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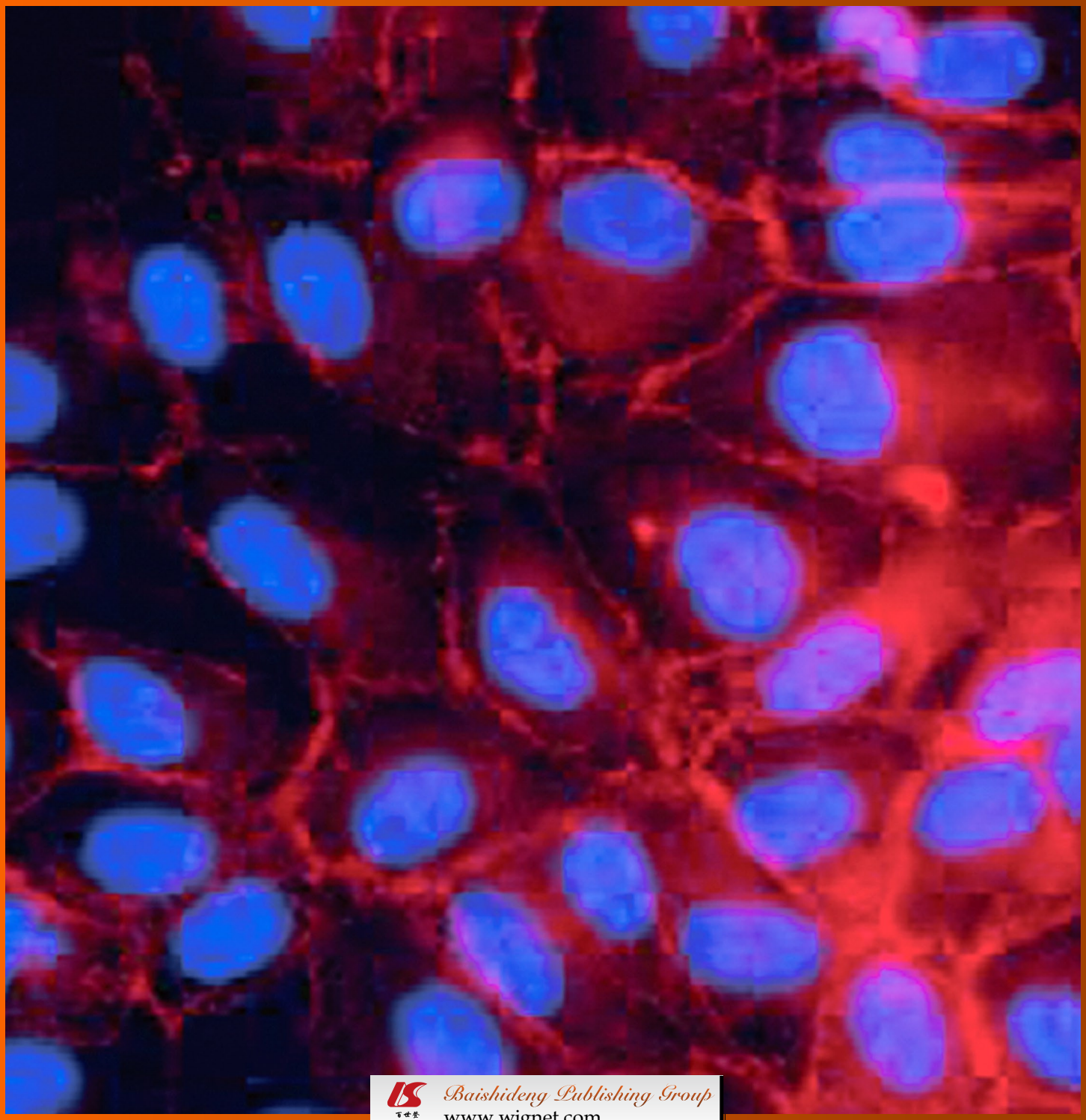

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EDITORIAL

Histone acetylation and its role in embryonic stem cell differentiation

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Abstract

The understanding of mechanisms leading to cellular differentiation is the main aim of numerous studies. Accessibility of DNA to transcription factors depends on local chromatin structure and chromatin compaction inhibits gene transcription. Histone acetylation correlates with an open chromatin structure and increased gene expression. Gene transcription levels are changed in early embryonic stem cells differentiation in a tissue-specific manner and epigenetic marks are modified, including increased global acetylation levels. Manipulation of histone deacetylases activity might be an interesting tool to generate populations of specific cell types for transplantation purposes. Thus, this review aims to show recent findings on histone acetylation, a post translational modification and its manipulation in embryonic stem cells differentiation.

Key words: Embryonic stem cells; Cellular differentiation; Epigenetics; Histone acetylation

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EMBRYONIC STEM CELLS

Embryonic stem cells (ES cells) are derived from inner mass cells within a blastocyst and can be maintained and proliferated *in vitro*[1,2]. The importance of embryonic stem cells for modern biology rests in two major properties which distinguish them from other cell types. Firstly, they are pluripotent cells, capable of developing into any type of cell from all three embryonic germ layers (endoderm, mesoderm and ectoderm) and, secondly, they have the ability to self-renew, going through numerous cycles of cell division while maintaining the undifferentiated state[3].

ES cells represent a suitable model for study of processes involved in lineage specification during mammalian development[4]. Also, ES cells can be genetically manipulated. Embryoid bodies or isolated differentiated cells are shown to be a useful tool to investigate the effects of gene insertion and deletion[5].

The first pluripotent cell line was isolated from a teratocarcinoma. Embryonal carcinoma cells served as a model to study cellular differentiation given their potential to participate in embryonic development[6], although their use in clinical research was not possible given its potential to form tumors. Finally, in 1981, ES cells were isolated from the inner cell mass of murine embryos[1,2] and in 1998 the derivation of ES cells from human em-

bryos[7] brought the concept of regenerative medicine and tissue engineering as possibilities for the treatment of degenerative diseases.

In the future, degenerative diseases caused by destruction or loss of function of certain cellular type could be treated with transplantation of differentiated ES cells[8]. Diabetes *mellitus*, a candidate for cellular therapy, is caused by selective destruction of pancreatic cells. Another candidate is Parkinson's disease in which dopaminergic neurons are destroyed in a particular region of the brain.

Also, the use of adult stem cells for replacing damaged cardiomyocytes is described, although clinical application of these cells may be hindered because of their limited ability to proliferate and differentiate in culture[9]. Therefore, considering the pluripotency and self-renewal abilities of ES cells, these cells can be expanded *in vitro*, an attractive source of stem cells for treatment of several cardiac diseases. The capacity of murine ES cells to differentiate into cardiac myocytes has been investigated intensively[10-13] and important findings were described, including the report of improvement of left ventricular function after myocardial infarction by transplanting murine ES cells in rodents[14,15].

The first step to develop efficient cellular therapies for human diseases is the establishment of *in vitro* differentiation protocols and methods for sorting large amounts of specific cell types from total population without cellular damage. Unfortunately, the heterogeneous nature of cellular differentiation *in vitro* has discouraged the use of ES cells in transplantation studies[8]. A better understanding of epigenetic events leading to lineage commitment and differentiation might clarify the pathways of heterogeneous ES cells differentiation and support the development of efficient protocols aiming at the achievement of lineage-restrictive differentiation.

REGULATION OF GENE EXPRESSION

Mammalian development occurs with the establishment of hundreds of cellular types, all of them derived from the same totipotent cell. Each differentiated cell relies on the same genetic material, although showing specific gene expression patterns, achieved by silencing and activation of tissue-specific genes[16]. The mechanisms of gene expression diversification are regulated by epigenetics. These heritable changes based in chromatin structure and not in DNA sequence, permit modulation of gene expression activities in response to external signs[17].

Eukaryotic cells contain approximately 6 million pair bases of DNA corresponding to almost 30 000 different proteins. The main part of the DNA sequence remains silenced in a compact chromatin form which makes transcription difficult or impossible[18]. Only a small part of DNA is used for gene expression in each tissue type.

The chromatin structure is established by epigenetic modifications, including DNA methylation, histone post translational modifications, chromatin remodelling and non-coding RNAs[19]. Studies have elucidated DNA methylation and histone post translational modifications as important events that play key roles in mammalian development and lineage specification[16].

Nucleosomes form the fundamental repeating units of eukaryotic chromatin and are composed by 147 DNA base pairs wrapped twice around eight core histone proteins: 2 H2A, 2 H2B, 2 H3 and 2 H4[20]. Each core histone protein possesses a globular domain and a long N-terminal tail protruding from the nucleosome which can be covalently modified. Such modifications include acetylation, phosphorylation, methylation and others[18]. Histone modifications act in chromatin condensation, replication, DNA repair and transcriptional regulation. Some post translational modifications are associated with euchromatin (histone H3 acetyl-lysine 9, mono-, di- and tri-methyl lysine 4 and histone H4 acetylation) while others are related to heterochromatin (mono-, di-, tri-methyl lysine 9 and histone H3 tri-methyl lysine 27)[21].

Among these post translational modifications, acetylation and methylation have been the most studied. Histone acetylation is related to transcriptionally-active domains and its levels correlate with gene expression[10]. Histone methylation can play a different role in gene expression events, depending on which residue is modified[22]. L*ocus*-specific histone modifications are proposed for description of a code defining the transcriptional potential state of a cell - the histone code[23].

When post translational modifications are blocked, development is affected. Histone-deacetylase 1 deficiency in mice leads to embryonic lethality[24]. Depletion of genes responsible for histone H3 lysine 9 methylation, *de novo* methylation and maintenance of methylated status also results in embryonic death[25-27].

The regulatory mechanisms for transcription and chromatin organization involved with histone modifications are not clearly defined. One hypothesis is that epigenetic factors, including modifying enzymes and remodelling factors, are capable of inducing *cis*- and *trans*- chromatin interactions[28]. Conformational changes could even be mediated by protein complexes recruited by post translational modifications. These interactions would promote structural changes on chromatin and related DNA, altering their physical properties and affecting higher order structures, leading to consequences in many aspects of genome function[29].

HISTONE ACETYLATION

Histone acetylation, precisely on lysine residues, promotes neutralization of its positive charge, weakening the interaction between the histone tail and the negatively charged local DNA. This mechanism induces exposure of local chromatin structure[30], permitting the binding of transcription factors and significantly increasing gene expression[31].

Two main enzymes control acetylation. Histone acetyltransferase (HAT) adds acetyl groups to the histone tails, neutralizing them and weakening their nucleosome interactions. Histone deacetylase (HDAC), on the other hand, removes acetyl groups from histones and drives chromatin compaction and gene silencing on the local DNA[11].

Over the past decade, more then a dozen HDAC were identified in mammalian cells. Based on their sequence similarities, HDACs were grouped into four functional classes: class I (HDAC1, HDAC2, HDAC3 and HDAC8), class II (HDAC4, HDAC5, HDAC6, HDAC7, HDAC9 and HDAC10), class III (SIRT1 and SIRT7) and class IV (HDAC11 and related enzymes)[32].

Studies in mice revealed that HDAC1 gene expression is stimulated by growth factors[33] and controlled by negative feedback mechanism of its own product[34]. HDAC1 plays a significant role in many biological processes, such as cell cycle progression, cell proliferation and differentiation, being essential for normal embryonic development[24].

