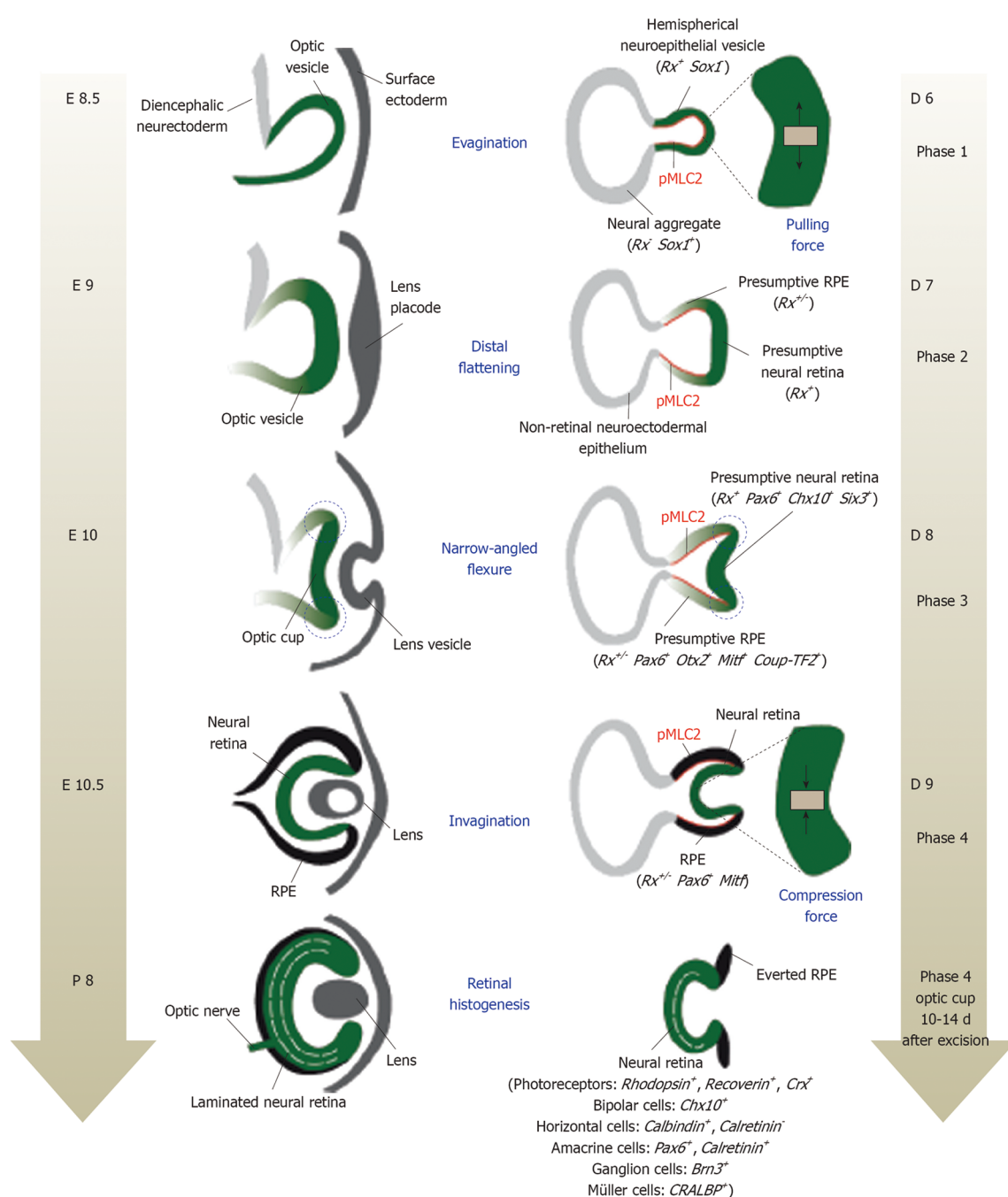


# World Journal of Stem Cells

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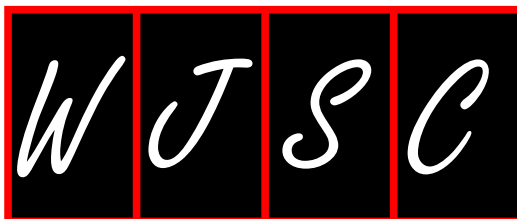
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## Shaping the eye from embryonic stem cells: Biological and medical implications

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### Abstract

Organogenesis is regulated by a complex network of intrinsic cues, diffusible signals and cell/cell or cell/matrix interactions that drive the cells of a prospective organ to differentiate and collectively organize in three dimensions. Generating organs *in vitro* from embryonic stem (ES) cells may provide a simplified system to decipher how these processes are orchestrated in time and space within particular and between neighboring tissues. Recently, this field of stem cell research has also gained considerable interest for its potential applications in regenerative medicine. Among human pathologies for which stem cell-based therapy is foreseen as a promising therapeutic strategy are many retinal degenerative diseases, like retinitis pigmentosa and age-related macular degeneration. Over the last decade, progress has been made in producing ES-derived retinal cells *in vitro*, but engineering entire synthetic retinas was considered beyond reach. Recently however, major breakthroughs have been achieved with pioneer works describing the extraordinary self-organization of murine

and human ES cells into a three dimensional structure highly resembling a retina. ES-derived retinal cells indeed assemble to form a cohesive neuroepithelial sheet that is endowed with the intrinsic capacity to recapitulate, outside an embryonic environment, the main steps of retinal morphogenesis as observed *in vivo*. This represents a tremendous advance that should help resolving fundamental questions related to retinogenesis. Here, we will discuss these studies, and the potential applications of such stem cell-based systems for regenerative medicine.

