explain their importance in stem cell biology; however, detailed investigation will be required to understand the specific role of miRNA for the maintenance and proper function of particular stem cell types.

CANCER STEM CELLS AND miRNA FUNCTIONS

Failure to repair errors in stem cells result in the accumulation of epigenetic abnormalities, initiate the signaling cascades that support tumorigenesis, allow the cells to escape the restrictions of its niche and transform them into cancer stem cells. These cells are structurally and functionally distinct from other cells within the tumor mass and are capable of self-renewing mitosis where one of the daughter cells functions as a stem cell while other becomes a progenitor cell[42]. Cancer stem cells are characterized by cell surface marker profiles, form tumorospheres and have increased resistance to chemo- and radio-therapeutic agents, a likely cause of cancer relapse in patients. Cancer stem cells have been isolated for hematological malignancies, mainly acute myelogenous leukemia, chronic myeloid leukemia, acute lymphoblastic leukemia (ALL), multiple myeloma and solid tumor organs of breast, brain, lung, prostate, testis, ovary, stomach, colon, skin, liver and pancreas[43]. Increased resistance to anti-cancer therapeutics, limitless proliferative capacity, aberrant differentiation and multidrug resistance trait associated with the overexpression of genes that code for transmembrane efflux pump proteins are the innate properties of cancer stem cells that offers a great challenge in long term remission[44].

Several profiling studies have determined potential implications of high percentage of miRNAs in cancer

Table 2 Aberrant miRNA expression in cancer stem cell

miRNA	Tumor type	Mechanism(s)	Ref.
miRNA as oncomiR			
miR-17-92 polycistron	Upregulated in lung, breast, stomach, prostate, colon and pancreatic cancers	Regulate c-Myc expression	[46,47]
miR-21, miR-205	Head and neck cancer	Target transcripts of tumor suppressive genes including kinesin family member 1B isoform α, hypermethylated in cancer 2, and pleomorphic adenoma gene 1	[71]
miR-372, miR-373	Testicular germ cells	Neutralize p53-mediated CDK inhibition, possibly through direct inhibition of the expression of the tumor-suppressor LATS2	[72]
miR-21	Breast cancer	Target tumor suppressor tropomyosin 1	[73]
miR-126	Gastric carcinoma	Targets SOX2, and PLAC1	[48]
Let-7	Hepatocellular carcinoma	Targets SOCS1, caspase-3	[56]
miR-181	Hepatocellular carcinoma	Targets RASSF1A, TIMP3 as well as nemo-like kinase	[56]
miR-495	Breast cancer	Modulated by transcription factor E12/E47, suppresses E-cadherin expression to promote cell invasion and inhibits regulated in development and DNA damage responses 1 expression to enhance cell proliferation in hypoxia through post-transcriptional mechanism	[74]
miRNAs as tumor suppressors			
Let-7	Colon adenocarcinomas	Target Lin-28b which promotes cell migration, invasion and transforms immortalized colonic epithelial cells	[50]
miR-15 miR-16 cluster	Chronic lymphocytic leukemia	Targets the apoptotic inhibitor Bcl-2	[47]
miR-29	Cholangiocarcinoma	Regulate the anti-apoptotic protein Mcl-1	[75]
miR200c	Head and neck squamous cell carcinoma	Negatively modulates the expression of BMI1 and ZEB1	[62]
miR-125b	Glioma	Decreases the cell cycle regulated proteins CDK6 and CDC25A	[76]

Let-7: Lethal-7; SOX2: Sex-determining region Y-box 2; PLAC1: Placenta-specific 1 gene.

due to its close proximity to chromosomal breakpoints; cancer associated genomic regions and/or fragile sites and dysregulated expression levels in many malignancies. Multiple functional studies on miRNAs using various algorithms and statistical methods validate their involvement, functions, characteristics, correlations and associations with cancer through targeting proto-oncogenes or tumor suppressor genes (Table 2)[45].

MiRNAs differentially regulate the key properties of cancer stem cells, including cell-cycle exit and differentiation, prosurvival and antistress mechanisms (e.g., resistance to anoikis) and epithelial-mesenchymal transitions (EMT), migration and invasion, which contribute to enhanced tumor initiation and metastatic potential (Figure 2). miR-17-92 polycistron has been reported as the first onco-miR that accelerates tumor development in lung, breast, stomach, prostate, colon and pancreatic cancers by regulating c-Myc expression[46,47]. MiR-126 mediated inhibition of sex-determining region Y-box 2 (SOX2) [SOX2, a crucial transcription factor for the maintenance of ESC pluripotency and the determination of cell fate] and placenta-specific 1 gene may contribute to gastric carcinogenesis[48]. Increased expression of 2 miRNA clusters, 106a-363 and in particular 302-367 in mouse fibroblasts, positively regulate the mesenchymal-to-epithelial transition, cell cycle and epigenetic functions and could allow potent increases in induced pluripotent stem cell generation efficiency[49].

The first functional evidence of tumor suppressive miRNAs was the miR-15/miR-16 cluster, located in a genomic region of chromosome 13 and often deleted in chronic lymphocytic leukemias (CLLs). These miRNAs are not expressed in CLLs but play an oncogenic role by accumulating oncogenic targets, the apoptotic inhibitor Bcl-2[47]. Lin-28 represses biogenesis of let-7 microRNAs and its overexpression has been correlated with reduced patient survival and increased probability of tumor recurrence in human colon adenocarcinomas[50].

In a systematic miRNA expression profiling analysis in human ALL patients, 77 miRNAs were up-regulated and 67 miRNAs were down-regulated in the patient group when compared to the control group with fold changes > 2.0. Among differentially expressed miRNAs, miR-9, miR-181a and miR-128 were of high significance, whereas miR-582-5p, miR-223, miR-143, miR-126, *etc.* displayed the least significance in patients[51]. Shimono *et al*[52] identified 37 differentially expressed miRNAs in $CD44^{+}CD24^{-/lo}$ breast cancer stem cells (BCSCs) and among these miR-200c-141, miR-200b-200a-429 and miR-183-96-182 clusters were significantly downregulated.

Knowing the functional role of miRNAs in a specific tumor, therapies can be targeted to cancer stem cells in order to correct their aberrant expression levels. miRNA based therapeutics aim to potentially reverse the tumorigenic properties of cancer stem cells by targeting its biogenesis pathways, restoring the tumor suppressor functions of and/or blocking the oncogenic properties of miRNAs *via* the RNAi pathway.

THERAPEUTIC IMPLICATIONS

Dysregulated miRNAs *via* modulating cancer stem cell properties are highly associated with tumor initiation,

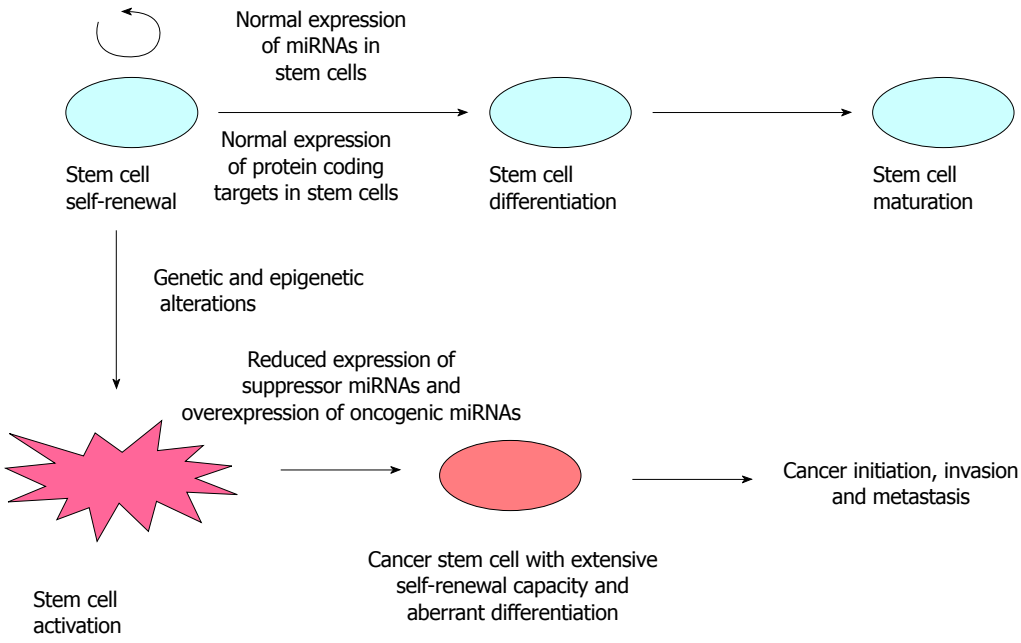

Figure 2 Stem cells express a unique set of miRNAs that maintain self-renewal, promote differentiation and maturation through various regulatory mechanisms. Distinct small sub population of cells arises from stem cells due to accumulation of genetic and epigenetic abnormalities that might function as cancer stem cells. These cells display differential expression of miRNAs which regulate the fundamental properties that contribute to enhanced tumor initiation and metastatic potential.

tumor maintenance, metastasis and therapy resistance. Studies have shown the potential implications of miRNA based therapeutics as a novel strategy to target therapy-resistant cancer stem cells. miRNAs identified as oncogenic that promote cancer, when targeted by locally administered antagomiRs, and miRNAs recognized as tumor suppressors can be downregulated using an appropriate viral vector system could eliminate the cancer stem cells significantly. Lack of tumor specificity and low transfection efficiency associated with the *in vivo* systemic delivery of pharmaceutical formulations of functional miRNA and/or its antagonists to tumor cells *via* non viral mediated gene transfer limits their use[53,54]. Among the current approaches of gene delivery, systemic administration of miRNA using adeno associated viral vectors, not only minimizes the risk of vector-related toxicities, but also increases gene transfer efficiency, could be a successful strategy[55].