In studies of chromatin function, including histone acetylation, ES cells differentiation represents a unique model during lineage commitment in DNA regions regulated throughout development[35]. Pluripotent ES cells exhibit chromatin domains with both transcriptionally active and silent histone modifications[36]. At the onset of differentiation, changes in morphology and gene expression pattern become evident[37]. During early differentiation, transcription levels of many genes are strongly altered in a temporal manner. Epigenetic changes involving post translational histone modifications are related to regulation of both local and global gene expression[38]. Histone acetyltransferases stimulate transcription through the acetylation of histones, resulting in relaxation of nucleosomes[39].

Histone deacetylases and acetylases transmit differentiation signals to initiate appropriate epigenetic modifications, such as erasure of pre-existing chromatin structure and establishment of new histone modification patterns during *in vitro* differentiation of ES cells[37].

According to Meshorer *et al*[40], ES cells undergo a wave of global acetylation with the beginning of differentiation process. Histone H3 lysine 9 acetylation is an active euchromatin-related modification. Its level is almost undetectable in pluripotent ES cells and dramatically increases when cells leave the undifferentiated state. Histone H3 lysine 9 trimethylation, a silent heterochromatin functional component, is almost undetectable in pluripotent ES cells and increases on day 7 of differentiation[40]. Another study identified higher levels of histone H3 lysine 9 acetylation on day 2 of differentiation which increased two fold on day 4 and was maintained until day 18, the last day evaluated. On the other hand, histone H3 lysine 9 trimethylation levels increased on day 2, corresponding to a deacetylation of this residue[35].

When pluripotent genes were evaluated, including *Oct4*, *Nanog, Utf1, Foxd3, Cripto* and *Rex1*, they revealed an opened chromatin on undifferentiated cells and condensed chromatin after differentiation. In contrast, neural-specific genes including *Pax3, Pax6, Irx3, Nkx2.9* and *Mash1* were revealed as transcriptionally silent on pluripotent cells and active after tissue-specific differentiation[41]. All these changes reveal the importance of epigenetic control over ES cells differentiation.

USE OF HDAC INHIBITORS TO INDUCE ES CELLS DIFFERENTIATION

HDAC inhibitors supplementation aims to evaluate histone acetylation effects on gene expression before and after cellular differentiation and elucidate molecular pathways controlling the loss of undifferentiated state and commitment to a specific cellular lineage[9,39,42-45].

Trichostatin A (TSA) is a reversible HDAC inhibitor that reacts at nanomolar concentrations with most class I and II HDACs[30], promoting histone hyperacetylation and strongly increasing cellular protein synthesis. This event leads to differentiation of tumor cells, preventing them from proliferation[46]. In some cases histone hyperacetylation can cause cell cycle arrest or even apoptosis[18]. Crystallography studies indicate that TSA blocks the enzyme catalytic site by chelating a zinc ion on the enzyme tubular structure base[46].

TSA treatment promotes histone H3 and H4 hyperacetylation even when performed in the presence of LIF, rapidly leading to morphological and molecular changes resembling those observed in the early phase of ES cells differentiation[47].

The increased acetylation levels caused by TSA induced in pluripotent ES cells the same cellular behaviour as those involved in differentiation processes. The authors also reported decreased levels of histone H3 lysine 9 acetylation in undifferentiated ES cells treated with TSA on pluripotent related genes: OCT3/4, REX1 and FGF4. In differentiation-related genes, histone H3 lysine 9 acetylation was higher in TSA treated cells.

HDAC inhibition is related to neuronal lineage progression[48-50]. Histone deacetylation is involved with repression of neuronal genes in non-neuron cells. HDAC 1 and 2 combine with co-repressors CoREST, N-CoR and mSin3A and are recruited by REST (also known as NRSF - *neuron-restrictive silencer factor*)[51]. REST blocks transcription of neuronal genes by linking to NRSE - *neuron-restrictive silencer element* - present on regulatory regions of many neuronal genes. HDAC inactivation inhibits REST mechanisms and prevents its role against neuronal differentiation. Studies using rat hippocampal progenitor cells revealed a neurogenic transcription factor (NeuroD - *neurogenic differentiation transcription factor*) increased after HDAC inhibition, leading to neuronal differentiation[52].

Neuronal differentiation in TSA-treated cultures seemed to be enhanced at the expense of oligodentrocytes which need HDAC activity for the progression of neural progenitors into mature oligodendrocytes[53]. Also, even though astrocyte differentiation involves HAT activity for glial fibrillary protein (GFAP) expression[53,54], TSA apparently decreases its proportion in comparison to neuron cells, suggesting that HDAC inhibitors enhances neuronal differentiation also at the expense of astrocytes[50].

Histone acetylation is also involved in striated myocyte differentiation. Class II HDACs are highly expressed in adult cardiomyocytes and skeletal myoblasts[15] where they bind and repress myocyte enhancer factors 2 (MEF2)

functions[55,56]. HDACs are also involved in the repression of neuronal genes in cells that are not committed to neuronal specification and the HDCAs inhibition is related to cellular commitment in many lineages[51].

In this respect, the control of global histone acetylation can be obtained by using HDAC inhibitors. Class Ⅱ HDAC are highly expressed in skeletal and cardiac muscle[15] and interact with myocyte enhancer factors (MEF2) inactivating them. Because of this property, HDAC are used clinically for cardiac hypertrophy treatment. Studies *in vitro* have demonstrated that TSA enhances striated myocyte population in undifferentiated stem cells when applied at day 6 or 7 of differentiation[9,57,58]. The last authors[9] verified that TSA induces the expression of p300, an intrinsic histone acetyltransferase that is co-activator of GATA-4 gene (a critical cardiac transcription factor), suggesting that mechanisms of TSA-induced cardiac-specific differentiation involve acetylation of specific transcription factors such as GATA-4.

TSA treatment induces entry of mesoderm cells into the cardiac muscle differentiation process in a dose-dependent manner, increasing Nkx-2, MEF2C, GATA4 and cardiac actin transcripts[58]. Authors believe that HDAC4 expression inhibits cardiomyogenesis, decreasing cardiac muscle related genes expression, and inhibition of HDAC activity is sufficient for increasing early cardiomyogenesis[9,57].

Nonetheless, there are studies[39] that show TSA detrimental effects to striated myogenesis when the HDAC inhibitor is applied at the onset of ES cell differentiation. HDACs have a critical role in cellular biology by controlling the expression of selective cell cycle inhibitors[24] and many studies have shown that HDAC inhibition leads to anti-proliferative effects on ES cells[39,47,59,60]. Thus, the effects of TSA over the cells appear to be completely dependent on dosage and stage of differentiation.

Also, HDAC inhibition causes an increase in the number of apoptotic cells by inducing the expression of pro-apoptotic genes[61] as well as histone hyperacetylation since the relaxed form of DNA is easily catalyzed by endonucleases[62]. Therefore, the HDACs inhibitors require attention on its use, aiming at minimum dosages.

CONCLUSION

Elucidation of mechanisms driving ES cells differentiation and the consequent control of these events are the objective of numerous studies in cellular and molecular biology. Unfortunately, this process is still not clear in many aspects and the same culture system drives cellular commitment in different ways.

During differentiation, levels of histone acetylation are increased, leading to rises in gene expression from all germ layers. But, in this early differentiation phase, cells are not committed yet to specific lineages[63]. The understanding of when these chromatin modifications can drive cellular differentiation and how they are achieved is an objective of many studies. For instance, studies inducing histone hyperacetylation in ES cells in order to establish a desired cellular phenotype are conducted in cardiomyocytes and neuronal cell types.

In ES cells differentiation, even though changes in gene expression patterns can alter phenotype and function of cells, more studies are needed in order to elucidate whether these transient changes in gene expression promoted by HDAC inhibitors can sustain differentiation or only a transitory phenotype. In addition, the establishment of adequate protocols in order to minimize antiproliferative effects and obtain desirable effects are needed, considering the dose and the stage of cellular differentiation.

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ORIGINAL ARTICLE

Gene targeting and Calcium handling efficiencies in mouse embryonic stem cell lines

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Abstract

AIM: To compare gene targeting efficiencies, expression profiles, and Ca^{2+} handling potentials in two widely used mouse embryonic stem cell lines.

METHODS: The two widely used mouse embryonic stem cell lines, R1 and HM-1, were cultured and maintained on Mitomycin C treated mouse embryonic fibroblast feeder cell layers, following standard culture procedures. Cells were incubated with primary and secondary antibodies before fluorescence activated cell sorting analysis to compare known pluripotency markers. Moreover, cells were harvested by trypsinization and transfected with a kinase-inactive murine Tyk2 targeting construct, following the BioRad and Amaxa transfection procedures. Subsequently, the cells were cultured and neomycin-resistant cells were picked after 13 d of selection. Surviving clones were screened twice by polymerase chain reaction (PCR) and finally confirmed by Southern blot analysis before comparison. Global gene expression profiles of more than 20400 probes were also compared and significantly regulated genes were confirmed by real time PCR analysis. Calcium handling potentials of these cell lines were also compared using various agonists.

RESULTS: We found significant differences in transfection efficiencies of the two cell lines (91% ± 6.1% *vs* 75% ± 4.2%, $P = 0.01$). Differences in the targeting efficiencies were also significant whether the Amaxa or BioRad platforms were used for comparison. We did not observe significant differences in the levels of many known pluripotency markers. However, our genome-wide expression analysis using more than 20 400 spotted cDNA arrays identified 55 differentially regulated transcripts ($P < 0.05$) implicated in various important biological processes, including binding molecular functions (particularly Ca^{2+} binding roles). Subsequently, we measured Ca^{2+} signals in these cell lines in response to various calcium agonists, both in high and low Ca^{2+} solutions, and found significant differences ($P < 0.05$) in the regulation of Ca^{2+} homeostasis between the investigated cell lines. Then we further compared the detection and expression of various membrane and in-

tracellular Ca^{2+} receptors and similarly found significant ($P < 0.05$) variations in a number of calcium receptors between these cell lines.

CONCLUSION: Results of this study emphasize the importance of considering intrinsic cellular variations, during selection of cell lines for experiments and interpretations of experimental results.

Key words: Embryonic stem cells; Microarray; Calcium; Agonists; Transfection; Cell culture; Pluripotency; Gene targeting

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INTRODUCTION

Embryonic stem cells (ESCs) are derived from the inner cell mass of the developing blastocyst with a retained potential to self-renew and to differentiate into diverse cell lineages[1,2]. Currently mouse ESCs are used in various research applications, including gene targeting, which involves the transfer of a designed alteration in an exogenous DNA sequence to the cognate DNA sequence in the living cell genome *via* homologous recombination[3]. Generation of knockout mouse models by targeted disruption of essential genes provides useful insights into genes that regulate development and allows investigators to dissect molecular developmental mechanisms[3,4]. In an effort to understand the therapeutic applications of stem cells, mouse models have been created for a number of human genetic diseases, and gene targeting in stem cells has also been common practice. The various methods of gene transfer technology and comparative efficiencies were described elsewhere[5-7]. However, most studies have focused mainly on comparing the efficiency of different gene delivery techniques, parameters or phenotypes of the offspring carrying the mutant gene. In the earlier studies, cell line differences were seldom described, although some reports acknowledged the existence of differences[8].

Despite the similarities observed in the expression of some classical pluripotent stem cell markers, such as Pou5f1 (formerly Oct4), Nanog and alkaline phosphatase (ALP)[8,9] variations were also evident between ESCs of different species[10-12], different tissue types[13,14] and distinct ESC lines derived from the same species[9]. Previous studies using different mouse cell lines have shown variations in growth performance and in some other phenotypes[9,15]. Undoubtedly, these studies have made significant contributions in advancing the field by unraveling the effects of various factors on the phenotype and performance of the cells. Better understanding is required to harness cellular potency, as it is fundamental in biology and also critical for future therapeutic uses of stem cells[16]. The HM-1 and R1 ESCs are two ESC lines of the same mouse strain (strain 129, but different sublines) that are widely used to decipher ESC biology and various applications.