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**Key words:** Retina; Optic cup; Embryonic stem cells; Retinal pigment epithelium; Three dimensional culture

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### OPTIC CUP MORPHOGENESIS *IN VIVO*

Harmonious formation and patterning of tissues and organs during development relies on complex cellular interactions that are tightly regulated in both time and space. Among well known processes is embryonic induction, in which a group of cells causes changes in adjacent cell morphology, proliferative capacities or destiny. Eye development is often taken as a paradigm for successive and often reciprocal inductions that

progressively shape an organ. By the end of neurulation, the optic vesicle (OV) evaginates from the anterior neural tube, comes in close contact with the head surface or “pre-lens” ectoderm, and instructs it to become a lens placode. As a consequence, this ectodermal region thickens and eventually invaginates to form the lens vesicle. Concomitant to this process, complex structural changes take place in the OV, which in turn invaginates into an optic cup (OC) (Figure 1A). Although still a matter of debate (see below), the pre-lens ectoderm seems to be required for OC morphogenesis<sup>[1]</sup>. Then, the OC inner wall forms the sensory neural retina while the outer one forms the retinal pigmented epithelium (RPE). The ectoderm was shown to specify the neural retina, while diffusible signals emanating from the perocular mesenchyme determine the RPE. Besides, opposing influences from the neural tube or optic stalk on one hand and from the dorsal OC region on the other hand contribute to dorso-ventral patterning of the OC. This extensive series of interactions ensures the coordinated development of different parts which, together, will constitute a functional eye<sup>[2]</sup>.

## FROM MURINE EMBRYONIC STEM CELLS TO OC MORPHOGENESIS *IN VITRO*

In the past few years, major progress has been made in setting up efficient procedures allowing the *in vitro* differentiation of RPE or neural retinal cells from embryonic stem (ES) cells or induced pluripotent stem (iPS) cells<sup>[3-9]</sup> (Figure 2). Members of Yoshiki Sasai's group are among great contributors. A first milestone was the generation of neural precursors from ES cells cultured under low growth factor conditions (serum-free floating culture of embryoid-body-like aggregates with quick reaggregation, or SFEBq)<sup>[10,11]</sup>. Then, these neuralized ES cells were shown to acquire a retinal-restricted identity when exposed to biological compounds inhibiting Wnt and BMP/Nodal pathways (a known requirement for eye field formation *in vivo*) in the presence of Activin and serum<sup>[12]</sup>. However, in these procedures, cells do not self organize into neuroepithelia. Researchers from Sasai's group then turned to a three dimensional (3D) culture system by adding basement-membrane matrix components (matrigel) to the SFEBq culture medium<sup>[13-15]</sup>. In these conditions, ES cells were not only efficiently “retinalized”, but also organized within aggregates into polarized neuroepithelia. A striking phenomenon then started to occur after 6-7 d of culture with the budding of vesicles, followed 2 d later by an invagination process, giving rise to OC-like structures (Figure 1B). These were shown, as *in vivo*, to be composed of a distal portion exhibiting features of neural retina, and a proximal one expressing characteristic markers of RPE. The entire process of this spontaneous OC morphogenesis, monitored in 3D live imaging with multi-photon optics, could be subdivided in 4 main steps, resembling the developmental phases occurring *in vivo*. The first one is the

evagination of hemispherical vesicles from neuralized aggregates. Phase 2 is characterized by the flattening of the distal portion of the vesicles. During phase 3 the angles at the junctions (hinge points) between prospective neural retina and RPE become acute. Finally, phase 4 marks the invagination event that shapes the OC. Noteworthy, this whole dynamic process occurs in the absence of a full embryonic environment and in particular without lens or any surface ectoderm. Whether OC formation *in vivo* is indissolubly linked to lens development and *vice versa* has been a large matter of debate<sup>[1,16,17]</sup>. The findings reported by Eiraku *et al.*<sup>[13]</sup> show that, at least in this simplified *in vitro* context, lens is dispensable for OC invagination.