Inhibition of let-7 results in the increased chemosensitivity of hepatocellular cancer stem cells (HSCs) to sorafenib and doxorubicin, while silencing of miR-181 leads to reduction in HSCs motility and invasion by controlling the aberrant expressions of cytokine IL-6 and transcription factor Twist[56]. Induction of the tumor-suppressive miRNAs let-7a and miRNA-96 and suppression of the TGFÎ²-induced oncogenic miRNA-181a in BCSCs epigenetically preserve the differentiated phenotype of mammary epithelium and prevent EMT-related cancer-initiating cell self-renewal[57]. Downregulation of miR-125b-2 expression in glioblastoma multiforme (GBM) derived stem cells could allow temozolomide, a chemotherapeutic agent, to induce apoptosis by increasing the cytochrome c release from mitochondria, induction of Apaf-1, activation of caspase-3, poly-ADP-ribose polymerase and proapoptotic protein Bax while decreasing the expression of Bcl-2[58]. Specific inhibition of miR-21 by an anti-miR-21 locked nucleic acid modulates its upstream regulator activator protein-1, composed of c-Jun and c-Fos family transcription factors and tumor suppressor programmed cell death 4, and thereby increases drug sensitivity of cancer stem cells to anticancer drugs[59].

Forced expression of miR-124 and miR-137 in human derived GBM-derived stem cells leads to loss of their self-renewal and oncogenic capacity, leaving normal stem and precursor cells unharmed[39]. Overexpression of miR-128 significantly blocked glioma CSC self-renewal by directly targeting BMI-1 and caused a decrease in histone methylation [H3K27me(3)] and Akt phosphorylation, and up-regulation of p21(CIP1) levels, whereas transfection of GBM cancer stem cells with miR-34a could cause cell-cycle arrest or apoptosis, inhibit xenograft growth, and mediated by downregulation of multiple oncogenic targets, including c-MET, Notch-1/2 and CDK6[60,61]. In another study, miR145 (a tumor-suppressive miRNA) has been studied as a negative regulator of GBM tumorigenesis by targeting Oct4 and Sox2 in GBM-CD133(+). miR 145 delivery, using polyurethane-short branch polyethylenimine as a therapeutic-delivery vehicle, to GBM-CD133(+) significantly inhibited their tumorigenic and CSC-like abilities and facilitated their differentiation into CD133(-)-non-CSCs[62]. miR-34a overexpressed in bulk prostate cancer cells ($CD44^+$) cells, when transfected with mature oligonucleotide mimics or infected with lentiviral vectors encoding pre-miR-34a, and exerted pronounced inhibitory effects on prostasphere establishment, migration and metastasis *in vivo*[63]. Restoration of miR-200c

may be a promising therapeutic approach in head and neck squamous cell carcinoma. It could significantly inhibit the malignant CSC-like properties of ALDH1(+)/CD44(+) cells by negatively modulating the expression of BMI1 and inhibiting the metastatic capability of EMT by reducing the expression of ZEB1, Snail and N-cadherin, but up-regulating the E-cadherin expression[64]. Overexpression of miR-328 directly targets ABCG2 and MMP16, reverses drug resistance, inhibits cell invasion of side population (SP) cells from colorectal cancer, and thereby decreases invasive and strong tumor formation ability[65].

Studies on the physiological and behavioral differences between cancer stem cells and normal stem cells are required to help in the identification of specific mRNAs in cancer stem cells which may regulate oncogenesis or suppression to influence tumor development or progression that could act as a suitable drug target for safe and effective therapeutics.

CONCLUSION

miRNAs, a newly identified class of regulatory non-coding endogenous RNAs, have pivotal functions in stem cell maintenance. A small SP of cells identified in a variety of cancers harbor stem cell properties called cancer stem cells which are responsible for relapse and treatment failure in many cancer patients. These cells express miRNAs aberrantly where they can function as oncogenes or tumor suppressor genes. Identification of miRNA as a signature molecule to CSCs and their potential role make them good therapeutic targets for next-generation anti-cancer drugs and directly impact the current efforts in the safe eradication of malignancies.

REFERENCES

1 **Vaish M**. Mismatch repair deficiencies transforming stem cells into cancer stem cells and therapeutic implications. *Mol Cancer* 2007; **6**: 26
2 **Hatfield S**, Ruohola-Baker H. microRNA and stem cell function. *Cell Tissue Res* 2008; **331**: 57-66
3 **Kwak PB**, Iwasaki S, Tomari Y. The microRNA pathway and cancer. *Cancer Sci* 2010; **101**: 2309-2315
4 **Lee RC**, Feinbaum RL, Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* 1993; **75**: 843-854
5 **Reinhart BJ**, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, Horvitz HR, Ruvkun G. The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. *Nature* 2000; **403**: 901-906
6 **Berezikov E**, Guryev V, van de Belt J, Wienholds E, Plasterk RH, Cuppen E. Phylogenetic shadowing and computational identification of human microRNA genes. *Cell* 2005; **120**: 21-24
7 **Bagasra O**, Prilliman KR. RNA interference: the molecular immune system. *J Mol Histol* 2004; **35**: 545-553
8 **Calin GA**, Sevignani C, Dumitru CD, Hyslop T, Noch E, Yendamuri S, Shimizu M, Rattan S, Bullrich F, Negrini M, Croce CM. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proc Natl Acad Sci USA* 2004; **101**: 2999-3004
9 **Bruchova H**, Yoon D, Agarwal AM, Mendell J, Prchal JT. Regulated expression of microRNAs in normal and polycythemia vera erythropoiesis. *Exp Hematol* 2007; **35**: 1657-1667
10 **Fiore R**, Schratt G. MicroRNAs in synapse development: tiny molecules to remember. *Expert Opin Biol Ther* 2007; **7**: 1823-1831
11 **Esau CC**, Monia BP. Therapeutic potential for microRNAs. *Adv Drug Deliv Rev* 2007; **59**: 101-114
12 **Latronico MV**, Catalucci D, Condorelli G. Emerging role of microRNAs in cardiovascular biology. *Circ Res* 2007; **101**: 1225-1236
13 **Perkins DO**, Jeffries CD, Jarskog LF, Thomson JM, Woods K, Newman MA, Parker JS, Jin J, Hammond SM. microRNA expression in the prefrontal cortex of individuals with schizophrenia and schizoaffective disorder. *Genome Biol* 2007; **8**: R27
14 **Sonkoly E**, Wei T, Janson PC, Sääf A, Lundeberg L, Tengvall-Linder M, Norstedt G, Alenius H, Homey B, Scheynius A, Ståhle M, Pivarcsi A. MicroRNAs: novel regulators involved in the pathogenesis of psoriasis? *PLoS One* 2007; **2**: e610
15 **Chen Y**, Cheng G, Mahato RI. RNAi for treating hepatitis B viral infection. *Pharm Res* 2008; **25**: 72-86
16 **Chen JF**, Callis TE, Wang DZ. microRNAs and muscle disorders. *J Cell Sci* 2009; **122**: 13-20
17 **Heneghan HM**, Miller N, Kerin MJ. Role of microRNAs in obesity and the metabolic syndrome. *Obes Rev* 2010; **11**: 354-361
18 **Karolina DS**, Wintour EM, Bertram J, Jeyaseelan K. Riboregulators in kidney development and function. *Biochimie* 2010; **92**: 217-225
19 **Lee Y**, Kim M, Han J, Yeom KH, Lee S, Baek SH, Kim VN. MicroRNA genes are transcribed by RNA polymerase II. *EMBO J* 2004; **23**: 4051-4060
20 **Borchert GM**, Lanier W, Davidson BL. RNA polymerase III transcribes human microRNAs. *Nat Struct Mol Biol* 2006; **13**: 1097-1101
21 **Cai X**, Hagedorn CH, Cullen BR. Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. *RNA* 2004; **10**: 1957-1966
22 **Gregory RI**, Chendrimada TP, Shiekhattar R. MicroRNA biogenesis: isolation and characterization of the microprocessor complex. *Methods Mol Biol* 2006; **342**: 33-47
23 **Lund E**, Dahlberg JE. Substrate selectivity of exportin 5 and Dicer in the biogenesis of microRNAs. *Cold Spring Harb Symp Quant Biol* 2006; **71**: 59-66
24 **Obernosterer G**, Leuschner PJ, Alenius M, Martinez J. Post-transcriptional regulation of microRNA expression. *RNA* 2006; **12**: 1161-1167
25 **Thomson JM**, Newman M, Parker JS, Morin-Kensicki EM, Wright T, Hammond SM. Extensive post-transcriptional regulation of microRNAs and its implications for cancer. *Genes Dev* 2006; **20**: 2202-2207
26 **Pratt AJ**, MacRae IJ. The RNA-induced silencing complex: a versatile gene-silencing machine. *J Biol Chem* 2009; **284**: 17897-17901
27 **Boutz PL**, Chawla G, Stoilov P, Black DL. MicroRNAs regulate the expression of the alternative splicing factor nPTB during muscle development. *Genes Dev* 2007; **21**: 71-84
28 **Vasudevan S**, Tong Y, Steitz JA. Switching from repression to activation: microRNAs can up-regulate translation. *Science* 2007; **318**: 1931-1934
29 **Kanellopoulou C**, Muljo SA, Kung AL, Ganesan S, Drapkin R, Jenuwein T, Livingston DM, Rajewsky K. Dicer-deficient mouse embryonic stem cells are defective in differentiation and centromeric silencing. *Genes Dev* 2005; **19**: 489-501
30 **Wang Y**, Medvid R, Melton C, Jaenisch R, Blelloch R. DGCR8 is essential for microRNA biogenesis and silencing of embryonic stem cell self-renewal. *Nat Genet* 2007; **39**: 380-385
31 **Peter ME**. Let-7 and miR-200 microRNAs: guardians against pluripotency and cancer progression. *Cell Cycle* 2009; **8**:

843-852
32 **Melton C**, Blelloch R. MicroRNA Regulation of Embryonic Stem Cell Self-Renewal and Differentiation. *Adv Exp Med Biol* 2010; **695**: 105-117
33 **Xu N**, Papagiannakopoulos T, Pan G, Thomson JA, Kosik KS. MicroRNA-145 regulates OCT4, SOX2, and KLF4 and represses pluripotency in human embryonic stem cells. *Cell* 2009; **137**: 647-658
34 **Melton C**, Judson RL, Blelloch R. Opposing microRNA families regulate self-renewal in mouse embryonic stem cells. *Nature* 2010; **463**: 621-626
35 **Burdon T**, Smith A, Savatier P. Signalling, cell cycle and pluripotency in embryonic stem cells. *Trends Cell Biol* 2002; **12**: 432-438
36 **Johnson CD**, Esquela-Kerscher A, Stefani G, Byrom M, Kelnar K, Ovcharenko D, Wilson M, Wang X, Shelton J, Shingara J, Chin L, Brown D, Slack FJ. The let-7 microRNA represses cell proliferation pathways in human cells. *Cancer Res* 2007; **67**: 7713-7722
37 **Schultz J**, Lorenz P, Gross G, Ibrahim S, Kunz M. MicroRNA let-7b targets important cell cycle molecules in malignant melanoma cells and interferes with anchorage-independent growth. *Cell Res* 2008; **18**: 549-557
38 **Bian S**, Sun T. Functions of noncoding RNAs in neural development and neurological diseases. *Mol Neurobiol* 2011; **44**: 359-373
39 **Papagiannakopoulos T**, Kosik KS. MicroRNAs: regulators of oncogenesis and stemness. *BMC Med* 2008; **6**: 15
40 **Rybak A**, Fuchs H, Hadian K, Smirnova L, Wulczyn EA, Michel G, Nitsch R, Krappmann D, Wulczyn FG. The let-7 target gene mouse lin-41 is a stem cell specific E3 ubiquitin ligase for the miRNA pathway protein Ago2. *Nat Cell Biol* 2009; **11**: 1411-1420
41 **Schwamborn JC**, Berezikov E, Knoblich JA. The TRIM-NHL protein TRIM32 activates microRNAs and prevents self-renewal in mouse neural progenitors. *Cell* 2009; **136**: 913-925
42 **Clarke MF**, Fuller M. Stem cells and cancer: two faces of eve. *Cell* 2006; **124**: 1111-1115
43 **Garg M**. Mismatch repair system: Therapeutic approaches to cancer stem cells. In: Singh SR, Mishra PK, Hou SX, editors. Stem cells: Organogenesis and cancer. Kerala: Transworld Research Network, 2010: 271-291
44 **Garg M**. Gain of antitumor functions and induction of differentiation in cancer stem cells contribute to complete cure and no relapse. *Crit Rev Oncog* 2009; **15**: 57-78
45 **Garg M**. MicroRNA profiling involved in human tumorigenesis using Bioinformatics tools. In: Tuteja R, editor. Bioinformatics: Genome Bioinformatics and Computational Biology. Germany: Nova Science Publishers Inc., 2011: In press
46 **O'Donnell KA**, Wentzel EA, Zeller KI, Dang CV, Mendell JT. c-Myc-regulated microRNAs modulate E2F1 expression. *Nature* 2005; **435**: 839-843
47 **Calin GA**, Croce CM. MicroRNA signatures in human cancers. *Nat Rev Cancer* 2006; **6**: 857-866
48 **Otsubo T**, Akiyama Y, Hashimoto Y, Shimada S, Goto K, Yuasa Y. MicroRNA-126 inhibits SOX2 expression and contributes to gastric carcinogenesis. *PLoS One* 2011; **6**: e16617
49 **Liao B**, Bao X, Liu L, Feng S, Zovoilis A, Liu W, Xue Y, Cai J, Guo X, Qin B, Zhang R, Wu J, Lai L, Teng M, Niu L, Zhang B, Esteban MA, Pei D. MicroRNA cluster 302-367 enhances somatic cell reprogramming by accelerating a mesenchymal-to-epithelial transition. *J Biol Chem* 2011; **286**: 17359-17364
50 **King CE**, Cuatrecasas M, Castells A, Sepulveda AR, Lee JS, Rustgi AK. LIN28B promotes colon cancer progression and metastasis. *Cancer Res* 2011; **71**: 4260-4268
51 **Zhang H**, Yang JH, Zheng YS, Zhang P, Chen X, Wu J, Xu L, Luo XQ, Ke ZY, Zhou H, Qu LH, Chen YQ. Genome-wide analysis of small RNA and novel MicroRNA discovery in human acute lymphoblastic leukemia based on extensive sequencing approach. *PLoS One* 2009; **4**: e6849
52 **Shimono Y**, Zabala M, Cho RW, Lobo N, Dalerba P, Qian D, Diehn M, Liu H, Panula SP, Chiao E, Dirbas FM, Somlo G, Pera RA, Lao K, Clarke MF. Downregulation of miRNA-200c links breast cancer stem cells with normal stem cells. *Cell* 2009; **138**: 592-603
53 **Pirollo KF**, Xu L, Chang EH. Non-viral gene delivery for p53. *Curr Opin Mol Ther* 2000; **2**: 168-175
54 **Xu L**, Pirollo KF, Chang EH. Tumor-targeted p53-gene therapy enhances the efficacy of conventional chemo/radiotherapy. *J Control Release* 2001; **74**: 115-128
55 **Kota J**, Chivukula RR, O'Donnell KA, Wentzel EA, Montgomery CL, Hwang HW, Chang TC, Vivekanandan P, Torbenson M, Clark KR, Mendell JR, Mendell JT. Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model. *Cell* 2009; **137**: 1005-1017
56 **Meng F**, Glaser SS, Francis H, DeMorrow S, Han Y, Passarini JD, Stokes A, Cleary JP, Liu X, Venter J, Kumar P, Priester S, Hubble L, Staloch D, Sharma J, Liu CG, Alpini G. Functional analysis of microRNAs in human hepatocellular cancer stem cells. *J Cell Mol Med* 2012; **16**: 160-173
57 **Oliveras-Ferraros C**, Cufí S, Vazquez-Martin A, Torres-Garcia VZ, Del Barco S, Martin-Castillo B, Menendez JA. Micro(mi)RNA expression profile of breast cancer epithelial cells treated with the anti-diabetic drug metformin: induction of the tumor suppressor miRNA let-7a and suppression of the TGFβ-induced oncomiR miRNA-181a. *Cell Cycle* 2011; **10**: 1144-1151
58 **Shi L**, Zhang S, Feng K, Wu F, Wan Y, Wang Z, Zhang J, Wang Y, Yan W, Fu Z, You Y. MicroRNA-125b-2 confers human glioblastoma stem cells resistance to temozolomide through the mitochondrial pathway of apoptosis. *Int J Oncol* 2012; **40**: 119-129
59 **Misawa A**, Katayama R, Koike S, Tomida A, Watanabe T, Fujita N. AP-1-Dependent miR-21 expression contributes to chemoresistance in cancer stem cell-like SP cells. *Oncol Res* 2010; **19**: 23-33
60 **Godlewski J**, Nowicki MO, Bronisz A, Williams S, Otsuki A, Nuovo G, Raychaudhury A, Newton HB, Chiocca EA, Lawler S. Targeting of the Bmi-1 oncogene/stem cell renewal factor by microRNA-128 inhibits glioma proliferation and self-renewal. *Cancer Res* 2008; **68**: 9125-9130
61 **Li Y**, Guessous F, Zhang Y, Dipierro C, Kefas B, Johnson E, Marcinkiewicz L, Jiang J, Yang Y, Schmittgen TD, Lopes B, Schiff D, Purow B, Abounader R. MicroRNA-34a inhibits glioblastoma growth by targeting multiple oncogenes. *Cancer Res* 2009; **69**: 7569-7576
62 **Yang YP**, Chien Y, Chiou GY, Cherng JY, Wang ML, Lo WL, Chang YL, Huang PI, Chen YW, Shih YH, Chen MT, Chiou SH. Inhibition of cancer stem cell-like properties and reduced chemoradioresistance of glioblastoma using microRNA145 with cationic polyurethane-short branch PEI. *Biomaterials* 2012; **33**: 1462-1476
63 **Liu C**, Tang DG. MicroRNA regulation of cancer stem cells. *Cancer Res* 2011; **71**: 5950-5954
64 **Lo WL**, Yu CC, Chiou GY, Chen YW, Huang PI, Chien CS, Tseng LM, Chu PY, Lu KH, Chang KW, Kao SY, Chiou SH. MicroRNA-200c attenuates tumour growth and metastasis of presumptive head and neck squamous cell carcinoma stem cells. *J Pathol* 2011; **223**: 482-495
65 **Xu XT**, Xu Q, Tong JL, Zhu MM, Nie F, Chen X, Xiao SD, Ran ZH. MicroRNA expression profiling identifies miR-328 regulates cancer stem cell-like SP cells in colorectal cancer. *Br J Cancer* 2012; **106**: 1320-1330
66 **Cartwright P**, McLean C, Sheppard A, Rivett D, Jones K, Dalton S. LIF/STAT3 controls ES cell self-renewal and pluripotency by a Myc-dependent mechanism. *Development* 2005; **132**: 885-896
67 **Delaloy C**, Liu L, Lee JA, Su H, Shen F, Yang GY, Young WL, Ivey KN, Gao FB. MicroRNA-9 coordinates proliferation and migration of human embryonic stem cell-derived