We transfected these ESCs with the non-isogenic DNA construct and compared the efficiencies of homologous recombination. The significant differences observed during this initial targeting efficiency comparison lead us, subsequently, to compare the genome-wide gene expression profiles of these ESCs, in order to further compare the intracellular calcium handling potentials and expression of calcium receptors. Calcium, an intracellular second messenger, is known to be a growth-regulating divalent cation[17], and a ubiquitous intracellular signal responsible for controlling numerous cellular processes[18]. Here we demonstrate significant differences in the gene targeting efficiency, the gene expression profiles, and the intracellular Ca^{2+} handling potentials of these embryonic stem cell lines. The results of the current study reveal significant variations in the examined parameters, and underscore the importance of understanding cellular variations (efficiency) for better results in gene targeting and other in vitro culture applications of the cells.

MATERIALS AND METHODS

Materials for the cell culture, sample preparation and hybridizations, unless otherwise indicated were purchased from Invitrogen (Carlsbad, USA), whilst nerve growth factor (NGF), epidermal growth factor (EGF), and caffeine were obtained from the same source (Invitrogen). Other materials for Ca^{2+} measurement and real time polymerase chain reaction (PCR) analysis, unless otherwise indicated, were purchased from Sigma-Aldrich Chem. Inc. (St. Louis, USA).

Embryonic stem cells and culture conditions

The R1[19] mouse ESC, at passage 9, was kindly provided by Mount Sinai Hospital and Samuel Lunenfeld Research Institute, Canada. The mouse HM-1[20] ESC, at passage 19, was kindly provided by Roslin Institute, UK. The genetic backgrounds of both ESC lines are described in Table 1, while their developmental potentials were earlier tested and confirmed by production of chimeras with germline transmission[21,22]. These ESC lines were further cultured and maintained on Mitomycin C treated (10 μg/mL Mitomycin C in standard ESC medium for 150 min) mouse embryonic fibroblast (MEF) feeder cell layers. Cells were grown in standard ESC medium changed daily [high

Table 1 Some characteristics of R1 and HM-1 embryonic stem cells and fluorescence activated cell sorting analysis

Cell line	R1	HM-1
Genetic background	(129X1/SvJ x 129S1/Sv) F1 (+/p+$^{tyr-c}$Kitl^{Sl-J}/+)[1]	129/Ola[2]
Culture conditions	DMEM[3] + 15%FBS + 1000 U ESGRO mLIF	DMEM[3] + 15%FBS + 1000 U ESGRO mLIF
Cell doubling (in our hands)	14-16 h	12-14 h
Pluripotency		
Germline competence[4]	+	+
In vitro differentiation to form three germ layers[5]	+	+
Karyotype (FISH)	XY (often XO)	XY more stabile
Euploidy (Hoest staining)	78% ± 4.4%	71% ± 5.3%
Telomerase (PCR)	+	+
FACS analysis (mean ± SE)		
Pou5f1 (FACS)	90.6% ± 5.8%	88.8% ± 5.7%
Nanog (FACS)	72.5% ± 6.2%	68.7% ± 2.8%
SSEA-1 (FACS)	58.8% ± 4.1%	65.1% ± 3.1%
Sox2 (FACS)	97.4% ± 1.2%	98.8% ± 0.2%
ALP (enzyme assay)	+	+

[1]http://www.mshri.on.ca/nagy; [2]HPRT (hypoxanthine phosphoribosyl transferase)-deficient mice derived; [3]DMEM media: DMEM(4.5 g/L glucose) + 2 mmol glutamine + 0.1 mmol MEM non-essential amino-acids + 0.1 β-mercaptoethanol + 1 mmol sodium pyruvate + penicillin and streptomycin; [4]Based on the literature; [5]Spontaneous and induced (DMSO for cardiac and RA for neuronal) differentiation was tested. PCR: Polymerase chain reaction; FACS: Fluorescence activated cell sorting.

glucose DMEM supplemented with 0.1 mmol/L 2-mercaptoethanol (Sigma-Aldrich), fetal bovine serum (15% v/v; HyClone, Logan, USA), 1000 U/mL ESGRO-LIF (CHEMICON International, Temecula, USA) and antibiotics (Penicillin: 50 U/mL, Streptomycin: 50 μg/mL)]. When in culture, the cells were routinely maintained at 37℃ in humidified air containing 5% CO_2 in an incubator and passaged every other day. For RNA preparation, 2×10^6 cells/mL were lysed in RLT buffer (Qiagen, Düsseldorf, Germany) and frozen in multiple vials prior to further experiments.

Fluorescence activated cell sorting and pluripotency markers analysis

In preparation for fluorescence activated cell sorting (FACS) sorting, cells were dissociated with cell dissociation buffer (Invitrogen), washed and fixed with 4% PFA (Paraformaldehyde) for 15 min. Following fixation, the cells were permeabilized with 0.1% Triton-X 100, and 0.1% BSA-containing (Bovine serum albumin) PBS (Phosphate buffered saline). In order to check the existence of some classical pluripotency marker genes [*Pou5f1* (*Oct4*), *Nanog*, *Sox-2,* and *Fut4* (*SSEA-1*)], cells were incubated with primary antibodies [(mouse polyclonal Oct4 (1:200), goat polyclonal nanog (1:200) and goat polyclonal Sox-2 (1:200), Santa Cruz Biotechnology, Santa Cruz, USA); mouse monoclonal SSEA-1 (1:100), MC-480, Developmental Studies Hybridoma Bank, Iowa City, USA)] at 37℃ for 2 h. ESCs were incubated with fluorescent secondary antibodies [anti-mouse IgM-Cy3 (1:200); anti-mouse IgG-FITC (1:200), anti-goat IgG-Cy3 (1:200), and anti-goat IgG-Cy5 (1:200), respectively, from JacksonImmuno Research Laboratories, West Grove, USA] for 1 h at room temperature. Nuclei were counterstained with bisbenzamide (Hoechst 333258, Sigma). Cells were washed, pelleted and resuspended in 1 mL PBS, and analyzed within 12 h, with FACS-CALIBUR flow cytometer (Becton Dickinson, Franklin Lakes, USA) using its own software.

Embryonic stem cells transfection

To transfect R1 and HM-1 ESCs and compare the homologous recombination efficiency, a kinase-inactive murine Tyk2 targeting construct was kindly provided by Dr. Mathias Mueller (Institute of Animal Breeding and Genetics, Veterinary University of Vienna, Vienna, Austria) and used with intact cells. The construct was made from mouse C57B1/6 strain DNA, and thus non-isogenic for both R1 and HM-1 ESC lines which derived from 129 mouse sub strains. For transfection, cells were harvested by trypsinization (5 min in 0.25% trypsin-EDTA solution) before following the established procedures of the Bio-Rad (Bio-Rad laboratories, Hercules, USA) or Amaxa (Amaxa Biosystems, Cologne, Germany) transfection systems. Briefly, for the Bio-Rad system, 3×10^7 cells with 10-μg linearized vector were resuspended in 0.8 mL of electroporation buffer [in mmol/L; 20 HEPES (pH 7.0), 137 NaCl, 5 KCl, 0.7 $NaHPO_4$, 6 glucose, and 0.1 of 2-mercaptoethanol] and electroporated with a single pulse (240 V and 500 μF), using Gene pulser Ⅱ (Bio-Rad) in a 4-mm cuvette. After electroporation, cells were incubated at room temperature for 10 min before plating. Similarly, for the nucleofection of cells using the Amaxa system, 3×10^6 cells and 5-μg linearized vector were resuspended in 100 μL nucleofector solution (mouse ES cell nucleofector Kit, Amaxa), and three different Amaxa programs (A23, A24 and A30) were compared for the efficiency. As a control, 5 μg EGFP (Enhanced Green Fluorescent Protein) plasmid DNA (Amaxa) was used with the same electroporation and nucleofection (program A23) protocols, as well for the homologous recombination assays.

Following electroporation or nucleofection, cells were plated onto antibiotic-free, mitotically inactivated neomy-

cin-resistant MEF feeder layers, in ESC culture medium. Neomycin selection was started 24 h post-electroporation or nucleofection, by applying 200 μg/mL of G418 (Sigma) in culture medium and neomycin-resistant cells were picked after 13 d of selection. Surviving clones were screened twice by PCR and correct targeting was further confirmed by Southern blot analysis of the genomic DNA. Finally, the results of different transfection systems and programs were compared for the percentage of positive clones (for the construct), after neomycin selection.

Southern blot analysis and PCR positive clone selection

To confirm the PCR positive clones for homologous recombination, Southern blot hybridization was carried out with DIG system (Roche Applied Science, Basel, Switzerland) by following the manufacturer's protocol. Briefly, 20 μg genomic DNA from individual drug-selected colonies was digested with *Bam*H I (Invitrogen), and screened on 1% agarose gel. Gels were blotted on Hybond-N+ membrane (GE Healthcare Bio-Sciences, Uppsala, Sweden) overnight in 20 × SSC buffer (Sigma). As a probe, the 472 bp long PCR fragments were labeled with DIG-11-dUTP using the PCR DIG Probe Synthesis Kit (Roche), and hybridized to the membrane at 45℃ overnight in the DIG Easy Hyb solution (Roche). Detection was performed using the DIG Wash and Block Buffer Set (Roche). Dig labeled probe was detected with Anti-Digoxigenin-AP Fab fragment (Roche) and visualized with NBT/BCIP Solution (Roche).

Microfluorimetric measurements of intracellular calcium signaling ($[Ca^{2+}]_i$)

For this experiment, adenosine 5'-triphosphate (ATP), bradykinin, histamine, thapsigargin, nerve growth factor (NGF), epidermal growth factor (EGF), and caffeine were used. Cells were seeded in 96-well black-well/clear-bottom plates (Greiner Bio-One, Frickenhausen, Germany) at a density of 40000 cells per well in ESC medium and cultured at 37℃ for 24-48 h. The cells were incubated with medium containing the cytoplasmic calcium indicator 2 μmol/L Fluo-4 AM (Invitrogen) at 37℃ for 40 min. Then cells were washed four times with and finally cultured in Hank's solution (in mmol/L; 136.8 NaCl, 5.4 KCl, 0.34 Na_2HPO_4, 0.44 KH_2PO_4, 0.81 $MgSO_4$, 1.26 $CaCl_2$, 5.56 glucose, 4.17 $NaHCO_3$, pH 7.2) containing 1% bovine serum albumin and 2.5 mmol/L Probenecid for 30 min at 37℃. The plates were then placed to a FlexStation Ⅱ ^{384} fluorimetric image plate reader (FLIPR, Molecular Devices, Sunnyvale, USA), and changes in $[Ca^{2+}]_i$ (reflected by changes in fluorescence; lEX = 494 nm, lEM = 516 nm) of the above Hank's medium was recorded. Ca-responses were also measured in low Ca (0.6 mmol/L) Hank's solution. When calculating dose-response curves[23] data were fitted to the Hill equation: $B/B_{max} = [X]^n/([EC_{50}]^n + [X]^n)$.

Where B is the actual fluorescence value, B_{max} is the theoretical maximum of B, × is the ligand in question, and n is the Hill coefficient.

Experiments were performed in quadruplets and the average values (mean ± SE) were used for calculations. When applicable, data were analyzed using a two-tailed un-paired *t*-test and $P < 0.05$ values were regarded as significant differences.