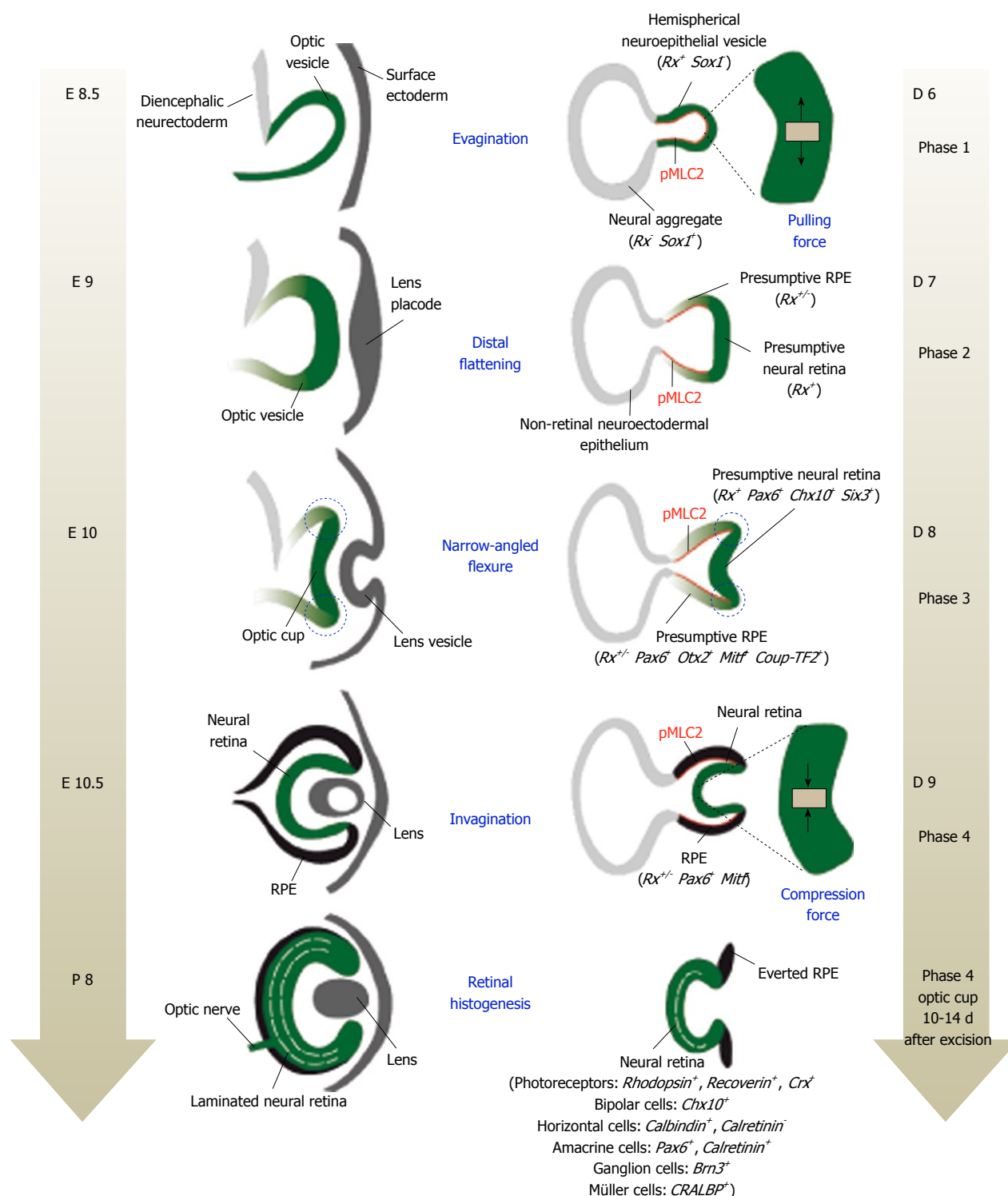
## CELL DIFFERENTIATION WITHIN MURINE ES-DERIVED RETINA

In the developing embryonic eye, newly generated OCs extensively grow as a result of cell proliferation. During this step, apico-basal interkinetic nuclear migration (INM) accompanies cell cycle progression of retinal progenitor cells. Such INM was also observed in ES-derived OC neuroepithelia, highlighting again the great similarity with the *in vivo* situation. Besides, when phase 4 OC were excised and maintained in long-term culture, they progressively acquired a typical lamination, reminiscent of the early postnatal retina. Accordingly, expression of differentiation markers of all retinal neurons and glia could be detected in a correct apico-basal pattern (Figure 1B). Additionally, the typical temporal histogenic sequence was conserved as well, with the birth of ganglion cells first, followed by the progressive genesis of amacrine, cone, horizontal, rod and, finally, bipolar cells. Thus, these synthetic retinas not only undergo spontaneous morphogenesis, but also self-regulate the spatial and temporal order of neuronal differentiation. However, it remains to be determined whether connectivity establishes between the different cell layers of the ES-derived OC and whether it leads to any signs of functionality.

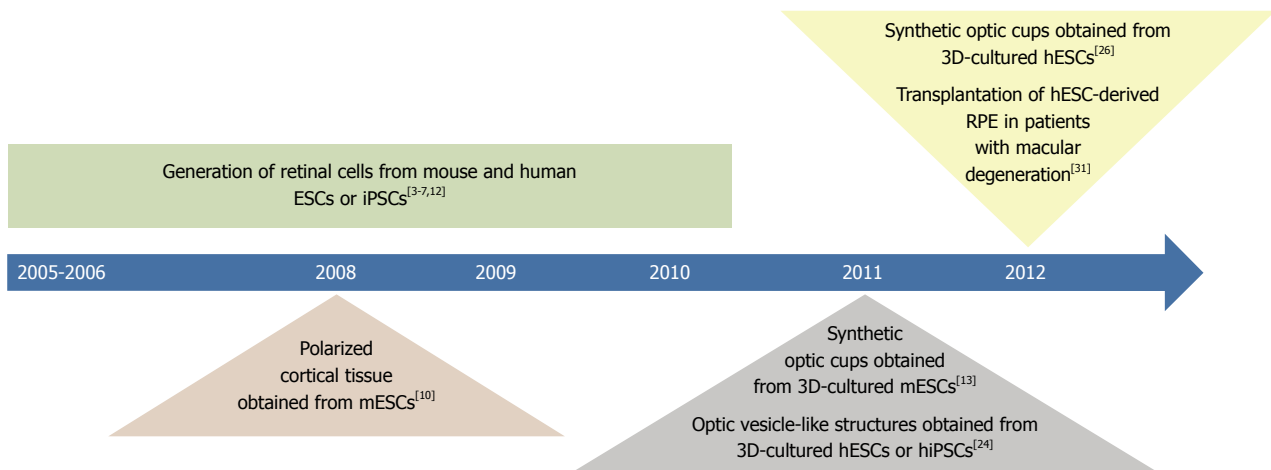
## MECHANISMS UNDERLYING MURINE OC MORPHOGENESIS

Certainly, *in vitro* generated OCs represent a powerful system for future studies on several aspects of retinal development. Eiraku *et al.*<sup>[13]</sup> started to address some questions and notably investigated the cellular and molecular mechanisms sustaining murine OC morphogenesis *in vitro*. They found that OC invagination requires the activation of the Rho-associated protein kinase (ROCK), a key regulator of actomyosin contraction in epithelial morphogenesis<sup>[18]</sup>. During phase 1, ROCK activity is uniformly distributed in the proximal and distal portions of the OV. It then progressively decreases from phase 2 to phase 4 in the neural retina and gets restricted to the prospective RPE and hinge points (Figure 1B). This likely confers them a higher





**Figure 1 Schematic representation of *in vivo* (A) and *in vitro* (B) retinal development.** A: During mouse embryonic development, the *Rx*-expressing optic vesicle (green) evaginates from the diencephalon at embryonic day 8 (E8). After contacting the surface ectoderm, the outer wall of the optic vesicle invaginates to form the optic cup. Its proximal layer forms the retinal pigmented epithelium (RPE), while the distal layer constitutes the neural retina. Retinal histogenesis and consecutive lamination then occurs. The process is completed postnatally (P0-P11); B: Retinal morphogenesis from mouse embryonic stem cells mimics this whole *in vivo* process and can be divided into 4 main phases. In phase 1, the *Rx*-expressing neuroepithelium (green) evaginates from the neural aggregate. In phase 2, its distal portion flattens. During phase 3, the angles at the hinge between the presumptive RPE and the neural retina become acute (dotted blue circles). Finally, a two-walled optic cup is formed during phase 4, following invagination of the retinal neuroepithelium. Pulling and compression forces that contribute to these morphogenetic changes are illustrated with black arrows in the neural retina magnifications. Progressive acquisition of neural retina- and RPE-specific identities is highlighted by the indication of selectively expressed set of genes. From phase 2, both tissues can also be distinguished in terms of mechanical properties, with the RPE (and hinge points) being more rigid than the NR. This correlates with higher levels of Rho-associated protein kinase activity, visualized by the expression of phosphorylated Myosin Light Chain 2 (pMLC2; red outline). Finally, following excision and further culture, phase 4 optic cup recapitulates retinal histogenesis, as observed *in vivo*. As a minor difference compared to the *in vivo* situation, synthetic retinas exhibit scattered ganglion cells and fewer cone photoreceptors. Note also that optic cup excision leads to RPE