neural progenitors. *Cell Stem Cell* 2010; **6**: 323-335
68 **Liu C**, Teng ZQ, Santistevan NJ, Szulwach KE, Guo W, Jin P, Zhao X. Epigenetic regulation of miR-184 by MBD1 governs neural stem cell proliferation and differentiation. *Cell Stem Cell* 2010; **6**: 433-444
69 **Smrt RD**, Szulwach KE, Pfeiffer RL, Li X, Guo W, Pathania M, Teng ZQ, Luo Y, Peng J, Bordey A, Jin P, Zhao X. MicroRNA miR-137 regulates neuronal maturation by targeting ubiquitin ligase mind bomb-1. *Stem Cells* 2010; **28**: 1060-1070
70 **Yu JY**, Chung KH, Deo M, Thompson RC, Turner DL. MicroRNA miR-124 regulates neurite outgrowth during neuronal differentiation. *Exp Cell Res* 2008; **314**: 2618-2633
71 **Tran N**, McLean T, Zhang X, Zhao CJ, Thomson JM, O'Brien C, Rose B. MicroRNA expression profiles in head and neck cancer cell lines. *Biochem Biophys Res Commun* 2007; **358**: 12-17
72 **Voorhoeve PM**, le Sage C, Schrier M, Gillis AJ, Stoop H, Nagel R, Liu YP, van Duijse J, Drost J, Griekspoor A, Zlotorynski E, Yabuta N, De Vita G, Nojima H, Looijenga LH, Agami R. A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors. *Cell* 2006; **124**: 1169-1181
73 **Zhu S**, Si ML, Wu H, Mo YY. MicroRNA-21 targets the tumor suppressor gene tropomyosin 1 (TPM1). *J Biol Chem* 2007; **282**: 14328-14336
74 **Hwang-Verslues WW**, Chang PH, Wei PC, Yang CY, Huang CK, Kuo WH, Shew JY, Chang KJ, Lee EY, Lee WH. miR-495 is upregulated by E12/E47 in breast cancer stem cells, and promotes oncogenesis and hypoxia resistance via downregulation of E-cadherin and REDD1. *Oncogene* 2011; **30**: 2463-2474
75 **Mott JL**, Kobayashi S, Bronk SF, Gores GJ. mir-29 regulates Mcl-1 protein expression and apoptosis. *Oncogene* 2007; **26**: 6133-6140
76 **Shi L**, Zhang J, Pan T, Zhou J, Gong W, Liu N, Fu Z, You Y. MiR-125b is critical for the suppression of human U251 glioma stem cell proliferation. *Brain Res* 2010; **1312**: 120-126

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ORIGINAL ARTICLE

Generation of a human embryonic stem cell line stably expressing high levels of the fluorescent protein mCherry

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Abstract

AIM: The generation and characterization of a human embryonic stem cell (hESC) line stably expressing red fluorescent mCherry protein.

METHODS: Lentiviral transduction of a ubiquitously-expressed human EF-1α promoter driven mCherry transgene was performed in MEL2 hESC. Red fluorescence was assessed by immunofluorescence and flow cytometry. Pluripotency of stably transduced hESC was determined by immunofluorescent pluripotency marker expression, flow cytometry, teratoma assays and embryoid body-based differentiation followed by reverse transcriptase-polymerase chain reaction. Quantification of cell motility and survival was performed with time lapse microscopy.

RESULTS: Constitutively fluorescently-labeled hESCs are useful tools for facile *in vitro* and *in vivo* tracking of survival, motility and cell spreading on various surfaces before and after differentiation. Here we describe the generation and characterization of a hESC line (MEL2) stably expressing red fluorescent protein, mCherry. This line was generated by random integration of a fluorescent protein-expressing cassette, driven by the ubiquitously-expressed human EF-1α promoter. Stably transfected MEL2-mCherry hESC were shown to express pluripotency markers in the nucleus (POU5F1/OCT4, NANOG and SOX2) and on the cell surface (SSEA4, TRA1-60 and TG30/CD9) and were shown to maintain a normal karyotype in long-term (for at least 35 passages) culture. MEL2-mCherry hESC further readily differentiated into representative cell types of the three germ layers in embryoid body and teratoma based assays and, importantly, maintained robust mCherry expression throughout differentiation. The cell line was next adapted to single-cell passaging, rendering it compatible with numerous bioengineering applications such as measurement of cell motility and cell spreading on various protein modified surfaces, quantification of cell attachment to nanoparticles and rapid estimation of cell survival.

CONCLUSION: The MEL2-mCherry hESC line conforms to the criteria of bona fide pluripotent stem cells and maintains red fluorescence throughout differentiation, making it a useful tool for bioengineering and *in vivo* tracking experiments.

Key words: Human embryonic stem cells; Fluorescent marker; mCherry; Pluripotency; Cellular motility

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INTRODUCTION

Human embryonic stem cells (hESC) hold great promise for use in regenerative medicine therapies because of their intrinsic properties of indefinite self-renewal and pluripotency (ability to differentiate into endoderm, ectoderm and mesoderm derivatives). As hESC steadily progress towards clinical applications there is a need to track hESC *in vitro* and *in vivo* and to optimise hESC culture expansion and differentiation protocols. To enable this, there is an increasing need for well-characterized, *bona fide*, genetically stable, fluorescently-tagged hESC lines that both fluoresce as undifferentiated cells and do not silence expression of the fluorescent protein upon differentiation. Unfortunately, one of the properties that distinguishes hESCs from most other cell lines is their tendency to progressively silence exogenous sequences, i.e., transgenes, that are integrated into the cell's genome[1,2]. Furthermore, because the chromatin in ESCs largely exists in a "poised" state marked by bivalent histone modifications, transgenes are frequently silenced upon differentiation when genetic programs for specific cell lineages are closed as cells progress to a terminally differentiated state. Perhaps for these reasons, at present only a limited number of cell lines with stable robust expression of a constitutively active green fluorescent transgene have been described and are available for use by the hESC research community[3,4]. We therefore set out to produce and characterize a hESC line that robustly expresses high levels of a red fluorescent protein, mCherry, that has an emission spectrum that is spectrally separated from most commonly used fluorophores such as FITC. Here we show that this line, which we have termed MEL2-mCherry, maintains all characteristics of the original MEL2 hESC line, such as expression of pluripotency markers, self renewal over prolonged periods of time, preservation of a normal euploid karyotype and differentiation into representative cell types of the three germ layers, while maintaining mCherry expression. We have further adapted the MEL2-mCherry line to single-cell passaging and demonstrate its utility in cell tracking experiments under various experimental settings. The MEL2-mCherry line, which was purposefully generated in an IP-unencumbered cellular background, will therefore prove a useful tool for the hESC research community.

MATERIALS AND METHODS

Tissue culture

We chose the MEL2 hESC female (XX) IP-unencumbered cell line, generated by Stem Cell Sciences Ltd and previously characterized by the International Stem Cell Initiative[5], as the host for a fluorescent protein-encoding transgene. MEL2 hESC were cultured in KOSR medium on mouse embryonic fibroblasts (MEFs) as described previously. For single cell passage adaptation, MEL2-mCherry hESC were cultured in mTeSR1 medium on Matrigel (1/100 dilution) and passaged weekly using gentle TrypLE (Invitrogen) trypsin substitute digestion (5 min at 25 ℃). ROCK inhibitor Y-27632 (Millipore) was included in the medium at 1 μmol/L on each first day after single cell passaging until the culture was single cell-adapted (approximately 3-4 passages).

Generation of stably-transfected hESC clones

Following standard passaging on MEFs, as described in[6], the MEL2 parental cell line was plated onto a Matrigel (BD Biosciences) substrate (1/100 dilution) and cultured in complete mTeSR1 medium without any additional supplements (Stem Cell Technologies, USA) for 1 d prior to transfection. About 2 μg of pEF6-mCherry plasmid (a gift from Dr. Sweet M, IMB, University of Queensland) (see Figure 1 for plasmid map) was transfected using FuGene6 reagent (1:3 DNA:FuGene6 ratio, Roche Biochemicals) using the manufacturer's recommendations. Selection with BlasticidinS-HCl (Gibco) at 2 μg/mL was started 3 d after transfection. Resistant colonies uniformly expressing high levels of the mCherry protein were isolated manually and culture expanded. Live FACS sorting of mCherry expressing cells was carried out (using FACSAria, BD Biosciences, USA) and pure sorted cells were reseeded and further expanded for full characterization and cryopreservation.