Microarray hybridization and functional data analyses

To examine the transcriptional profiles and identify differentially regulated genes with major biological and molecular functional differences that may serve to explain the variations, a cDNA array containing about 20400 probes on a glass-surface was used. Total RNA was isolated using an RNeasy Midi kit (Qiagen), and samples used for real time PCR were additionally on-column treated with RNase-Free DNase I (Qiagen), following the manufacturer's instructions. For hybridization, 15 μg of total RNA from each ESC sample was labeled either with Cy3 or Cy5 fluorescent dyes, and the dyes were swapped during the hybridization. All procedures of microarray processing were carried out as described in earlier studies[24,25].

The hybridized slides were scanned and MIAME-compliant gene expression data were submitted to the Gene Expression Omnibus (GEO) database (GSE 7173). A full description of the DNA-chip platform is available from the same database (GPL 3697). Similar to the earlier studies, genes were ranked according to the lowest absolute ratio of signal intensities regardless of reproducible up- or down-regulation. The false discovery rate among the top ranked and reproducibly regulated genes was calculated by random permutations of genes and expression ratios. For further functional characterizations, the resulting lists of regulated genes were imported into the Expression Analysis Systematic Explorer (EASE) and the Database for Annotation, Visualization, and Integrated Discovery softwares (DAVID)[26,27]. These software tools systematically mine the functional information associated with the generated microarray data[28], and analyze for overrepresentation in the gene ontology (GO) biological process, molecular function and cellular component. However, it is important to note that overrepresentation does not refer to the abundance of gene expression but rather describes a class of genes that have similar functions, regardless of their expression level[27].

Real time PCR analysis and data validation

For validation of the results, independent samples of these ESCs were prepared from the same passage aliquots. Equal amounts of total RNA from each sample were reverse transcribed to cDNA, and real time PCR was used for validation. Gene specific primers for a subset of differentially regulated genes from the microarray analysis, additional primers for various membrane and intracellular receptors involved in Ca^{2+} homeostasis and two reference genes were designed. The details of cDNA synthesis, real-time PCR and analysis procedures were as described earlier[29].

RESULTS

FACS analysis and pluripotency markers

First we determined the proportion of cells expressing the main pluripotency marker genes *Pou5f1, Nanog, Fut4*

Table 2 Comparative targeting efficiencies of R1 and HM-1 mouse embryonic stem cells

Parameters	Cell line (passage number)									
	R1 (p12)					HM-1 (p21)				
	Amaxa			BioRad	Σ	Amaxa			BioRad	Σ
Electroporation program	A23[1]	A24[1]	A30[1]			A23[1]	A24[1]	A30[1]		
Cell number		3×10^6		3×10^7			3×10^6		3×10^7	
DNA concentration (μg)		5		10			5		10	
Number of selected clones	120	192	168	356	836	168	168	24	384	744
Positive clones after 1st PCR screen	3	10	11	15	39	5	4	0	15	24
Percentage (%)	2.5	5.2	6.5	4.2	4.7	2.9	2.4	0	3.9	3.2
Positive clones after 2nd PCR screen	3	10	11	15	39	5	4	0	15	24
Homologous recombination confirmed by Southern blot analysis										
Clone number	3	9	10	7	29	4	4	0	5	14
Efficiency (%)	2.5	4.7	6	2	3.5	2.4	2.4	0	1.3	1.9
Transfection efficiency										
Transient	No data on Amaxa system			91% ± 6.1%		No data on Amaxa system			75% ± 4.2%	
Stable	No data on Amaxa system			34% ± 2.9%		No data on Amaxa system			25% ± 5.3%	

[1]Amaxa program numbers. PCR: Polymerase chain reaction.

and *Sox-2* in mouse R1 and HM-1 ESCs based on FACS (Fluorescent Activated Cell Sorting) analysis. The results revealed the expression of these classical pluripotency marker genes in both cell lines. As shown in Table 1, the average percentages of positive cells were comparable with no significant difference in the levels of *Pou5f1*, *Nanog*, *Fut4* and *Sox-2* between R1 and HM-1 ESCs. The detected comparable profiles, in the percentage of positive cells, confirmed pluripotency of the two ESCs used for the study and gave us confidence in the subsequent results derived from the analysis.

Transfection and targeting efficiencies

To examine the potentials of R1 and HM-1 mouse ESCs for gene targeting and generation of genetically modified mice, we compared the efficiency of homologous recombination in these ESCs using the same non-isogenic targeting construct. The targeting efficiency was calculated by the ratio of positive clones (homologous recombined clones), confirmed by Southern blot analysis, to the total number of screened clones. The results of analysis revealed significant differences between these cell lines (R1 *vs* HM-1), transfection platforms (Amaxa *vs* Bio-Rad) and Amaxa program numbers (A23, A24 and A30).

Furthermore, major variations in the efficiency of the two ESC lines were also observed with the different Amaxa programs. Using program A23 of Amaxa, there was no significant difference, in the percentage of positive clones, between the R1 (2.5%) and HM-1 (2.4%) ESCs. However, based on the Southern blot confirmed positive clones formation efficiency from program A24 of Amaxa, the R1 ESCs outperformed the HM-1 (4.7% *vs* 2.4%). Similarly, when comparing the percentage of positive clones from program A30 of Amaxa, the R1 ESCs performed best (6.0%), while HM-1 ESCs had no positive clones at all, indicating another major difference between the two ESC lines. Similar comparison was also made with Bio-Rad system, and the results revealed better performances of R1 ESCs compared to HM-1 (2.0% *vs* 1.3%). However, the latter difference was not statistically significant.

Moreover, the numbers of PCR positive clones obtained from Bio-Rad system were significantly ($P < 0.05$) reduced during Southern blot confirmation analysis, while more than 90% of similar clones from the Amaxa system were further confirmed, indicating higher incidence of false positives in the former. The transfection efficiency of both ESCs was also compared using BioRad system. The results of transient transfection was significantly better for R1 ($P < 0.01$) than for HM-1 ESCs (Table 2).

Gene expression profiles and reproducibility analysis

We compared the genome-wide expression profiles of the two ESCs, using microarrays to examine the possible causes of variations observed in targeting efficiency. During analysis, genes were ranked according to the lowest ratio of expression (HM-1/R1) in four independent chip hybridization experiments, and the ranking was independent of consistent up- or down-regulation. However, the vast majority of the top selected genes were reproducibly regulated in all four hybridization experiments. Following consideration of the reproducibility analysis and the estimation of false positive genes, the significant analysis resulted in the selection of 55 differentially regulated genes. Only 8 transcripts were up-regulated, while 47 transcripts were down-regulated in the HM-1 compared to R1 ESCs (Table 3).

Functional classifications of the differentially regulated genes

To search for important biological clusters that may serve to explain the functional roles of differentially regulated genes, we used the overrepresentation analysis modules

Table 3 Differentially regulated transcripts between R1 and HM-1 embryonic stem cells

Lion ID	Gene symbol	Gene name	Locus ID	Unigene ID	Reference sequence	Fold change			Chrom location
						Min	Aver	Stdv	
Up regulated in HM-1 compared to the R1 embryonic stem cells									
MG-4-li7	Rps15a	Ribosomal protein S15a	267019	Mm.288212	NM_170669	1.44	1.5	0.08	7 F1
MG-3-6b3	HSPa9a	Heat shock protein 9a	15526	Mm.209419	NM_010481	1.4	1.48	0.05	18 15cM
MG-14-78c20	B930046C15Rik	RIKEN cDNA B930046C15Rik gene	544998	Mm.327147	XM_987063	1.39	1.53	0.1	14 A1
MG-13-43a6	Lefty1	Left right determination factor	13590	Mm.378911	NM_010094	1.33	1.5	0.14	1 F
MG-3-1 1116	LOC544988	Hypothetical protein LOC544988	544988	Mm.350858	NM_001024712	1.29	1.4	0.08	14 A1
MG-3-32g7	Nsmce1	Non.SMC element 1 homolog	67711	Mm.4467	NM_026330	1.28	1.43	0.1	7 F3
MG-11-2g16	Glo1	Glyoxylase 1	109801	Mm.261984	NM_025374	1.26	1.33	0.05	17 16.0 cM
MG-3-223f10	EST					1.26	2.35	1.26	
Down regulated in HM-1 compared to the R1 embryonic stem cells									
MG-15-102f2	Dab2	Disabled homolog 2 drosophila	13132	Mm.240830	NM_001008702	1.99	2.13	0.13	15 6.7cM
MG-12-140m7	Spink3	Serine protease inhibitor Kazal-type 3 precursor	20730	Mm.272	NM_009258	1.92	2.1	0.14	18 B3
MG-3-251i14	Sparc	secreted acidic cystein rich glycoprotein	20692	Mm.291442	NM_009242	1.86	2.05	0.13	11 29.9 cM
MG-15-55i8	Amot	Angiomotin	27494	Mm.100068	NM_153319	1.76	1.9	0.14	X F2
MG-8-118g22	Krt2-8	Keratin complex 2 basic gene 8	16691	Mm.358618	NM_031170	1.75	1.93	0.13	15 58.86cM
MG-13-5n3	Cryab	Crystallin, α B	12955	Mm.178	NM_009964	1.7	1.78	0.1	9 29.0cM
MG-15-261m9	BC024814	cDNA sequence BC024814	239706	Mm.214953	NM_146247	1.69	1.88	0.15	16 A1
MG-8-17b12	Prph1	Peripherin 1	19132	Mm.2477	NM_013639	1.66	1.98	0.25	15 55.5 cM
Table 3b. cont.	Gpc4	Glypican-4 precursor (K-glypican)	14735	Mm.1528	NM_008150	1.62	2.1	0.37	X 16.0 cM
MG-8-34a20									
MG-13-91a5	Serpinh1	Serine (Cystein) proteinase inhibitor, clade H, member 1	12406	Mm.22708	NM_009825	1.56	1.63	0.05	7 E1
MG-14-101o20	Ctgf	Connective tissue growth factor precursor	14219	Mm.1810	NM_010217	1.56	1.73	0.1	10 17 cM
MG-12-197o17	Podxl	Podocalyxin like protein 1 precursor	27205	Mm.89918	NM_013723	1.46	1.58	0.15	6 10.0 cM
MG-6-75c23	Lamc1	Laminin gamma 1	226519	Mm.1249	NM_010683	1.45	1.5	0.08	1 81.1 cM
MG-8-118f19	Anxa2	Annexin 2	12306	Mm.238343	NM_007585	1.45	1.5	0.08	9 37.0 cM
MG-16-54i3	9630046K23Rik	RIKEN cDNA 9630046K23 gene	224143	Mm.284366	NM_172380	1.44	1.58	0.13	16 B3
MG-6-2i22	Timp2	tissue inhibitor of metalloproteinase 2	21858	Mm.206505	NM_011594	1.42	1.5	0.08	11 72.0 cM
MG-13-1c9	S100a10	Calpactin I light chain	20194	Mm.1	NM_009112	1.41	1.48	0.05	3 41.7 cM
MG-14-79j4	Lhfp12	Lipoma HMGIC fusion partner-like 2, mRNA	218454	Mm.316553	NM_172589	1.41	1.58	0.21	13 C3-D1
MG-3-38p22	Ctsl	Cathepsin L precursor	13039	Mm.930	NM_009984	1.38	1.63	0.15	13 30.0 cM
MG-3-91d9	Kcnh2	Potassium voltage-gated channel, sub family H (eagralated), member 2	16511	Mm.6539	AC113055	1.38	1.53	0.1	5
MG-3-45k24	Gsn	Gelsoin	227753	Mm.21109	NM_146120	1.37	1.48	0.15	2 24.5 cM
MG-6-31m16	Clec21	C-type lectin domain family, member 1	381758	Mm.349066	XM_355753	1.37	1.4	0	6 B1
MG-13-2e2	EST	EST			CR517795	1.36	1.48	0.1	
MG-3-171n1	Iap1-3	Intracisternal A particles, Eya 1 linked	15601		AF097546	1.35	1.5	0.08	1 10.4 cM
MG-3-85d19	Sfrsi	Splicing factor, arginine/serine-rich (ASF/SFZ)		Mm.45645	CR518131	1.34	2.05	0.89	11
MG-12-195b2	Timp3	Metalloproteinase inhibitor 3 precursor (TIMP3) tissue inhibitor	21859	Mm.4871	NM_011595	1.33	1.45	0.13	10 47.0 cM
MG-14-58h24	Col8a1	Procollagen type Ⅷ, α 1	12837	Mm.370175	BC058281	1.33	1.45	0.1	16 C1.1
MG-3-23b6	Tpm1	Tropomyosin 1 α	22003	Mm.121878	M22479	1.32	1.38	0.05	9 40.0 cM
MG-13-1k15	S100a4	S100 calcium binding protein A4	20198	Mm.3925	BC051214	1.31	1.45	0.13	3 43.6 cM
Table 3b. cont.	S100a1	S100 calcium binding protein A1	20193	Mm.24662	NM_011309	1.31	1.35	0.06	3 43.6 cM
MG-3-72e5									
MG-68-98m4	Ctsz	Cathepsin Z precursor	64138	Mm.156919	NM_022325	1.31	1.4	0.08	2 103.5 cM
MG-13-117j9	Ahnak	AHNAK nucleoprotein (desmoyokin), mRNA	66395	Mm.203866	BC006892	1.29	1.4	0.08	19 A
MG-15-217f23	A930026I22Rik	RIKEN cDNA A930026I22 gene	77970	Mm.259790	BX632387	1.28	1.38	0.05	19 C3
MG-6-30n6	S100a6	S100 calcium binding protein A6 (Calcyclin)	20200	Mm.100144	NM_011313	1.28	1.45	0.1	3 43.6 cM
MG-6-30o19	Clstn3	calsyntenin 3	232370	Mm.193701	NM_153508	1.28	1.35	0.06	6 F2
MG-6-31b18	Camkk2	Calcium/calmodulin-dependent protein kinase kinase 2 β	207565	Mm.289237	NM_145358	1.28	1.3	0	5 F
MG-12-3f11	S100g	S100 calcium binding protein G	12309	Mm.6891	NM_009789	1.27	1.3	0	X F4
MG-6-1g19	My16	myosin, light polypeptide 6, alkali, smooth muscle and non-muscle	17904	Mm.337074	NM_010860	1.27	1.35	0.06	10
MG-6-31b24	4933433P14Rik	RIKEN cDNA 4933433P14 gene	66787	Mm.248019	BC031494	1.27	1.4	0.08	12 E
MG-12-42l4	Cotl1	Coactosin-like 1 (Dictyostelium)	72042	Mm.141741	XM_489250	1.26	1.45	0.13	8 E1
MG-3-254m20	A430065p19	Riken A430065P19 gene	329421	Mm.99648	NM_177376	1.26	1.45	0.13	2 C2