**Figure 2** Timeline indicating the major advancements over the past 7 years in deriving retinal cells or three dimensional retinal-like structures from embryonic stem or induced pluripotent stem cells *in vitro*. (m/h)ESCs: Mouse/human embryonic stem cells; (m/h)iPSCs: Mouse/human induced pluripotent stem cells; 3D: Three dimensional.

rigidity compared to that of the neural retina, as inferred by elasticity measures with an atomic force microscope. Such an acquisition of regionally distinct mechanical properties within the OC likely constitutes a major requirement for the invagination process to occur in phase 3. Cell proliferation-dependent tissue expansion in phase 4 then provides the pushing force that contributes to the bending of the neural retina under the more rigid RPE. To support these analyses, the authors performed elegant cell ablation experiments using a 3D-targeted multiphoton laser beam and observed the subsequent changes of the created gap area. This revealed that pulling tensions occur in phase 1 and 2 whereas compression forces are at work during phase 4 (Figure 1B). Based on these observations, a “relaxation-expansion” model for self-driven OC morphogenesis was proposed and validated *in silico* through computer simulation<sup>[19]</sup>. It stipulates the need of three sequential rules: (1) mechanical relaxation of the neural retina, in contrast to the RPE which remains rigid; (2) apical constriction of hinge cells, that likely directs the orientation of the neural retina epithelium bending; and (3) tangential expansion of the neural retina that promotes its complete invagination.

The authors next asked whether neural retina and RPE fates were irreversibly determined at the OV stage. Phase 1 vesicles excised from the aggregates and cultured without non-retinal tissue retained a neural retina identity, but both RPE differentiation and OC invagination failed to occur. Of note, both processes were restored when the isolated epithelia were exposed to biological or chemical activators of canonical Wnt signaling, consistent with the known requirement of this pathway in RPE specification *in vivo*<sup>[20,21]</sup>. Conversely, when whole aggregates were cultured in the presence of Wnt inhibitors, RPE differentiation was reduced and OC invagination was hampered. Thus, while the neural retina can autonomously differentiate at this stage, the presumptive RPE still requires inductive signals from the neighboring neural (non reti-

nal) tissues to be determined. Furthermore, these results also highlight again that the invagination process is RPE-dependent.

### 3D RETINAS FROM HUMAN ES CELLS

Several groups attempted to reconstitute retinal tissue *in vitro* using human cells. For this purpose, different 3D systems, like rotary cultures or cultures in matrix on top of RPE cells<sup>[22,23]</sup>, have been developed. Although the resulting cell aggregates or sheets differentiated towards retinal cell types, no self-organization of OV- or OC-like structures were reported.

In 2011, an important breakthrough has been obtained towards the generation of optic structures using human ES (hES) and iPS cells<sup>[24]</sup>. Meyer and colleagues took advantage of a stepwise procedure capable of inducing efficient expression of eye field markers such as *Rx* and *Pax6*<sup>[25]</sup>. Basically, pluripotent cells were cultured as free-floating aggregates, exposed to a neural induction medium and then prompted to form adherent neural clusters on laminin-coated dishes. After 16 d of differentiation, neural rosettes were isolated and grown in suspension. Strikingly, by day 20, most of the resulting aggregates remained spherical but a subset (around 20%) adopted a vesicle-like structure appearance. Importantly, immunofluorescence and comparative gene microarray analyses showed that these OV-like aggregates harbored molecular features of retinal progenitor cells, while the non-vesicular ones expressed forebrain markers<sup>[24]</sup>. Of note, longitudinal analysis of OV-like structures revealed progressive expression of several differentiation markers, in a temporal order resembling that observed *in vivo*. In addition, spatial cellular segregation reminiscent of retinal lamination could be observed in these aggregates. Finally, both morphological and electrophysiological criteria suggested that photoreceptors derived from such OV-like structures were capable of phototransduction. This work

thus undoubtedly demonstrated that homogeneous 3D populations of neural retinal cells can be obtained and isolated from both human ES and iPS cells. However, despite the high degree of neuroretinal differentiation, RPE was rarely observed in OV-like structures, unless they were treated with Activin A. Furthermore, they never underwent morphological changes to form OCs and even lost with time their vesicular shape. A possible explanation is the lack of both RPE and non-retinal tissue within aggregates.

It is only very recently that formation of complete hES-derived OCs was achieved, again by Sasai and co-workers<sup>[26]</sup>. Among differences with the protocol applied for murine SFEBq/matrigel cultures are (1) an improvement of the re-aggregation step, using V-bottomed instead of U-bottomed wells; (2) the addition of a Wnt inhibitor (IWR1e) to favor rostral neural cell fates at the expense of caudal ones; and (3) the use of a ROCK inhibitor (Y-27632) to prevent dissociation-induced apoptosis of hES cells<sup>[27,28]</sup>. Following a 12 d-culture in these conditions, supplementation of aggregates with fetal bovine serum and with the Hedgehog agonist SAG, was found to induce a high degree of “retinalization”.

However, although most of the neuroepithelium generated in this way was positive for the neural retina markers Chx10 and Pax6, no sign of RPE differentiation could be detected. As a consequence, self-formation of OC did not occur. This could be circumvented by adding the Wnt agonist CHIR99021 in a specific time window (between days 15-18). Under these conditions, as seen with murine ES cells, the neural retina epithelium first evaginated into an OV-like structure and then spontaneously invaginated to form a double walled OC. Importantly, hES-derived retinas, like their murine counterparts, were also able to acquire a typical layered structure, following proper spatio-temporal differentiation of retinal cell types. In particular, photoreceptors displayed characteristics of differentiated cells, like the presence of inner segments and connecting cilia, although outer segments were not clearly observed.