Pluripotency marker expression in the MEL2-mCherry cell line

To confirm appropriate expression of pluripotency-associated genes in the MEL2-mCherry cell line after transgenesis and associated manipulations, we performed immunofluorescence staining as well as flow-cytometric (FACS) analysis, essentially as described previously[7,8]. The primary antibodies used for immunofluorescence were: anti-Oct3/4 (C10, sc5279 SantaCruz, USA) at 1/75 (IF), anti-Sox2 (AB5603, Millipore, USA) at 1/100 (IF), anti-NANOG (9220 Millipore) at 1/150 (IF). Secondary antibodies were used in 1/1000 dilution and were anti-mouse IgG (H + L) AlexaFluor488 (Molecular Probes/Invitrogen). Isotype controls at identical concentrations as the primary antibodies were used to assess non-specific binding. No labeling was detected in isotype control-incubated samples (not shown). Counter-staining of the nuclei was

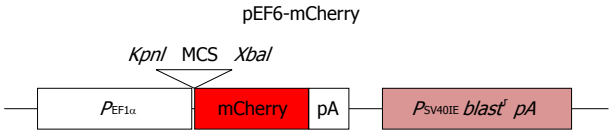

Figure 1 A schematic diagram of the pEF6-mCherry plasmid. MCS-multiple cloning site of the pEF6-V5/His vector.

performed using DAPI (4,6-diamidino-2-phenylindole) at 0.1 μg/mL in PBS. For flow cytometry, cells were brought to a single cell suspension using TrypLE (Invitrogen) and stained live with anti-Tra1-60 1/200 (MAB4360, Millipore) or anti-Tra1-81 1/200 (MAB4381 Millipore) or anti-SSEA4 1/400 (MAB4304, Millipore) or anti-TG30 1/400 (MAB4427, Millipore), essentially as described previously (Chung *et al*[7], 2010). For flow cytometry secondary antibodies, anti-mouse IgG (H + L) AlexaFluor488 or anti-mouse IgM AlexaFluor488 (Molecular Probes/Invitrogen, Carlsbad, USA) were always used in 1/1000 dilution. Isotype control antibodies used at identical concentrations as the primary antibodies were used to set the gates (not shown). Flow cytometry data were collected and analyzed using WEASEL JAVA-based software package (www.wehi.edu.au/other_domains/cytometry/).

MEL2-mCherry cell line differentiation

The differentiation potential of the MEL2-mCherry cell line was assessed using both an *in vitro* embryoid body formation assay and *in vivo* teratoma formation. To generate embryoid bodies (EBs), 5×10^4 cells were placed as a single-cell suspension in KOSR medium [20% knockout serum replacement in DMEM/F12 medium (Gibco/Invitrogen, USA)], as described previously[9] in a well of a 6 well ultra low-attachment polystyrene plate (Falcon, USA) and cultured for 2 wk. For the teratoma formation assay, a pellet of 5×10^5 cells was mixed with Matrigel matrix at 1:50 dilution and injected intramuscularly into the thigh muscle of a NOD/SCID mouse. Teratomas were harvested within 4-8 wk; half of it was fixed and processed for paraffin embedding and histological analysis. Haematoxylin/eosin-stained 5 μm sections were permanently mounted, microscopically analysed and imaged on an Olympus IX51 inverted microscope equipped with MicroPublisher 3.3 RTV CCD camera (QImaging, USA). The other half of the teratoma was embedded in OCT compound (Sakura Biotek, USA) *via* overnight incubations in the 10%-20%-30% gradient of sucrose in PBS and frozen at -80 ℃. Sections (6 μm) were cut using a Leica (Leica) cryostat on Superfrost slides (Fisher Scientific). Expression of mCherry was detected using rabbit polyclonal anti-RFP antibody (PM005) from Medical and Biological laboratories (MBL, USA) at 1:500 dilution and secondary anti-rabbit IgG AlexaFluor568 (1:1000 dilution, Molecular Probes/Invitrogen, USA).

Analysis of cellular behavior on various substrates using the MEL2-mCherry cell line

To analyse the behavior of the MEL2-mCherry cell line on various substrates, a single-cell suspension of 4×10^4 MEL2-mCherry cells was plated in 100 μL of StemPro (Invitrogen) hES medium in a well of a 96 well plate coated with various protein substrates and on an untreated tissue culture plastic as a control not capable of maintaining efficient hES cell attachment and growth (Substrate 1 in Figure 2). Phase-contrast and fluorescence images were captured using an inverted compound microscope Olympus IX51 (Olympus, Japan) equipped with MicroPublisher 3.3 RTV CCD camera (QImaging. USA).

Colony formation and cell tracking experiments using the MEL2-mCherry cell line

In order to track and compare hESC colony formation, the MEL2-mCherry cell line was mixed with equal numbers of cells of the parental MEL2 hES line (1×10^4) and seeded into a 6 well plate with either MEFs or Matrigel (BD Biosciences) coating matrix at 1/100 dilution. Images were then captured using Olympus IX81 Corvus-automated microscope equipped with carbon dioxide levels and temperature-controlled chamber (Solent Inc., USA) at 25 min intervals (Figure 2A and B).

Image analysis for quantification of MEL2-mCherry cells on various substrates

To assess the degree of attachment of cells to various substrates, a simple image analysis algorithm was applied to the analysis of the red channel fluorescent image of the MEL2-mCherry cells 16 h after plating as a single-cell suspension. All analyses were performed using an open-source Java-based freeware ImageJ (v. 1.43 used). Firstly, the area of cell spreading was defined [by utilising the automated background subtraction option (Process > Subtract background), with Rolling Ball diameter set at 40.0 and Smoothing set to "Off"] and manual threshold gating (Figure 2E-M). To define the area occupied by dead cells (retaining high levels of mCherry fluorescence within this time frame), the area occupied by spheroid, and thus perceptually higher mCherry-fluorescent apoptotic or necrotic bodies, was established utilizing the manual threshold gating approach so that normal attached live cells with lower fluorescence density were excluded (Figure 2H-J, shown in yellow).

RESULTS

Generation of the MEL2-mCherry cell line

For a fluorescently-tagged hESC line to be useful for cell tracking *in vitro* and *in vivo*, it is important to obtain high levels of fluorescent protein expression in both undifferentiated pluripotent and differentiated cells. In order to achieve this, we chose to utilise the promoter of the human translation elongation factor 1 α (*EF-1α*) gene to drive expression of the red fluorescent mCherry protein. This promoter was previously shown to offer a superior consistency in a wide array of cell types, including hESCs[4], and thus offers the best combination of good expression levels in hESCs and consistent