MG-3-37p2	Qscn6	Quiescin Q6	104009	Mm.27035	NM_001024945	1.26	1.38	0.05	1 G3
MG-6-31h10	Map3k11	mitogen activated protein kinase kinase kinase 11	26403	Mm.185020	NM-022012	1.26	1.33	0.05	19 0.5 cM
MG-6-82c15	Dbp	D site albumin promoter binding protein	13170	Mm.378235	NM_016974	1.26	1.3	0	7 23.0 cM
MG-8-17n2	Stard9	START domain containing 9	211824	Mm.246506	XM_619801	1.26	1.38	0.05	2 F1
MG-14-95h3	Ggta1	Glycoprotein galactosyltransferase α 1	14594	Mm.281124	NM_010283	1.25	1.38	0.15	2 25.0 cM
MG-3-6912	AC116589				AC116589	1.25	1.4	0.08	13

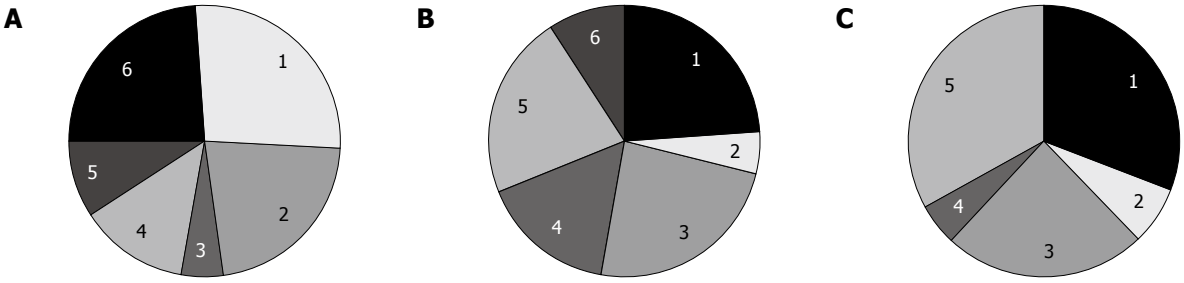

Figure 1 Overrepresentation analysis of the differentially regulated genes between R1 and HM-1 embryonic stem cells. Panels represent analysis results based on (A, 1: Morphogenesis and development, 15, 27%; 2: Organogenesis, 12, 22%; 3: Temperature response, 3, 5%; 4: Response to abiotic stimulus, 7, 13%; 5: Miscellaneous, 5, 9%; 6: Unclassified, 13, 24%) GO biological process, (B, 1: Calcium ion binding, 14, 24%; 2: Heat shock protein activity, 3, 5%; 3: Protein binding, 14, 24%; 4: Structural molecule activity, 9, 16%; 5: Unclassified, 13, 22%; 6: Enzyme regulator activity, 5, 9%) GO molecular function, (C, 1: Extracellular metrix, 17, 31%; 2: Basement membrane, 4, 7%; 3: Unclassifed, 13, 24%; 4: Muscle fiber, 3, 5%; 5: Miscellaneous, 18, 33%) GO cellular components.

of EASE and classified them according to gene ontology (GO) annotation. Despite the multiple roles played by some genes, the analysis revealed five major biological processes. In general, 27% of the regulated genes were annotated with morphogenesis and development, and 22% with organogenesis. Most of the differentially expressed ESTs and some other genes, which together account for 24% of regulated ones were not classified. Those genes that are overrepresented in response to abiotic stimulus and temperature constitute 13% and 5% of the differentially regulated genes, respectively (Figure 1A).

To examine the existence of specific molecular roles that can be related to the variations, the molecular functions of these genes were also analyzed. The majority of classified transcripts possess molecular binding functions with protein binding and calcium ion binding in equal proportions (24% each). The molecular functions of about 22% of the transcripts were not known. The remaining regulated genes were annotated as structural molecules (16%) and regulators of enzyme activity (9%) (Figure 1B). Moreover, the cellular localizations of the differentially expressed genes were also examined. 31% of the differentially regulated transcripts were overrepresented in the extracellular region. The cellular localizations of about a quarter (24%) of genes were not known with the rest annotated to different cellular locations (Figure 1C).

Validation of the microarray results with real time PCR analysis

We used real time PCR as an independent analysis tool to investigate a subset of the differentially regulated genes and validate the results of microarray analysis. Primer sequences and product sizes are listed in Table 4. The examined subset of genes include Ca^{2+} binding cluster genes such as *Camkk2* and S100 family (*S100a4*, *S100a6*, and *S100a10*) genes and some other up- and down regulated transcripts (Figure 2). The results of the real time PCR analysis fully confirmed the trends and patterns of expression observed during microarray analysis with minor ratio variations, compared to the microarray results.

Intracellular Ca^{2+} handling and homeostasis

Because of the significant variations observed during molecular function analysis (for the calcium binding proteins) we further examined the calcium handling potentials of these ESCs. A previous study[30] described the functional existence of multiple Ca^{2+} signaling pathways on ES-D3 mouse ESCs. Therefore, we compared the characteristics of intracellular Ca^{2+} handling potentials of R1 and HM-1 ESCs in high Ca^{2+} solutions (i.e. 1.8 mmol/L), using various agonists and FLIPR (FlexStation Ⅱ ^{384} fluorimetric image plate reader). First, we tested the effect of ATP, a well-known activator of P2 purinergic receptors[31], on the responsiveness of the cells. The application of ATP (0.01-100 μmol/L) resulted in very similar, dose-dependent elevations of $[Ca^{2+}]_i$ in both ESCs, with comparable EC_{50} values (Figure 3A). We also examined the performances in high and low Ca solutions. In HM-1 ESCs, the suppression of extracellular calcium concentration $[Ca^{2+}]_e$ did not modify the responsiveness of the cells to ATP (Figure 4A). In contrast, in R1 ESCs, 200 μmol/L ATP induced a significantly smaller elevation of $[Ca^{2+}]_i$ in low-Ca^{2+} solution than under high-Ca^{2+} conditions (Figure 4A). Similarly, the responsiveness of the two cell types to bradykinin (0.02-20 μmol/L), an agonist of metabotropic bradykinin receptors[32], was very similar (Figure 3B). In addition, when the $[Ca^{2+}]_e$ was decreased, both cell types responded with significantly

Table 4 Primer sequences and polymerase chain reaction conditions of genes selected for real time polymerase chain reaction assays