## DIFFERENCES BETWEEN MOUSE AND HUMAN *IN VITRO* GENERATED OCs

Despite the aforementioned similarities, several specific features of human compared to mouse artificial OCs were observed by the authors<sup>[26]</sup>. In particular, the former took longer to develop (about 24 d *vs* 9 d for mouse) and were bigger (550  $\mu$ m *vs* 250  $\mu$ m in diameter) and thicker (approximately 120  $\mu$ m *vs* 60  $\mu$ m). This obviously reflects the differences observed *in vivo* during fetal development of the two species, and therefore suggests that both retinal size and developmental timing are intrinsically controlled.

Another intrinsic property harboured by hES-derived retinal neuroepithelium is its ability to spontaneously evert from an apically concave (OV) to an apically convex

(OC) structure. While murine synthetic retinas require RPE and hinge points for this process, human ones are able to bend in an apically convex manner independently. Notably, this property was found to be associated with a preferential localization of cell bodies to the apical side of the neuroepithelium and to be integrin-dependent, two features that are specific to human retinas.

Finally, differences were also observed concerning photoreceptor development: much more cones were generated in hES-derived OCs and the overall differentiation process of photoreceptor cells was consistently slower (as observed again during fetal development). Importantly, photoreceptor genesis could be accelerated by counteracting Notch activity through the addition of the  $\gamma$ -secretase inhibitor DAPT.

## PERSPECTIVES IN REGENERATIVE MEDICINE

The findings reported in this review not only represent major advances for basic research but also bear profound medical implications, above all in the field of regenerative medicine. One obvious conclusion is that such artificial retinas constitute potential homogeneous sources of stage-selected retinal cells for transplantation in patients affected by neurodegenerative diseases like retinitis pigmentosa or age-related macular degeneration<sup>[29]</sup>. Of note, photoreceptor transplantation has been shown to be a valid therapeutic strategy, able to restore vision in a mouse model of rod degeneration<sup>[30]</sup>, paving the way for similar treatments in humans. Such a possibility is well illustrated by a recent clinical trial, where hES cell-derived RPE was transplanted in patients affected by different forms of macular degeneration<sup>[31]</sup>. This study shows for the first time successful attachment and survival of the graft, associated with significant improvement of visual acuity and no tumorigenicity nor transplant rejection after 4 mo. Although further follow-up of the patients is required, this represents an encouraging and promising milestone. It should, however, be noted that such a strategy may not be sufficient to treat retinopathies that primarily result from alterations of the vascular microenvironment, like that occurring in diabetic retinopathy. In these cases, therapeutic revascularization<sup>[32]</sup> must be considered prior to retinal cell replacement.

As far as genetic retinal disorders are concerned, a fascinating application would be to produce patient's iPS cells, correct the disease-linked genetic mutation, and promote their differentiation into a fully functional retina<sup>[33]</sup>. *In vitro* generated organs from iPS cells also constitute powerful systems to model human diseases and perform large-scale drug screening. The study by Meyer and collaborators gives an interesting proof of concept of such potential uses through the description of OV-like structure formation from iPS cells derived from a patient affected by gyrate atrophy<sup>[24]</sup>. This pathology is an autosomal recessive disorder primarily affecting RPE.

Importantly, RPE dysfunction was observable *in vitro* and could be corrected through both pharmacological and gene therapy approaches<sup>[24]</sup>. This study thus validates iPS-derived 3D retinal models as versatile systems for personalized drug testing and gene rescue. However, in a concomitant work, the authors described a substantial mutational load in the iPS cell line occurring at the time of derivation<sup>[34]</sup>. Some evidence points to the reprogramming process as a possible critical mutagenic step and many mutations are found enriched in genes known to have causative effects in cancers<sup>[35,36]</sup>. Thus, extensive genetic characterization of the iPS cell clones to be used will be critical before considering them as potential sources for transplantation and regenerative medicine.

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## Reprogramming somatic cells by fusion with embryonic stem cells does not cause silencing of the *Dlk1-Dio3* region in mice

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### Abstract

**AIM:** To examine the imprinted *Dlk1-Dio3* locus in pluripotent embryonic stem (ES) cell/fibroblast hybrid cells.

**METHODS:** *Gtl2*, *Rian*, and *Mirg* mRNA expression in mouse pluripotent ES cell/fibroblast hybrid cells was examined by real-time reverse transcription-polymerase chain reaction. Pyrosequencing and bisulfate sequencing were used to determine the DNA methylation level of the *Dlk1-Dio3* locus imprinting control region.

**RESULTS:** The selected hybrid clones had a near-tetraploid karyotype and were highly pluripotent judging from their capacity to generate chimeric embryos and adult chimeras. Our data clearly demonstrate that *Gtl2*,

*Rian*, and *Mirg*, which are imprinted genes within the *Dlk1-Dio3* locus, are active in all examined ES cell/fibroblast hybrid clones. In spite of interclonal variability, the expression of the imprinted genes is comparable to that of ES cells and fibroblasts. Quantitative analysis of the DNA methylation status of the intergenic differentially methylated region (IG DMR) within the *Dlk1-Dio3* locus by pyrosequencing and bisulfite sequencing clearly showed that the DNA methylation status of the imprinted region in the tested hybrid clones was comparable to that of both ES cells and fibroblasts.

**CONCLUSION:** Reprogramming process in a hybrid cell system is achieved without marked alteration of the imprinted *Dlk1-Dio3* locus.