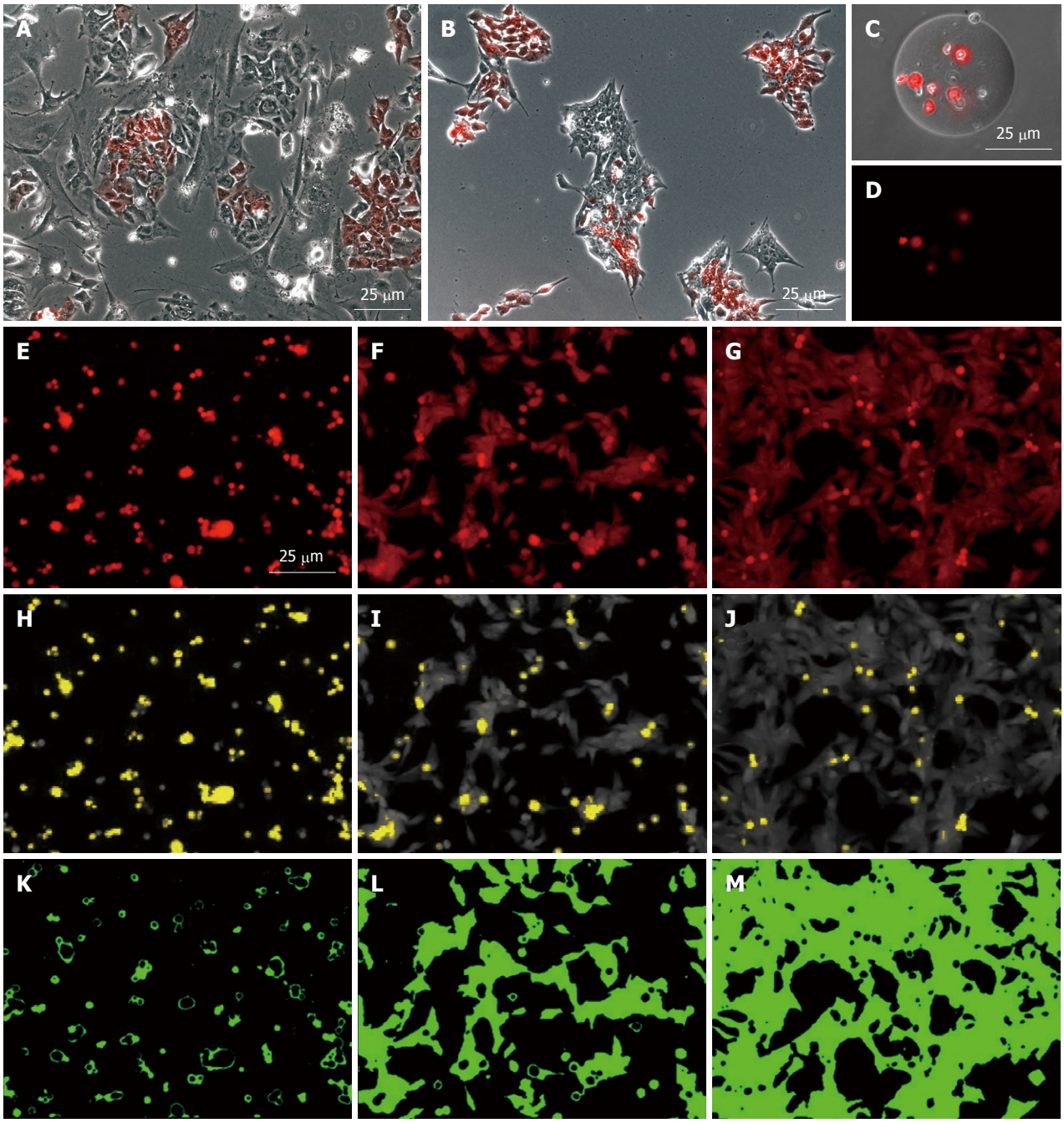

Figure 2 Utility of the MEL2-mCherry line in various analyses of human embryonic stem cell behavior. Robust mCherry expression of the MEL2-mCherry line allows for analysis of cellular behavior such as (A) mobility on various substrates or (B) mode of colony formation after mixing with non-fluorescent parental MEL2 human embryonic stem (hES) cells or (C, D) following encapsulation into transparent microcarriers; E-M: Utility of the MEL2-mCherry line for the analysis of cellular adhesion to various substrates. Uniformity and robustness of mCherry expression, in combination with its uniform distribution throughout the cell, allows the use of fluorescence level distribution to quantify cell adhesion (E-G), to identify dead, unattached cells (yellow in H-J), and accurately determine the area of substrate coverage by live hES cells (K-M). From left to right, three different ECM molecules were tested. In this substrate set relative areas of surface coverage were 4.4%, 41.7% and 76.2%.

expression across derivatives of the main three basic germ layers[10]. We strategically chose the MEL2 hESC line as a host for transduction with the EF-1α promoter-driven mCherry as this line is IP unencumbered and can be freely distributed. Following transfection of MEL2 hESC with the pEF6-mCherry plasmid, we observed that between 30%-50% of the hESC expressed various levels of mCherry. We next selected highly mCherry expressing clones using Blasticidin resistance and sub-cultured these clones for up to 15 passages. We observed that over these initial passages, individual cells within the colonies inactivated their transgene expression at a low frequency (data not shown), consistent with the previously reported tendency of hESC to inactivate foreign transgenes[1]. However, manual selection of uniformly-red sections of colonies combined with FACS sorting of mCherry-MEL2

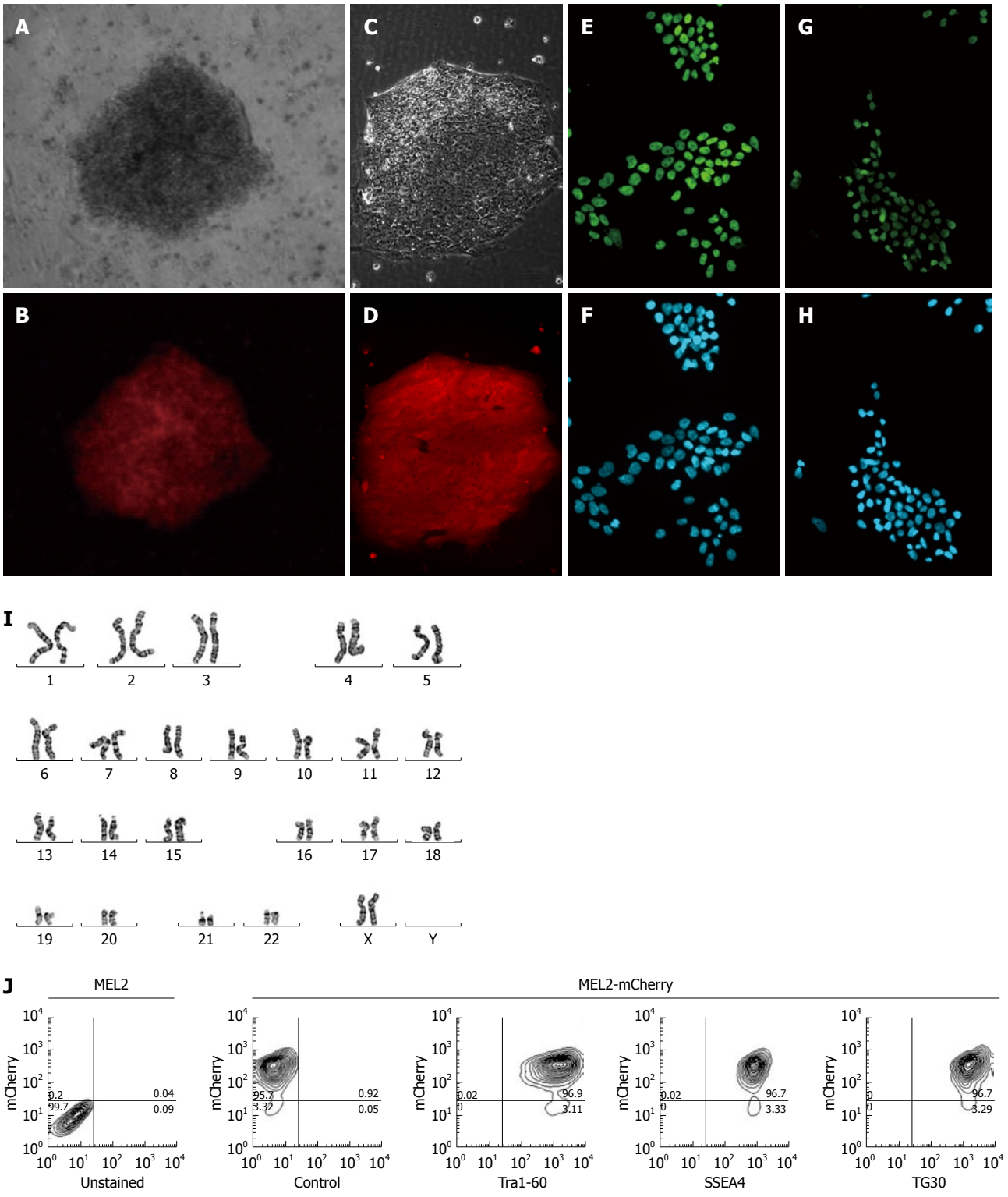

Figure 3 Characterization of the MEL2-mCherry human embryonic stem cell line. (A) Brightfield and (B) red mCherry fluorescence of a colony of MEL2 human embryonic stem cell (hESC) *transfected with Ef1a-mCherry plasmid* 12 d after Blasticidin selection (scale bar = 100 μm). (C) Brightfield and (D) red mCherry fluorescence of a colony of single-cell passaging-adapted MEL2-mCherry hESC grown on Matrigel (scale bar = 100 μm). (E) POU5F1/OCT4 expression and (F) corresponding DAPI nuclear staining of MEL2-mCherry hESC. (G) NANOG protein expression and (H) corresponding DAPI nuclear staining in nuclei of MEL2-mCherry cells. (I) Normal human female karyotype of MEL2-mCherry hESC at passage 15 (Giemsa stain of a representative metaphase chromosome spread shown). (J) FACS analysis showing high levels of mCherry fluorescence (95.7%, i.e.,) and the presence of high levels of pluripotency-associated surface antigens Tra1-60, SSEA4 and TG30(CD9). Control-a representative primary isotype control antibody staining.

cultures allowed us to select subclones that stably and uniformly expressed mCherry fluorescence (Figure 3A-D). In order to make the stable mCherry MEL2 line, which we called MEL2-mCherry, more useful for bioreactor and

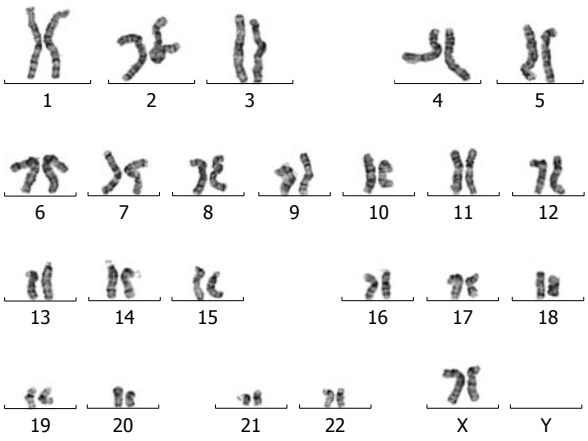

Figure 4 Giemsa-stained representative chromosomal spread of the MEL2-mCherry cell line after 35 passages.

cell tracking experimentation, we next adapted the line to single cell growth as described in Materials and Methods.