Gene symbol	Gene name	Primer sequence (5' to 3')	Product size (bp)	Annealing T°
S100a4	S100 calcium binding protein A4	For-TCAAGCTGAACAAGACAGAGC Rev-ACTTCATTGTCCCTGTTGCTG	131	56
S100a6	S100 calcium binding protein A6	For-TCCACAAGTACTCTGGCAAGG Rev-GGTCCAGATCATCCATCAGC	138	56
S100a10	S100 calcium binding protein A10	For-AGCTCTTCCAAGGACTGCTG Rev-TTTGTCAAGTGGTCTTTGTCG	138	60
Camkk2	Calcium/calmodulin-dependent protein kinase kinase2, β	For-GTCACACCACGTCTCCATTAC Rev-ACTTTCATTGCGTAATAAGTATTGTC	144	56
Tpm1	Tropomyosin 1 α	For-ATGCCCGTGTTCCTTAAAGC Rev-CCCTGACATGGAGAACTGGG	157	59
Sparc	Secreted acidic cystein rich glycoprotein	For-ATCCCCATGGAACATTGCAC Rev-TCCTTGTTGATGTCCTGCTCC	122	59
Trh	Thyrotropin releasing hormone	For-GGTTCTTCCACGCCTCCTAAG Rev-AACCTTGGAGGATGCGCTG	135	59
Lefty1	Left right determination factor 1	For-GCAAACCAAGGACAGAATCCC Rev-TGTTTGCCCAACTTGTGGC	108	59
P2rx1	Purinergic receptor P2X, ligand-gated ion channel 1	For-GTCTCCAGGCTTCAACTTC Rev-GAGCCGATGGTAGTCATAGT	156	56
P2rx2	Purinergic receptor P2X, ligand-gated ion channel 2	For-GTAGAGCAAGCAGGAGAGA Rev-AGACAAGTCCAGGTCACAG	89	60
P2rx3	Purinergic receptor P2X, ligand-gated ion channel 3	For-CCCGCTAAGACCTGAATCT Rev-AGTCCCTGGTATGTGGTAGG	142	60
P2rx4	Purinergic receptor P2X, ligand-gated ion channel 4	For-CTGTGTGACGTCATAGTCC Rev-GCTCGTAGTCTTCCACATAC	85	60
P2rx5	Purinergic receptor P2X, ligand-gated ion channel 5	For-GCTTTCTTCTGTGACCTG Rev-ATCTTCCTCCTTCTGACC	99	60
P2rx6	Purinergic receptor P2X, ligand-gated ion channel 6	For-CCCAAAGACGACTACCAA Rev-CAAACCACTCCTAGGACACT	86	60
P2rx7	Purinergic receptor P2X, ligand-gated ion channel 7	For-GGAAGTTAACCGTTCCTG Rev-TGGGCTAGACCTACTTCC	98	60
P2ry1	Purinergic receptor P2Y, G-protein coupled 1	For-GGTCTAGCAAGTCTCAACAG Rev-GTAAATTGGCCTCACTCC	121	60
P2ry2	Purinergic receptor P2Y, G-protein coupled 2	For-CCAAGCATGGAGAGGAGT Rev-GAATTGCTTGCACCACAG	156	60
P2ry4	Purinergic receptor P2Y, G-protein coupled 4	For-ACTAACTGCAGGCAGAGG Rev-CCAGCAAAGAGTACTGAGG	170	60
P2ry5	Purinergic receptor P2Y, G-protein coupled 5	For-CATGTACCCGATCACTCTC Rev-GAACCTGGAGTCACTTCTTC	136	60
P2ry6	Purinergic receptor P2Y, G-protein coupled 6	For-GAGTTCTGCGTGTGTGTG Rev-GTCAGCCTTTCCTATGCT	172	60
P2ry10	Purinergic receptor P2Y, G-protein coupled 10	For-GACATTTGGTATGCAGGCAAG Rev-TGGTCCCTTCCTCTTCCTTAGT	82	56
P2ry12	Purinergic receptor P2Y, G-protein coupled 12	For-TGCTGAGGTGCTCAAACTCTAC Rev-GGGTCTCTTCGCTTGGTTC	84	56
P2ry13	Purinergic receptor P2Y, G-protein coupled 13	For-AACAGAGCACCAGAAGAGAG Rev-AGGATGCAGATGCTGTTG	119	60
P2ry14	Purinergic receptor P2Y, G-protein coupled 14	For-CACAAAGAGTCAGACGGAAGG Rev-ACATTGGCAGCCGAGAGTAG	96	60
Bdkrb1	Bradykinin receptor, β 1	For-AGTCGTCCCTGATCTGAA Rev-GTTCAACTCCACCATCCT	85	56
Bdkrb2	Bradykinin receptor, β 2	For-CACGCAGATCAGTTCCTAC Rev-ACCTCTCGGGACTTCTTC	99	60
Hrh1	Histamine receptor H1	For-GGGCTACATCAACTCCAC Rev-CCCTCTTGGACATCAGAC	153	60
Hrh2	Histamine receptor H2	For-CCTCTCCTTCCTCTCTATTC Rev-CCACCAGTCCATATACCTC	110	60
Hrh3	Histamine receptor H3	For-ACAGTCAGCAGGAGGGAGAG Rev-TGTCTTCACATTGGCAGAGG	135	56
Hrh4	Histamine receptor H4	For-CTTGGAAGAACAGCACGAAC Rev-GAGATGACAGGAAGCAGGAA	106	56
Ryr1	Ryanodine receptor 1, skeletal muscle	For-ACTTTGTACCCTGTCCTGTG Rev-CATAGGTCCATCCTTGCTC	132	60
Ryr2	Ryanodine receptor 2, cardiac muscle	For-GGATGAATGTCTCACTGTCC Rev-CTTATGTGGCTTCCACTCC	147	60
Ryr3	Ryanodine receptor 3	For-CAGGTATCTTGGAGGTCTTG Rev-GCCCATGCTTATCCAGTAG	111	60

Itpr1	Inositol 1,4,5-triphosphate receptor 1	For-GGTTGATGACCGTTGTGTTG Rev-GAACTGTTTCTGTGCGGAGT	121	60
Itpr2	Inositol 1,4,5-triphosphate receptor 2	For-GTGAGGATGGCATAGAAAG Rev-AGAAGAACAGGAGGTCGTAG	165	60
Itpr3	Inositol 1,4,5-triphosphate receptor 3	For-GGCTTCATCAGCACTTTGG Rev-GCAATCTCGGAACTTCTTGG	96	60
H2afz	H2A histone family, member Z	For-ACAGCGCAGCCATCCTGGAGTA Rev-TTCCCGATCAGCGATTTGTGGA	202	60
Ppia	Peptidylprolyl isomerase A	For-CGCGTCTCCTTCGAGCTGTTTG Rev-TGTAAAGTCACCACCCTGGCACAT	150	60

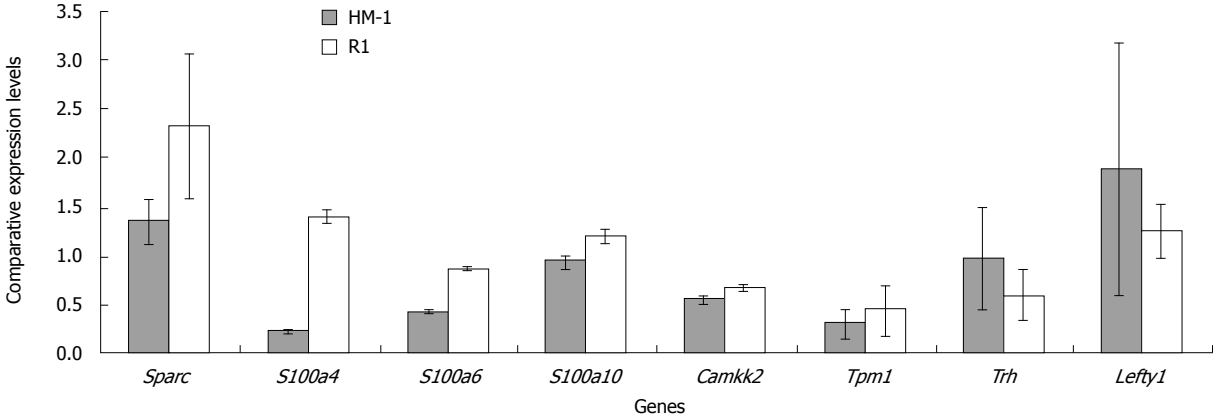

Figure 2 Relative expression analysis results of real time polymerase chain reaction for subsets of genes in R1 and HM-1 embryonic stem cells. Independently prepared samples were compared to validate the results of microarray. The analysis of real time polymerase chain reaction results supports the earlier microarray results with minor ration variations.

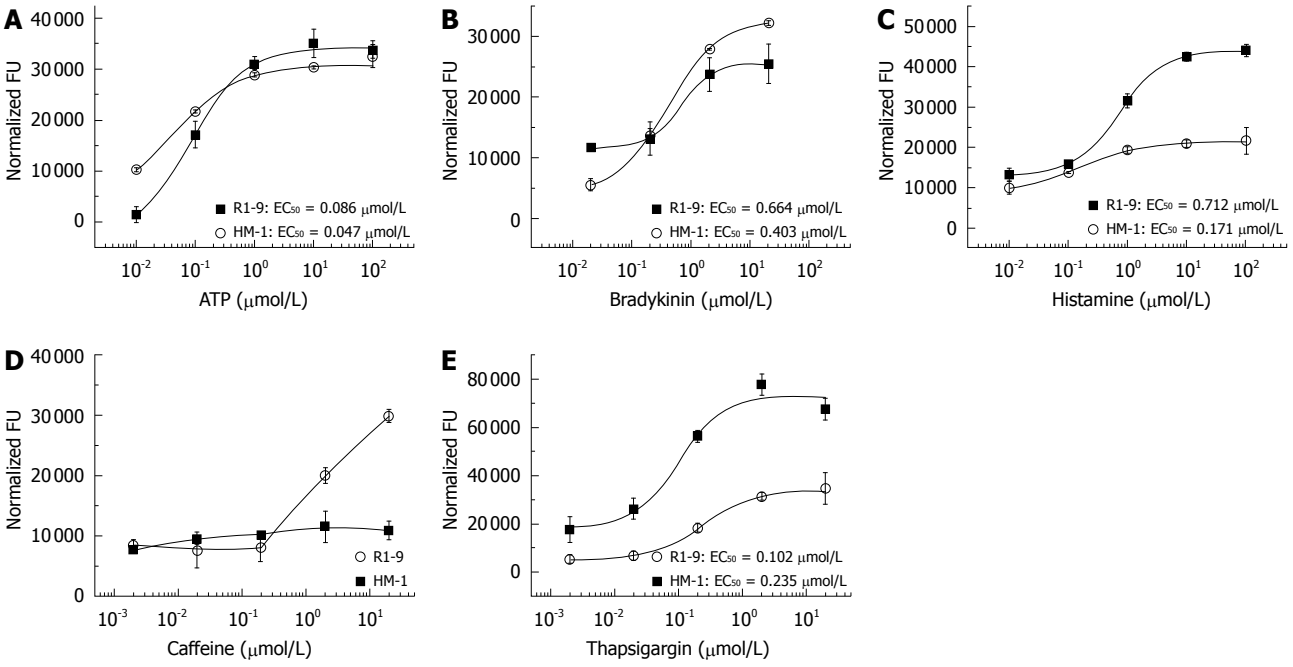

Figure 3 Comparison of agonist-induced changes in $[Ca^{2+}]_i$ in mouse R1 and HM-1 embryonic stem cells. The plates were placed into a FlexStation II384 (FLIPR) to monitor cell fluorescence (IEX = 494 nmol/L, IEM = 516 nmol/L, FU, fluorescence units) before and after the addition of various concentrations of agents in high-Ca (1.8 mmol/L) Hank's medium. A: ATP; B: Bradykinin; C: Histamine; D: Caffeine; E: Thapsigargin.

smaller Ca-transients to high (20 μmol/L) concentrations of bradykinin (Figure 4B).