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**Key words:** Cell fusion; Embryonic stem cells; Reprogramming; Pluripotency; Imprinted *Dlk1-Dio3* region; DNA methylation

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### INTRODUCTION

Cell fusion is one approach that has been used to dem-



onstrate nuclear reprogramming of somatic cells to a pluripotent-like state. In fact, embryonic stem (ES) hybrid cells obtained by the fusion of ES cells with various somatic cell types have characteristics similar to ES cells<sup>[1-5]</sup>. The great potential of ES cell/somatic cell hybrids was confirmed by the generation of chimeric embryos<sup>[6,7]</sup> and chimeric adults<sup>[1,8,9]</sup>. In addition, reprogramming in ES cell/somatic hybrid cells occurs rapidly, generally within 5-10 d<sup>[5,10]</sup>. Such remarkable reprogramming effects could be explained by the presence of numerous reprogramming factors in ES cells compared to the limited numbers usually used in the generation of induced pluripotent stem (iPS) cells<sup>[5,11]</sup>.

iPS cell derivation by forced expression of a few reprogramming factors is now considered to be a promising method of reprogramming<sup>[11-17]</sup>. Recently, several groups, using a number of different parameters, have shown that iPS cells differ from ES cells, which are considered to be the “gold standard” of pluripotency<sup>[11,18,19]</sup>. In other words, several errors can occur during the generation of iPS cells. One such “reprogramming error” is aberrant silencing of the imprinted *Dlk1-Dio3* locus located on mouse chromosome 12 caused by DNA hypermethylation of key imprinting control regions<sup>[20,21]</sup>. Dysregulation of this locus leads to altered gene expression that drastically limits developmental capacity so that such iPS cells after their injection into tetraploid blastocysts do result in the birth of “all iPS-cell derived” mice but rather generate chimeras with a low contribution of the tested cells. This phenomenon was observed in 95% of mouse iPS cell lines<sup>[21]</sup>. It should be noted that methylation level of CpG-sites in the imprinted *Dlk1-Dio3* locus is usually about 50% in both somatic and ES cells.

We have previously established ten stable hybrid clones, three of which are ES cell/embryonic fibroblast cell type and seven that are ES cell/adult fibroblast cell type<sup>[9]</sup>. Based on cytogenetic analysis, four of ten clones in which more than 80% of cells contained 76-80 chromosomes were selected; in other words, the hybrid cells had a near-tetraploid chromosome set. Injection of the GFP-labeled hybrid cells into blastocysts demonstrated that all four hybrid clones were able to give rise to chimeric embryos with a high contribution of GFP-labeled hybrid cell descendants. Furthermore, three clones resulted in the birth of about two dozen adult chimeras<sup>[9]</sup>. Taken together, the selected hybrid clones had highly pluripotency comparable with parental ES cells. It is important to note that cytogenetic and microsatellite analyses have demonstrated that the initial near-tetraploid karyotype of the hybrid cells remained stable during the development of the chimeras<sup>[9]</sup>.

This study examined the imprinted *Dlk1-Dio3* locus in pluripotent ES cell/fibroblast hybrid clones. The aim was to determine whether alterations of the *Dlk1-Dio3* locus observed in iPS cells are common in other reprogramming systems, particularly cell fusion, or whether the alternations are caused by the lack of some reprogramming factors used in generating iPS cells.

## MATERIALS AND METHODS

### Cells and culture conditions

We used the following cell lines in this study: (1) the murine ES cell line E14Tg2aSc4TP6.3 (tauGFP)<sup>[22]</sup>, in which the hypoxanthine phosphoribosyl transferase gene has been deleted, the pTP6 transgene contains a tau-tagged green fluorescent protein (GFP) and the puromycin resistance (*Puro*) gene. Culture conditions of this ES cell line were described previously in detail<sup>[9]</sup>; (2) the MA01 ES cell line, prepared from a blastocyst derived from the 129/Ola x BALB mouse using a previously published protocol<sup>[23]</sup>. MA01 cells had morphology and growth characteristics typical of ES cells, were positive for *Oct4* and *Nanog* expression, and had a diploid karyotype without visible chromosomal rearrangements. MA01 cells had undergone five to eleven passages; (3) mouse embryonic fibroblast (MEF) cultures from 13.5 dpc embryos derived from DD/c mice, prepared and cultures as described previously<sup>[9]</sup>; and (4) a set of hybrid cell clones: *tef4*, *taf2*, *taf5*, and *taf9* produced by fusing diploid tauGFP ES cells and diploid embryonic (series *tef*) or adult (series *taf*) fibroblasts. As mentioned above, these hybrid clones had near-tetraploid chromosome complements that indicated on absence of marked segregation of parental chromosomes. The ES cells and hybrid clones were cultured without feeder in Glasgow Modified Eagle's Medium MEM (Gibco/BRL-Life Technologies, UK) containing 10% fetal bovine serum (FBS; PAA, Austria), 1% non-essential amino acids (Gibco/BRL-Life Technologies), 10<sup>-4</sup> mol/L β-mercaptoethanol, 100 μg/mL penicillin and streptomycin, and 10<sup>3</sup> U/mL murine leukemia inhibitory factor (mLIF; Chemicon, UK). The cells were cultured in plastic dishes coated with 0.1% gelatin (Fluka, Germany) at 37 °C in a 5% CO<sub>2</sub> atmosphere with high humidity. The medium was changed every 2-3 d. Preparation of the hybrid cell clones was previously described<sup>[9]</sup>.

### Microsatellite analysis marking parental chromosomes in hybrid clones

To distinguish the parental chromosomes in hybrid cells, a set of microsatellite markers that allow unambiguous marking of each parental chromosome was used. Primers and polymerase chain reaction (PCR) conditions for the microsatellites were previously described<sup>[9]</sup>.