Characterization

To ascertain whether insertion of the mCherry transgene(s) had interfered with the properties of the parental MEL2 hESC line, we analyzed a number of key pluripotency properties of the newly-generated line. Firstly, we confirmed by immunostaining that the MEL2-mCherry line expresses high levels of nuclear pluripotency-associated transcription factors, such as POU5F1/OCT4 and NANOG (Figure 3E and G). The MEL2-mCherry line also retains high levels of expression of the pluripotency surface markers (Tra1-60, SSEA4, TG30 shown in Figure 3J), as assessed by flow cytometric analysis. MEL2-mCherry hESC display a normal karyotype [analysis of > 15 metaphase spreads at weekly passages 15 (Figure 3I) and 35 (Figure 4)]. Importantly, the MEL2-mCherry cell line continues to express mCherry following extensive differentiation into EBs (Figure 5A and B) and in cells derived from plated EBs (Figure 5C and D). Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of mRNA isolated from EBs differentiated for 0, 1 and 2 wk exemplifies the progressive down-regulation of *POU5F1/OCT4* and *NANOG* mRNA expression and confirms that these genes are no longer detectable in these entirely red fluorescent EBs (Figure 5B and D), suggesting that the line should be useful for *in vivo* tracking of differentiated hESC derivatives. Furthermore, the MEL2-mCherry line readily formed teratomas in NOD/SCID mice with easily identifiable derivatives of all three primitive germ layers, including ectodermally-derived neural epithelium and melanised retinal epithelium-like structures (Figure 5F), mesoderm-derived cartilage and bone (Figure 5E), and endodermally-derived gut-like epithelium and intestinal crypt-like structures (Figure 5G). Importantly, red fluorescence was maintained throughout the teratoma, indicating that the transgene is not silenced following terminal differentiation into multiple cell types (Figure 5H-L). For example, red fluorescence is clearly detected in chondrocytes (Figure 5I and J), as identified by their morphology and expression of Collagen II (Figure 5I). No significant contributions of undifferentiated cells were observed in these teratomas, as expected for karyotypically-normal hESC. We conclude that MEL2-mCherry hESC retain the key properties of pluripotency, long term self-renewal, differentiation into cell types of the three germ layers and karyotype stability, while maintaining high levels of mCherry expression both before and after differentiation.

Experimental applications of MEL2-mCherry MEL2 hESC

Because MEL2-mCherry hESC are highly red fluorescent, this line is extremely well-suited for use in cell-tracking and cell-mixing experiments. It can, for example, be used to explore the mode of colony formation of hESC. At present, it remains to be determined whether hESC form colonies by clonal growth or whether this occurs through cell migration and cell sorting of hESC. This can be elegantly addressed by mixing MEL2-mCherry cells with single cell adapted unlabeled hESC followed by real time assessment of cell behavior over time (Figure 2A and B) (Turner J. Pers comm, manuscript in prep.). The MEL2-mCherry line can also be used to compare and de-convolute cell proliferation, differentiation ability and gene expression of abnormal hESC by simply mixing such cells with the MEL2-mCherry line and then manipulating such mixtures under identical experimental conditions, followed by sorting of the cells on the basis of mCherry expression at the end of each experiment.

MEL2-mCherry hESC are also extremely suitable for analysing hESC behavior and survival after encapsulation within hydrogels or seeding on microcarriers. In such experiments (Figure 2), real time assessment and quantification of cell proliferation, attachment and viability is readily performed (since red fluorescence is rapidly lost following rupture of the plasma membrane) and can be easily quantified by measuring total red fluorescence in culture wells (Sin D. Pers comm, manuscript in prep.). The high level of red fluorescence of the MEL2-mCherry line also greatly aids in the assessment of cellular substrates used in bioengineering applications. As shown in Figure 2, immobilization of MEL2-mCherry cells on engineered surfaces coated with three different extracellular matrix molecules (from left to right) allows for direct, simple and potentially automated assessment of cellular adhesion (Figure 2E-G), cell death (Figure 2H-J) and cell spreading (Figure 2K-M).

DISCUSSION

HESCs possess great potential for enabling stem cell based therapies and advancing our understanding of very early human development[11]. However, efficient and cost effective methods for expansion and differentiation of these cells compatible with clinical applications are currently lacking, their inherent genetic and epigenetic instability remains a poorly understood problem, immune rejection must be circumvented and their efficacy and long term safety in pre-clinical models still needs to

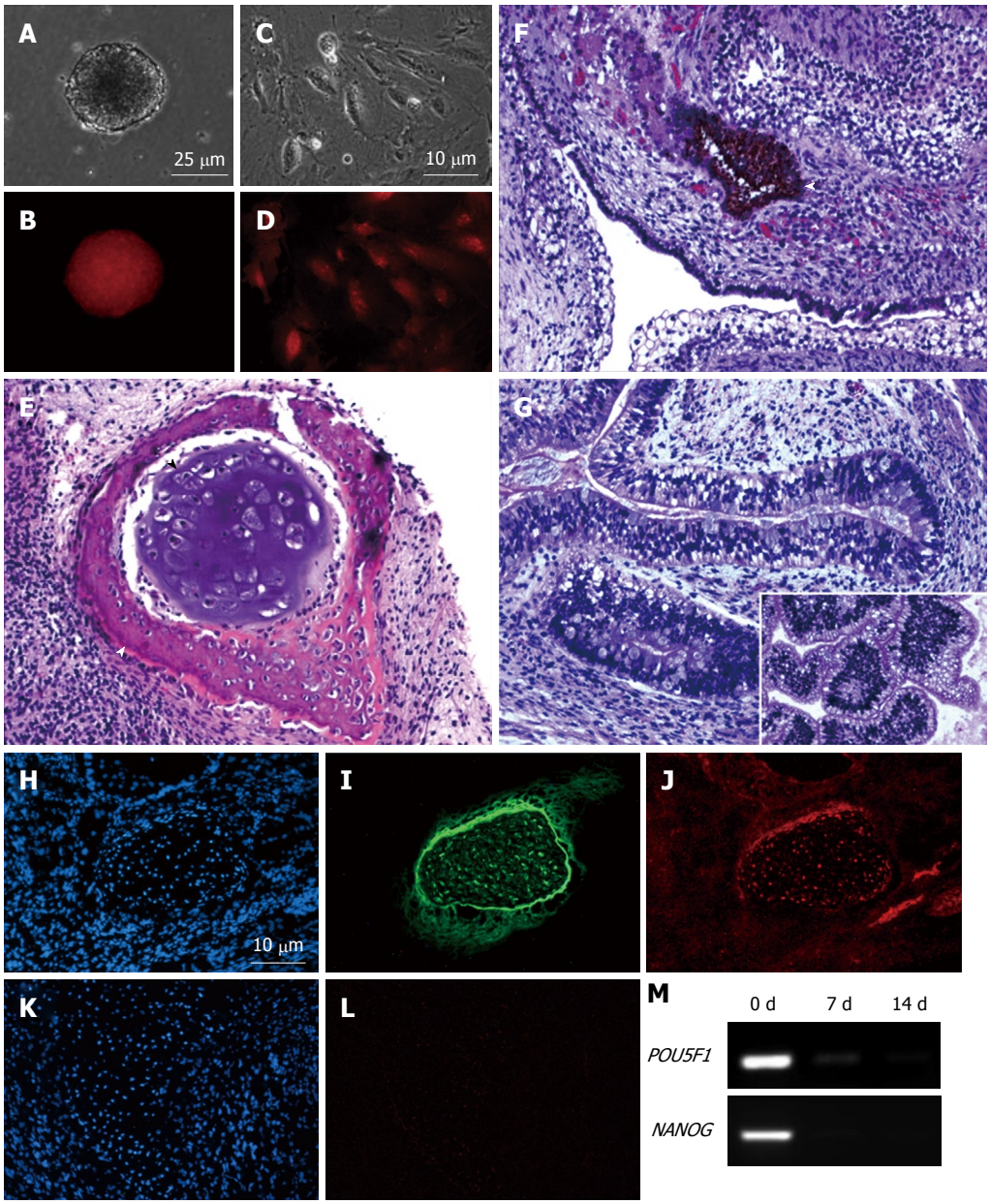

Figure 5 MEL2-mCherry line is capable of full-spectrum differentiation and retains high levels of mCherry fluorescence. (A) Brightfield and (B) Red fluorescence images of a typical EB derived from the MEL2-mCherry line showing robust uniform fluorescence. (C) Brightfield image and (D) Red mCherry fluorescence of cells dissociated from MEL2-mCherry human embryonic stem cell (hESC) derived embryoid bodies. (E-G) Teratoma sections showing the presence of derivatives of all three major germ layer: (E) Mesoderm-derived cartilage (black arrowhead) and bone (white arrowhead); (F) Ectodermally-derived neural epithelium, including melanised retinal epithelium-like structures and (G) Endodermally-derived gut-like epithelium and (inset shows transverse section through the intestinal crypt-like structures). (J) Immunofluorescent detection of the mCherry protein in teratoma cryosections reveals uniform red fluorescence in all cells, including for instance, differentiated chondrocytes expressing high levels of the type Ⅱ collagen (I). DAPI staining shows blue fluorescence of all nuclei in the sections (H and K). (L) Red fluorescence image of a cryosection incubated with an isotype control. (M) RT-PCR analysis showing down-regulation of pluripotency-associated genes POU5F1/OCT4 and NANOG mRNA expression in EBs following 1 and 2 wk withdrawal of the pluripotency-maintaining factors.