We then investigated the effect of histamine, another agent which activates metabotropic G-protein-coupled receptor pathways[30,33]. Histamine (0.01-100 μmol/L) induced markedly different Ca^{2+} release response patterns in the two cell types (Figure 3C). Namely, although the EC_{50} values were comparable, the amplitudes of the maximal histamine-evoked $[Ca^{2+}]_i$ elevations (B_{max} values) of R1 ESCs were more than two-fold higher than those measured on

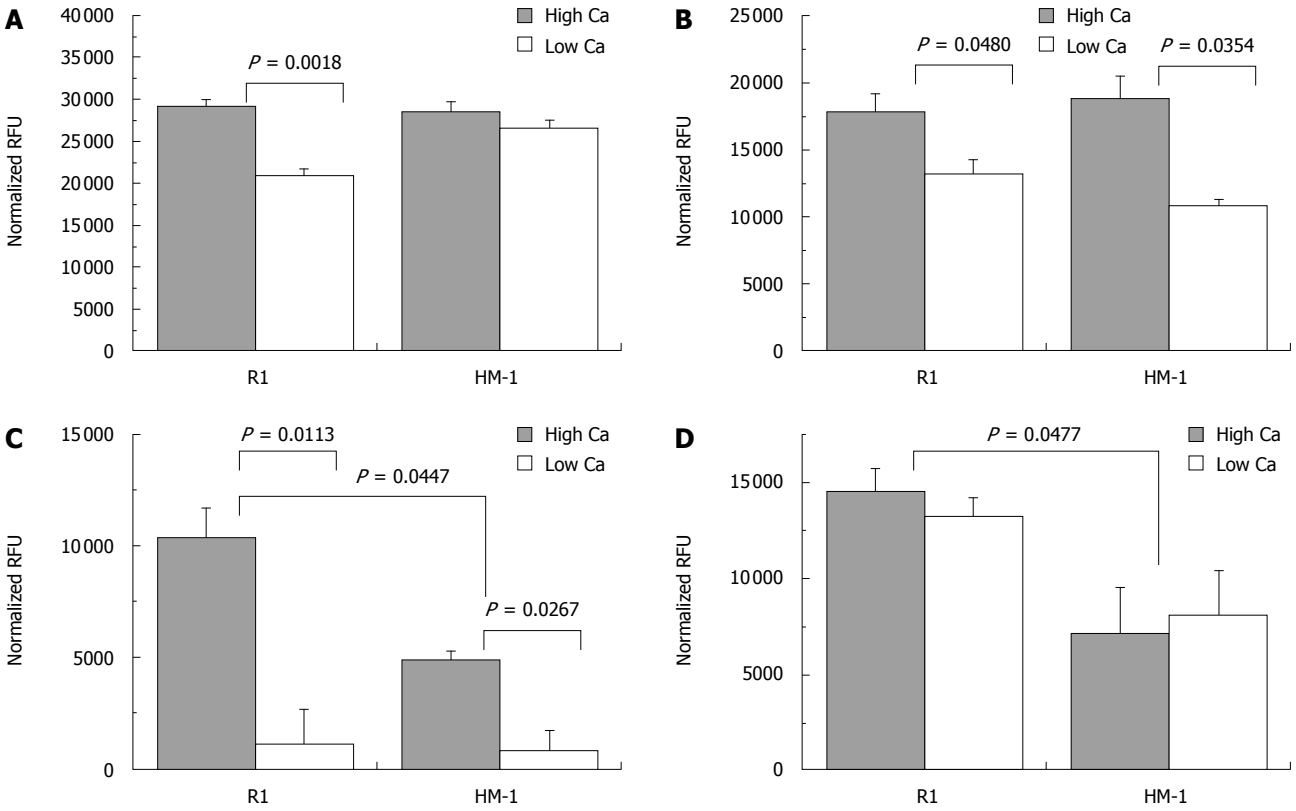

Figure 4 Comparison of agonist-induced changes of $[Ca^{2+}]_i$ in high- and low-Ca solutions. Cell fluorescence was monitored before and after addition of agents in high-Ca (1.8 mmol/L) and low-Ca (0.6 mmol/L) Hank's medium. Panels summarize effects of (A) 200 mmol/L ATP (B), 20 mmol/L Bradykinin (C), 100 mmol/L Histamine (D) 20 mmol/L Thapsigargin. Asterisks mark significant ($P < 0.05$) differences.

HM-1 ESCs. Importantly, however, the responsiveness of both cell types was strongly dependent on the $[Ca^{2+}]_e$ since we observed only minimal $[Ca^{2+}]_i$ elevation upon histamine (100 μmol/L) administration in low-Ca solutions (Figure 4C). Previous studies have also shown that certain tyrosine kinase coupled (metabotropic) receptors may also modulate $[Ca^{2+}]_i$[34]. Therefore, we also investigated the effect of two growth factors, NGF and EGF, on Ca-homeostasis of the cells. We found that none of the agonists modified $[Ca^{2+}]_i$ in either cell type (data not shown).

Functional intracellular Ca^{2+} stores may possess various Ca^{2+} release channels[18,35,36]. To investigate the presence of ryanodine receptors (RyRs), we employed caffeine (0.002-20 mmol/L), an activator of RyRs[30,37]. In high-Ca solution, R1 ESCs did not respond to caffeine (up to 20 mmol/L) (Figure 3D). However, under similar conditions, higher doses of caffeine (2-20 mmol/L) markedly ($P < 0.05$) elevated $[Ca^{2+}]_i$ in HM-1 ESCs (Figure 3D). As expected, the response of HM-1 ESCs to caffeine (20 mmol/L) was not affected by the suppression of $[Ca^{2+}]_e$ (data not shown). Finally, we investigated the Ca^{2+} content of the intracellular stores using thapsigargin, an inhibitor of the Ca^{2+} pump[30,38]. Thapsigargin (0.002-20 μmol/L) effectively elevated $[Ca^{2+}]_i$ in a dose-dependent manner in both cells types in high-Ca solution (Figure 3E). However, importantly, the maximal Ca^{2+} response was remarkably greater in R1 than in HM-1 ESCs. Furthermore, as expected, we found that suppression of $[Ca^{2+}]_e$ did not modify the amplitude of $[Ca^{2+}]_i$ elevations evoked by 20 μmol/L thapsigargin (Figure 4D).

Expression profiles of membrane and intracellular receptors involved in Ca-homeostasis

Since cell type-dependence was also observed when measuring the efficiency of the above agents in elevating $[Ca^{2+}]_i$ in R1 and HM-1 cells (Figure 3), it can be postulated that the receptor-mediated signaling mechanisms (including Ca-handling) are different in the different ESCs. In order to investigate this hypothesis, we performed a large number of experiments to identify the expression patterns of those receptors which either (1) function as molecular targets of the above agents that modulate $[Ca^{2+}]_i$ and the targeting efficiency; or (2) participate in the intracellular Ca-handling of the cells. For this we used qPCR (quantitative real time PCR) to measure and compare the expression of various receptors. Primer sequences and product sizes are listed in Table 4.

Indeed, as shown in Figure 5, qPCR analysis of gene expression levels of various surface membrane receptors (i.e. ionotropic P2X purinoreceptors; metabotropic P2Y purinoreceptors; metabotropic Bradykinin (B) receptor; metabotropic histamine (H) receptors) as well as of intracellular Ca-release channel receptors (i.e. ryanodine receptors; IP3 receptors) revealed that, as expected, HM-1 and R1 ESCs express markedly different patterns of receptors/molecules that participate in the regulation of Ca-

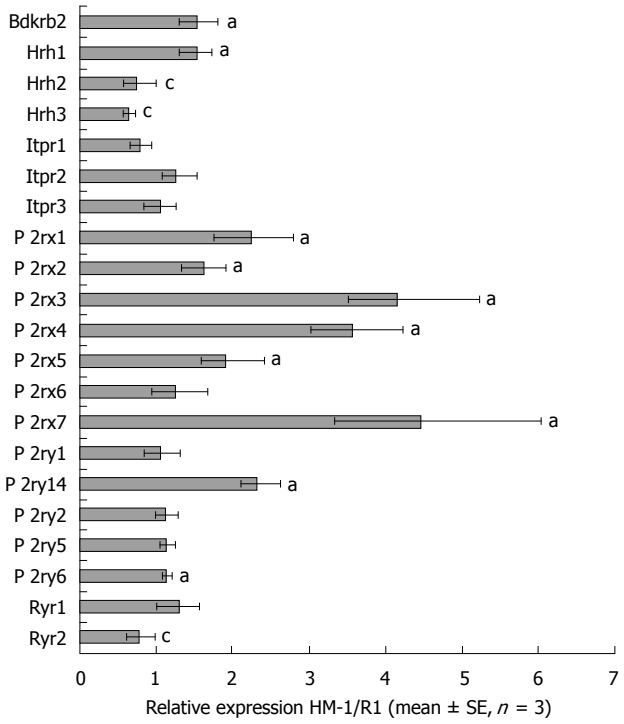

Figure 5 Differential expression of genes encoding surface membrane and intracellular receptors involved in the regulation of Ca-homeostasis of R1 and HM-1 embryonic stem cells. Independently prepared samples were used to measure the expression of various receptors by real time polymerase chain reaction and relative expression levels were presented. [a]Represent genes significantly ($P < 0.05$) up regulated; [c]Represent genes significantly ($P < 0.05$) down regulated in HM-1 compared to R1 embryonic stem cells.

homeostasis of these cells. The variations in the levels for most of the receptors was significant ($P < 0.05$) between these cell lines, as shown in Figure 5. Generally, the analysis revealed that the detection and expression intensity of these receptors was cell line dependent. This correlates well with our earlier observations and, thus, may contribute to the performance of these ESC lines.

DISCUSSION

Extensive molecular analysis was suggested as a prerequisite for the elucidation of the complex and interrelated processes that occur in biological systems[39]. In this study, we examined the gene targeting efficiency, the gene expression profiles and the Ca^{2+} homeostasis of two mouse embryonic stem cell lines, and found significant differences between them.

A number of research papers[40-42] have addressed the different aspects of DNA transfer and subsequent efficiency. A maximum DNA transfer into the cytoplasm of 7%, with only 4.5% reaching the nucleus, and further degradation of this fraction (in both cytoplasm and the nucleus) was described earlier[40]. To understand the causes of this and to increase the efficiency, different studies[3,8], have examined the effects of various factors, including contents of the medium, time of adding DNA and other ingredients, sizes of various molecules, DNA concentration, temperature, type of the construct, and extent of homology. One of these studies[42] examined the effects of various concentration gradients of calcium, phosphate solutions and other parameters on the kinetics and reproducibility of precipitate formation during transfection. Our study with one of its objectives to examine the inherent Ca^{2+} handling potentials of the transfected cells complementary and fills a knowledge gap.

As these ESCs were derived from the mouse strain129, the use of a non-isogenic DNA construct (C57B1/6) has enabled us to compare these ESCs under identical conditions. In our study, the targeting efficiency of 1.3% to 2% with BioRad and 2.4% to 6% with Amaxa systems is comparable to the previously reported values with non-isogenic mouse DNA constructs[8,43]. In line with our results, better performance using nucleofection was also reported by other studies[7,44]. Generally, R1 ESCs outperformed HM-1 ESCs in targeting efficiency irrespective of the transfection system and program types. Although details of the different Amaxa program packages (A23, A24, and A30) and the contents of the reagents are not known, we assume the existence of factors that elicited differential cellular responses. For the same program and conditions used in parallel, the two ESCs responded differently.

Following observation of the variations in targeting efficiencies, we then compared the global gene expression profiles using microarrays. Considering the genetic background from which the lines were derived, similar culture conditions, stringency of our filtering procedures and to avoid the possibility of missing informative genes with subtle differences, we decided to use at least 1.25 fold changes as bottom line for significance. The over-representation analysis in the biological process and in the molecular functions pointed to some functional roles of differentially regulated genes. The GO biological process defines the broad biological goals that are accompanied by ordered assemblies of molecular functions[28]. We focused mainly on the genes with the binding molecular functions, as they constituted the major categories of annotation for the differentially regulated genes (Figure 1B). Earlier studies[45,46] in different cell types have indicated the buffering capacity of calcium binding proteins in rendering the cells tolerant to the calcium load. Thus, the lower performances of HM-1 ESCs during transfection may be linked to a lower intrinsic potential. Comparison of these ESCs in high and low Ca^{+2} solutions confirmed this difference. Using the examined agonists, while no significant differences were observed in lower Ca^{+2} solutions, significant differences were observed in high Ca^{+2} solution comparisons (Figure 4). In order to further test the above observations, we used a range of agonists and examined the calcium handling potentials of these ESCs both in high and low calcium solutions (Figure 4). Evaluation of the effects of various agents, known as key regulators of intracellular homeostasis, revealed marked differences in the Ca-handling potentials of R1 and HM-1 ESCs. ATP is a well-known activator of P2 purinergic receptors[30,31]. Among these receptors, P2X purinoreceptors function as ionotropic ligand-gated with a marked permeability to calcium. P2Y metabotropic G-protein coupled purinorecep-

tors, however, initiate a phospholipase-C (PLC)-mediated intracellular signaling pathway which, eventually, results in the liberation of Ca^{2+} from the intracellular stores by acting on inositol-1,4,5-trisphosphate receptors (InsP3Rs), i.e. Ca^{2+} release channels[18,30,31,35,36]. Our FLIPR (FlexStation Ⅱ^{384} fluorimetric image plate reader) experiments revealed that whereas R1 and HM-1 ESCs responded very similarly in high-Ca solution, the ATP responsiveness of R1 ESCs but, importantly not of HM-1 cells, was significantly lower in low-Ca solution. These findings strongly suggest that in HM-1 ESCs, the elevated $[Ca^{2+}]_i$ originated exclusively from the intracellular stores whereas in R1 ESCs, a significant part of the Ca elevation was due to Ca-influx from the extracellular space.