### Bisulfite DNA methylation analysis

Genomic DNA extraction and bisulfite mutagenesis sequencing analysis were conducted using the EZ DNA Methylation-Direct kit (Zymo Research, Orange, CA, USA), as described previously<sup>[24]</sup>. The genomic DNA was eluted with 10 μL of elution buffer and used for two successive rounds of PCR with nested primer pairs (inner and outer), which were specific for the bottom strand of the mutagenized DNA (first-round primer set: IGDMR\_out\_for 5'-AAGGTATATTATGTTAGTGT-TAGGAAGGATTGTGA-3' and IGDMR\_out\_rev 5'-CAAAACATTCTCCATTAACAAAATAATA-CAACCT-3'; second-round primer set: IGDMR\_in\_for 5'-TGTGGTTTGTATGGGTAAGTTTATG-

GTTTATTG-3' and IGD<sub>MR</sub>\_in\_rev 5'-AATA-CAACCTTCCCTCACTCCAAAAATTAATAA-3'). The conditions for the first round of PCR were as follows: initial denaturation at 94 °C for 3 min, followed by 30 cycles of 94 °C for 15 s, 58 °C for 15 s, 72 °C for 30 s, and a final extension at 72 °C for 3 min. The first round PCR product was purified using an affinity column to remove the outer primers. The PCR products were eluted in 10 µL of water and used for the second PCR round: initial denaturation at 94 °C for 3 min, 30 cycles of 94 °C for 15 s, 58 °C for 15 s, and 72 °C for 20 s, and a final extension at 72 °C for 3 min. All PCRs were performed with Taq DNA polymerase. The PCR products were run on a 2% agarose gel to verify the amplification of specific bands, which were then excised from the gel and purified with the Min-Elute Gel Extraction Kit (Qiagen, Hilden, Germany). The purified PCR products were subcloned using the pGEMT Easy kit (Promega, Madison, WI, USA), and then individual clones were sequenced. Sequencing of individually cloned products was performed using the M13 reverse and forward primers. Clones were accepted only with  $\geq 90\%$  cytosine conversion. Resultant sequences were aligned to the DNA sequences of the selected loci of the gene of interest using the online quantification tool for methylation analysis (<http://quma.cdb.riken.jp/top/index.html>).

### Pyrosequencing procedure

Amplicons for pyrosequencing reactions were generated in a 50 µL reaction volume with 300 nmol/L forward and reverse PCR primers (5'-GTTATGGATTGGTGTTAAGGT-3', biotin-5'-TACAACCTTCCCTCACTC-3'), 10 mmol/L Tris-HCl (pH 8.9), 55 mmol/L KCl, 2.5 mmol/L MgCl<sub>2</sub>, 0.05% Tween-20, 0.2 mmol/L dNTP, 0.5 U of Hot start Taq DNA polymerase (Biosan, Novosibirsk, Russia), and 2 µL of purified first round PCR products, as described for bisulfite DNA methylation analysis. Single-stranded biotinylated PCR products were prepared for sequencing using the Vacuum Prep Tool and Streptavidin Sepharose™ HP according to the manufacturer's instructions. Pyrosequencing reactions were performed using the PSQ 96 SNP Reagent Kit (Pyrosequencing, Uppsala, Sweden). The degree of methylation at each CpG site was determined by Pyro Q-CpG Software (Biotage, Uppsala, Sweden). The methylation index for each sample was calculated as the mean value of methC percentage for all 12 CpGs examined. It is known that differences in sequence between methylated and unmethylated samples after bisulfite treatment can influence amplification efficiency. We constructed a calibration curve to verify that the pyrosequencing assay provided unbiased quantification. Unmethylated and 100% methylated DNA samples after bisulfite treatment were quantified and mixed at different ratios. The samples were PCR-amplified and subjected to pyrosequencing. Actual and expected methylation percentages are expected to be linearly correlated with an  $r^2 \geq 0.8$ .

### Real-time quantitative PCR

Total RNA from cultured cells was isolated using the SV Total RNA Isolation System (Promega, Madison, WI,

USA) following the manufacturer's instructions. After DNaseI treatment, 0.25 µg of total RNA was reverse transcribed using the oligo-dT primers and ImProm-II Reverse Transcription System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Amplification reactions were run in triplicate on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Bedford, MA, USA). First, primer efficiency was validated with a standard curve of serial dilution points of a scraped section cDNA pool and a no-template control (NTC). qPCR amplification mixture was a total volume of 25 µL, which contained 5 µL single strand cDNA template diluted 20 times after reverse-transcription, 10 µL 2.5 × SYBR Green I qPCR Master Mix (Syntol, Moscow, Russia), and 1.2 µL forward and reverse primers (5 µmol/L) so that the final primer concentration was 300 nmol/L. The sequence of the primers used in the study were as follows: *Ppia* (reference gene) 5'-CGCGTCTCCTTCGAGCTGTTTG-3' and 5'-TGTAAGATCACCACCCTGGCACAT-3'; *Rian* 5'-TCGAGACACAAGAGGACTGC-3' and 5'-ATTGGAAGTCTGAGCC-3'; *Mirg* 5'-TTGACTCCAGAAGATGCTCC-3' and 5'-CCTCAGGTTCTTAAGCAAGG-3'; *Gtl2* 5'-TTGCACATTTTCCTGTGGGAC-3' and 5'-AAGCACCATGAGCCACTAGG-3'; *Oct4* 5'-TAGGTGAGCCGTCCTTCCAC-3' and 5'-GCTTAGCCAGGTTTCGAGGAT-3'; *Nanog* 5'-TTGCTTACAAGGGTCTGCTACT-3' and 5'-ACTGTAGAAGAATCAGGGCT-3'. Cycle conditions were as follows: after an initial 5 min denaturation at 95 °C, the samples were amplified using 35 cycles at 95 °C for 15 s, 58 °C for 15 s, and 72 °C for 20 s. After optimization of qPCR systems, we used a relative expression software tool (REST 2009 V2.0.13©, Qiagen) to compare gene expression between tauGFP ES cells, MEFs, and ES cell/fibroblast hybrid clones.

## RESULTS

### Microsatellite analysis of hybrid clones

As mentioned above, four highly pluripotent hybrid clones: *tef4*, *taf2*, *tef9*, and *taf5* had near-tetraploid chromosome sets. To identify parental chromosome origin in hybrid clones, we analyzed a set of microsatellites marking all parental chromosomes except chromosomes 11 and X (Table 1). This analysis showed that all four hybrid clones retain chromosomes from both parental cell types. The *Dlk1-Dio3* imprinted locus is localized on chromosome 12, which was present in all analyzed hybrid clones. Also, microsatellite and repeated cytogenetic analyses at different passages from 8 to 14 demonstrated that chromosome numbers and microsatellite markers remained stable during that period.