be established[12]. These hurdles are currently being addressed through the development of microcarrier and/or cell encapsulation based culture and differentiation methods[13], engineering of smart surfaces, high throughput screening of small molecules and in depth single cell analysis technologies[14,15]. In order to enable the development of these technologies and allow tracking and interrogation of hESC behavior and gene expression *in vitro* and *in vivo*, fluorescently tagged hESC lines are extremely useful as this allows flow cytometric sorting of the cells at will. Here we report on the development of a mCherry expressing MEL hESC line and demonstrate that this line conforms to the criteria expected from *bona-fide* hESC and maintains red fluorescence both before and after differentiation. As hESC usually efficiently silence exogenous sequences in the undifferentiated state[1,2] and differentiation is known to silence large numbers of genes, e.g.,[16], identifying hESC lines that exhibit stable robust

transgene expression in both differentiated and undifferentiated cells, as we have reported here for the MEL2-mCherry line, is a low frequency event. We hypothesise that our protocol has allowed for selection of clones in which transgene(s) insertion(s) into the host DNA is not, or only minimally, detected by the methylation machinery of the hES cell. It will therefore be of interest to map the insertion sites of the E1α-mCherry transgene in the future so as to identify such "safe" harbour sites in the human genome. We next show that the MEL2-mCherry line can be adapted to single cell culture and in this state is extremely useful in examining hESC behavior in both standard culture conditions as well as bioreactor and encapsulation conditions. Indeed, mixing MEL2-mCherry hESC with unlabeled cells allows empirical determination (with potential mathematical deconvolution) of the distribution of compositional variations within hES colonies as a function of the nature of the substrate, plating density, abnormal genotype or other culture conditions. One can also envisage that this line will find utility in toxicity studies, which currently largely rely on staining with viability dyes since red fluorescence is rapidly lost when plasma membrane integrity is lost. By virtue of its persistent red fluorescence following short term *in vitro* (embryoid body) and long term *in vivo* differentiation (teratoma), the MEL2-mCherry hESC will further be useful for investigating efficacy, functional integration and safety of hESC based cellular grafts, and constitutes a valuable, freely available IP unencumbered tool for the hESC research community.

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COMMENTS

Background

Human embryonic stem cells (hESCs) are stem cells that can generate every cell type of the human body and can proliferate indefinitely. For these reasons they are attractive stem cells for use in regenerative medicine therapies. To test therapeutic approaches and analyse the behavior of these cells in the dish in real time, one needs to be able to track hESCs *in vitro* and *in vivo*. This can be achieved by stably delivering a fluorescent protein to the cells. It is important to show that the expression of such a molecular tracking protein is stable, that it remains expressed after differentiation of the cells and that it does not interfere with the basic properties of hESCs.

Research frontiers

hESCs are notoriously difficult to genetically modify and even when an exogenous gene has been inserted the cells have a propensity to silence the delivered transgene. For this reason only very few fluorescently tagged hESC have been reported to date. Here the authors report on the generation of a red fluorescently tagged hESC line that stably expresses high levels of a red fluorescent protein in their undifferentiated and differentiated state.

Innovations and breakthroughs

The immediate advantage of the generation of a red (mCherry)-fluorescently tagged hESC line, as reported in the paper, is that it allows double labeling with commonly used fluorophores such as FITC (green fluorescence). The authors have deliberately chosen to express mCherry in the MEL-2 hESC line that is IP-unencumbered and can therefore be distributed to stem cell researchers worldwide. The authors further demonstrate that this line can be readily adapted to single cell growth, making it extremely useful for analyzing hESC behavior in many applications.

Applications

The authors show that the mCherry Mel-2 hESC line can be used for investigating the effect of the cell culture substrate, plating density, genotype or other culture conditions. This line will also find utility in toxicity studies and should prove a be useful tool for investigating efficacy, functional integration and safety of hESC based cellular grafts.

Peer review

Ovchinnikov *et al* present the establishment of a hESC line that constitutively expresses mCherry. The advantages of developing such lines are numerous including assessment of proliferation, migration, and *in vivo* cell tracking. Overall, it is a well organized manuscript with adequate data to support the claims. The hESC line will be a useful tool for the research community.

REFERENCES

1 **Xia X**, Zhang Y, Zieth CR, Zhang SC. Transgenes delivered by lentiviral vector are suppressed in human embryonic stem cells in a promoter-dependent manner. *Stem Cells Dev* 2007; **16**: 167-176
2 **Liew CG**, Draper JS, Walsh J, Moore H, Andrews PW. Transient and stable transgene expression in human embryonic stem cells. *Stem Cells* 2007; **25**: 1521-1528
3 **Costa M**, Dottori M, Ng E, Hawes SM, Sourris K, Jamshidi P, Pera MF, Elefanty AG, Stanley EG. The hESC line Envy expresses high levels of GFP in all differentiated progeny. *Nat Methods* 2005; **2**: 259-260
4 **Liu J**, Jones KL, Sumer H, Verma PJ. Stable transgene expression in human embryonic stem cells after simple chemical transfection. *Mol Reprod Dev* 2009; **76**: 580-586
5 **Adewumi O**, Aflatoonian B, Ahrlund-Richter L, Amit M, Andrews PW, Beighton G, Bello PA, Benvenisty N, Berry LS, Bevan S, Blum B, Brooking J, Chen KG, Choo AB, Churchill GA, Corbel M, Damjanov I, Draper JS, Dvorak P, Emanuelsson K, Fleck RA, Ford A, Gertow K, Gertsenstein M, Gokhale PJ, Hamilton RS, Hampl A, Healy LE, Hovatta O, Hyllner J, Imreh MP, Itskovitz-Eldor J, Jackson J, Johnson JL, Jones M, Kee K, King BL, Knowles BB, Lako M, Lebrin F, Mallon BS, Manning D, Mayshar Y, McKay RD, Michalska AE, Mikkola M, Mileikovsky M, Minger SL, Moore HD, Mummery CL, Nagy A, Nakatsuji N, O'Brien CM, Oh SK, Olsson C, Otonkoski T, Park KY, Passier R, Patel H, Patel M, Pedersen R, Pera MF, Piekarczyk MS, Pera RA, Reubinoff BE, Robins AJ, Rossant J, Rugg-Gunn P, Schulz TC, Semb H, Sherrer ES, Siemen H, Stacey GN, Stojkovic M, Suemori H, Szatkiewicz J, Turetsky T, Tuuri T, van den Brink S, Vintersten K, Vuoristo S, Ward D, Weaver TA, Young LA, Zhang W. Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. *Nat Biotechnol* 2007; **25**: 803-816
6 **Ellerström C**, Strehl R, Noaksson K, Hyllner J, Semb H. Facilitated expansion of human embryonic stem cells by single-cell enzymatic dissociation. *Stem Cells* 2007; **25**: 1690-1696
7 **Chung TL**, Turner JP, Thaker NY, Kolle G, Cooper-White JJ, Grimmond SM, Pera MF, Wolvetang EJ. Ascorbate promotes epigenetic activation of CD30 in human embryonic stem cells. *Stem Cells* 2010; **28**: 1782-1793
8 **Whitworth DJ**, Ovchinnikov DA, Wolvetang EJ. Generation and Characterization of LIF-dependent Canine Induced

Pluripotent Stem Cells from Adult Dermal Fibroblasts. *Stem Cells Dev* 2012; **21**: 2288-2297
9 **Chung TL**, Brena RM, Kolle G, Grimmond SM, Berman BP, Laird PW, Pera MF, Wolvetang EJ. Vitamin C promotes widespread yet specific DNA demethylation of the epigenome in human embryonic stem cells. *Stem Cells* 2010; **28**: 1848-1855
10 **Ben-Dor I**, Itsykson P, Goldenberg D, Galun E, Reubinoff BE. Lentiviral vectors harboring a dual-gene system allow high and homogeneous transgene expression in selected polyclonal human embryonic stem cells. *Mol Ther* 2006; **14**: 255-267
11 **Pera MF**, Tam PP. Extrinsic regulation of pluripotent stem cells. *Nature* 2010; **465**: 713-720
12 **Pera MF**. Stem cells: The dark side of induced pluripotency. *Nature* 2011; **471**: 46-47
13 **Levenberg S**, Huang NF, Lavik E, Rogers AB, Itskovitz-Eldor J, Langer R. Differentiation of human embryonic stem cells on three-dimensional polymer scaffolds. *Proc Natl Acad Sci USA* 2003; **100**: 12741-12746
14 **Wheeler AR**, Throndset WR, Whelan RJ, Leach AM, Zare RN, Liao YH, Farrell K, Manger ID, Daridon A. Microfluidic device for single-cell analysis. *Anal Chem* 2003; **75**: 3581-3586
15 **Di Carlo D**, Lee LP. Dynamic single-cell analysis for quantitative biology. *Anal Chem* 2006; **78**: 7918-7925
16 **Ait-Si-Ali S**, Guasconi V, Fritsch L, Yahi H, Sekhri R, Naguibneva I, Robin P, Cabon F, Polesskaya A, Harel-Bellan A. A Suv39h-dependent mechanism for silencing S-phase genes in differentiating but not in cycling cells. *EMBO J* 2004; **23**: 605-615

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Regenerative Biology: From Stem Cells to Tissues
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Till & McCulloch Meetings
Montreal, QC, Canada

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World Journal of Stem Cells (*World J Stem Cells*, *WJSC*, online ISSN 1948-0210, DOI: 10.4252), is a monthly, open-access (OA), peer-reviewed journal supported by an editorial board of 284 experts in stem cell from 28 countries.

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In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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