Bradykinin activates metabotropic bradykinin receptors[32] which are similar to P2Y metabotropic purinoreceptors and were shown to initiate the G-protein - PLC - InsP3R intracellular signaling pathway to release Ca^{2+} from the intracellular stores[18,35,36]. Therefore, it was unexpected to observe the bradykinin-induced Ca-transients, similar for both ESCs, that showed a reasonable dependence on the $[Ca^{2+}]_e$. These intriguing findings suggest that the initiation of the bradykinin receptor-coupled signaling pathway, which apparently does exist in both ESC types, also results in an opening of a plasma membrane channel population, which is permeable for calcium. Such mechanisms were previously described in various cell types such as vascular endothelial cells and corneal epithelial cells[47,48]. Similar responses were observed in both cell types when histamine, the endogenous agonist of metabotropic histamine receptors (most of which also functioning *via* the G-protein - PLC - InsP3R -Ca-release pathway), was investigated[30,33]. An even more pronounced suppression of the amplitude of the histamine-evoked Ca-transients was measured in low-Ca medium suggesting that, intriguingly, most of the calcium originated from the extracellular space *via* Ca-permeable channels (similar to previous findings on other cell types)[47,49]. Moreover, we also found that R1 ESCs responded with much greater $[Ca^{2+}]_i$ elevations in response to histamine than HM-1 ESCs.

The above data have unambiguously demonstrated that both ESCs possess functional InsP3R-mediated Ca-release mechanisms. In contrast, we found that only HM-1 ESCs responded with $[Ca^{2+}]_i$ elevations to caffeine application. However, it was intriguing to observe (especially in light of the above data with caffeine) that thapsigargin, an inhibitor of the Ca^{2+} pump[30,38], was able to induce a markedly higher $[Ca^{2+}]_i$ elevation in R1 than in HM-1 ESCs. These data may indicate that the content of the (thapsigargin-sensitive) Ca-pools is much greater in R1 than in the other ESCs.

Finally, it is important to note that none of the agonists (NGF and EGF) of tyrosine kinase coupled (metabotropic) receptor pathways, caused changes in $[Ca^{2+}]_i$ suggesting the lack of functional tyrosine kinase receptor coupled signaling in R1 and HM-1 ESCs.

Taken together, our experiments revealed significant differences in the regulation of Ca^{2+} homeostasis of R1 and HM-1 ESCs and suggested that these differences may contribute to the different targeting efficiencies of the two cell types as measured in various functional *in vivo* and *in vitro* assays. The various agents differentially modulated the performances of these cell lines. These findings suggest that other components of the receptor-mediated signaling pathways may also differ in the various ESCs and hence may also play roles in determining the functional characteristics of the cells. Indeed, a series of gene expression analyses revealed that R1 and HM-1 ESCs exhibit markedly different expression patterns of genes encoding various surface membrane and intracellular receptors involved in the regulation of Ca-homeostasis of the cells. Hence, when a given agent is administered to different mouse ESCs, the activation of different receptor patterns (expressed on these cells) may induce differential elevation of $[Ca^{2+}]_i$ as well as initiation of distinct receptor-mediated intracellular signaling mechanisms. Collectively, this results in differential cellular effects (e.g. transfection efficiency) in the different cell types.

In summary, our study revealed some similarities and significant differences in targeting efficiency, Ca^{2+} homeostasis, and gene expression profiles between R1 and HM-1 ESCs. Despite the variations between the technique (Bio-Rad *vs* Amaxa) and program types (A23, A24 and A30) used, the cells with better potential (R1) performed better. Thus, our findings emphasize the significance of inherent cellular potentials for increased targeting efficiency, and are in line with the results of some previous studies[9,13,12,15,50] that have also acknowledged contributions of cellular differences to the experimental outcomes. The knowledge of intrinsic cellular differences in Ca^{2+} signals and their possible impacts on the targeting efficiency are significant inputs towards filling the current knowledge gap. A similar recent study[9,51] described the existence of marked variations in the differentiation propensity of the different human ESC lines. Differences in their potential may reflect the underlying genetic variations of the embryos from which the lines were derived, some other differences in the initial culture, and/or an interaction of the two factors. However, understanding these variations contributes to the selection of better performing cell lines for realizing the potentials of the ESCs for various applications. The approach of combining molecular profiles and *in vitro* culture performance will give better insight into the different cell lines. This is increasingly important for the characterization of human ESC lines before therapeutic use.

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COMMENTS

Background

Generation of knockout mouse models by targeted disruption of essential genes provides useful insights into genes that regulate development and allows investigators to dissect molecular developmental mechanisms. In an effort to understand the therapeutic applications of stem cells, mouse models have been created for a number of human genetic diseases, and gene targeting in stem cells has also been common practice. However, most studies have focused mainly on comparing the efficiency of different gene delivery techniques, parameters or phenotypes of the offspring carrying the mutant gene, but not the efficiency of different cell lines.

Research frontiers

Previous studies have acknowledged the existence of similarities and differences in the performances of different stem cell lines. In the area of improving the therapeutic applications of stem cells, the research hotspot is to increase the technical efficiencies of various genetic modifications and transfers in embryonic stem cells, in order to increase the chances of getting more colonies with required genetic information and recombination efficiency.

Innovations and breakthroughs

Currently mouse embryonic stem cells are used in various research applications, including gene targeting, with designed alterations in an exogenous DNA sequence and transfer to the DNA sequence in the living cell genome *via* homologous recombination. However, the procedures are laborious and performance of various stem cells differ markedly. Despite the acknowledged existence of variations among the different stem cell lines, most studies have focused mainly on comparing the efficiency of different gene delivery techniques, parameters or phenotypes of the offspring carrying the mutant gene. In the present study, the authors compare the two widely known mouse embryonic stem cell lines and show how the intrinsic qualities of these cells can affect the gene targeting performances and calcium handling potentials of these stem cells.

Applications

Results of this study emphasize the importance of considering intrinsic cellular variations, during selection of cell lines for experiments and interpretation of experimental results.

Terminology

Embryonic stem cells: Embryonic stem cells are cells derived from the inner cell mass of the developing blastocyst with a retained potential to self-renew and to differentiate into diverse cell lineages; gene targeting: Gene targeting is the transfer of designed alteration in an exogenous DNA sequence to the cognate DNA sequence in the living cell genome *via* homologous recombination; calcium homeostasis: Calcium homeostasis is the mechanism by which the body maintains adequate calcium levels.

Peer review

The manuscript entitled "Gene targeting and Ca^{2+} handling efficiencies in mouse embryonic stem cell lines" raises important questions to the scientific community and emphasizes the importance of considering intrinsic cellular variations during selection of cell lines for experiments and interpretations of experimental results. The novelty relies on Calcium handling efficiencies.

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Meetings

Events Calendar 2010

February 15-16, 2010
Stem Cells 2010
Crowne Plaza Hotel, St James, London, United Kingdom

May 11-13, 2010
World Stem Cells and Regenerative Medicine Congress
London, United Kingdom
http://www.terrapinn.com/2010/stemcells/index.stm

May 12-15, 2010
Stem Cells, Tissue Homeostasis and Cancer
EMBL Heidelberg, Germany
http://www.embl.de/training/courses_conferences/conference/2010/STM10-01/

June 16-19, 2010
ISSCR 8th Annual Meeting
Moscone West, San Francisco, CA United States
http://www.isscr.org/meetings/index.cfm

June 27-July 2, 2010
The 9th Gordon Conference on Cell Biology of the Neuron
Waterville Valley Resort, NH, United Kindom

July 11-14, 2010
3rd International Congress on Stem Cells and Tissue Formation
Dresden, Germany
http://www.stemcellcongress-dresden.org/

August 22-27, 2010
The 2010 Gordon Conference on Cell Death
Salve Regina University, Newport, RI, United Stated

October 4-6, 2010
World Stem Cell Summit 2010
Detroit Marriott Renaissance Center, Detroit, Michigan, United States
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Instructions to authors

GENERAL INFORMATION

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

The biggest advantage of the OA model is that it provides free, full-text articles in PDF and other formats for experts and the public without registration, which eliminates the obstacle that traditional journals possess and usually delays the speed of the propagation and communication of scientific research results. The open access model has been proven to be a true approach that may achieve the ultimate goal of the journals, i.e. the maximization of the value to the readers, authors and society.

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The role of academic journals is to exhibit the scientific levels of a country, a university, a center, a department, and even a scientist, and build an important bridge for communication between scientists and the public. As we all know, the significance of the publication of scientific articles lies not only in disseminating and communicating innovative scientific achievements and academic views, as well as promoting the application of scientific achievements, but also in formally recognizing the "priority" and "copyright" of innovative achievements published, as well as evaluating research performance and academic levels. So, to realize these desired attributes of *WJSC* and create a well-recognized journal, the following four types of personal benefits should be maximized. The maximization of personal benefits refers to the pursuit of the maximum personal benefits in a well-considered optimal manner without violation of the laws, ethical rules and the benefits of others. (1) Maximization of the benefits of editorial board members: The primary task of editorial board members is to give a peer review of an unpublished scientific article *via* online office system to evaluate its innovativeness, scientific and practical values and determine whether it should be published or not. During peer review, editorial board members can also obtain cutting-edge information in that field at first hand. As leaders in their field, they have priority to be invited to write articles and publish commentary articles. We will put peer reviewers' names and affiliations along with the article they reviewed in the journal to acknowledge their contribution; (2) Maximization of the benefits of authors: Since *WJSC* is an open-access journal, readers around the world can immediately download and read, free of charge, high-quality, peer-reviewed articles from *WJSC* official website, thereby realizing the goals and significance of the communication between authors and peers as well as public reading; (3) Maximization of the benefits of readers: Readers can read or use, free of charge, high-quality peer-reviewed articles without any limits, and cite the arguments, viewpoints, concepts, theories, methods, results, conclusion or facts and data of pertinent literature so as to validate the innovativeness, scientific and practical values of their own research achievements, thus ensuring that their articles have novel arguments or viewpoints, solid evidence and correct conclusion; and (4) Maximization of the benefits of employees: It is an iron law that a first-class journal is unable to exist without first-class editors, and only first-class editors can create a first-class academic journal. We insist on strengthening our team cultivation and construction so that every employee, in an open, fair and transparent environment, could contribute their wisdom to edit and publish high-quality articles, thereby realizing the maximization of the personal benefits of editorial board members, authors and readers, and yielding the greatest social and economic benefits.

Aims and scope

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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

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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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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]

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

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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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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

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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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