### Quantitative estimation of Oct4 and Nanog gene expression in ES cell/fibroblast hybrid clones

The pluripotent state of ES cells is supported by the activity of a small number of key genes such as *Oct4*, *Nanog* and *Sox2*. Therefore, we performed additional quantitative analysis of *Oct4* and *Nanog* expression in hybrid

Table 1 Microsatellite analysis of hybrid cell clones

Microsatellite marker <sup>1</sup>	Hybrid cell clones							
	<i>tef4</i>		<i>taf2</i>		<i>tef9</i>		<i>taf5</i>	
	129	DD	129	DD	129	DD	129	DD
D1Mit200	+	+	+	+	+	+	+	+
D2Mit9	+	+	+	+	+	+	+	+
D3Mit257	+	+	+	+	+	+	+	+
D4Mit11	+	+	+	+	+	+	+	+
D5Mit346	+	+	+	+	+	+	+	+
D6Mit201	+	+	+	+	+	+	+	+
D7Mit309	+	+	+	+	+	+	+	+
D8Mit155	+	+	+	+	+	+	+	+
D9Mit181	+	+	+	+	+	+	+	+
D10Mit109	+	+	+	+	+	+	+	+
D12Mit270	+	+	+	+	+	+	+	+
D13Mit78	+	+	+	+	+	+	+	+
D14Mit38	+	+	+	+	+	+	+	+
D15Mit14	+	+	+	+	+	+	+	+
D16Mit4	+	+	+	+	+	+	+	+
D17Mit36	+	+	+	+	+	+	+	+
D18Mit36	+	+	+	+	+	+	+	+
D19Mit10	+	+	+	+	+	+	+	+

<sup>1</sup>Numerals after letter D of microsatellites indicate number of marked chromosomes + indicates a presence of microsatellites specific for 129 and DD mice.

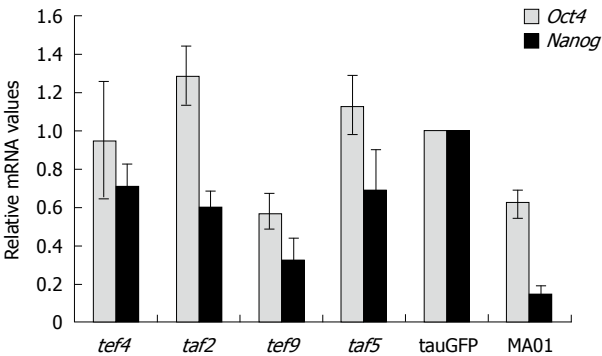


Figure 1 Relative expression levels of the pluripotency markers, *Oct4* and *Nanog*, in embryonic stem cell lines tauGFP and MA01 and in the hybrid cell clones *tef4*, *taf2*, *tef9*, and *taf5*. The values from tauGFP embryonic stem cells were set at 1.

cells, as shown in Figure 1. The ES cell lines tauGFP and MA01 were used as controls. As shown in Figure 1, *Oct4* and *Nanog* expression in the *tef4*, *taf2*, and *taf5* hybrid cells was similar to that in tauGFP ES cells and even somewhat higher than in MA01 ES cells. Expression of both genes was lower in *tef9* cells than in tauGFP ES cells but was comparable with that of MA01 ES cells. Interestingly, expression of *Oct4* and *Nanog* genes was higher in tauGFP ES cells (passed more than 40 passages) than MA01 cells analyzed at the eleventh passage (Figure 1).

### Quantitative estimation of *Gtl2*, *Rian*, and *Mirg* gene expression located within the *Dlk1-Dio3* locus of ES cell/fibroblast hybrid cells

The main focus of our study was to determine whether alterations in *Dlk1-Dio3* locus imprinting occurred in the

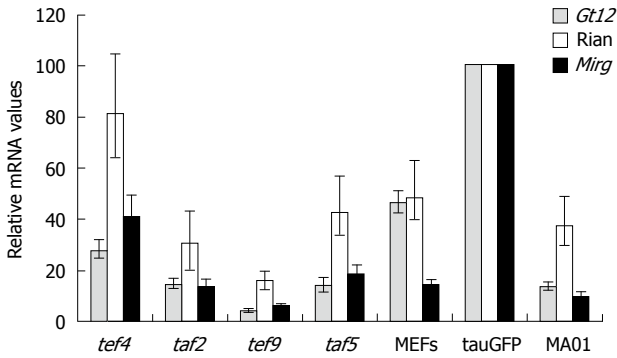


Figure 2 Relative expression levels of the maternally imprinted genes *Gtl2*, *Rian*, and *Mirg* in the *tef4*, *taf2*, *tef9*, and *taf5* hybrid clones, tauGFP and MA01 embryonic stem cell lines, and MEFs. The values from tauGFP embryonic stem cells were set at 1.

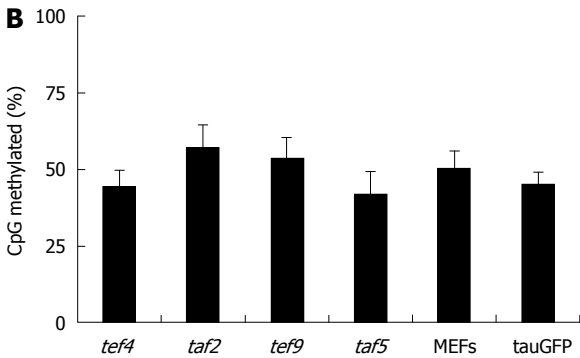
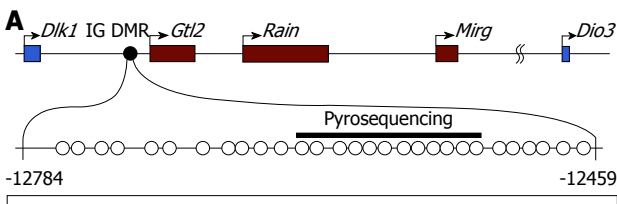


Figure 3 DNA methylation of intergenic differentially methylated region in embryonic stem cells (tauGFP), MEFs, *tef4*, *taf2*, *tef9*, and *taf5*. (A) Structure of the *Dlk1-Dio3* locus with the position of the intergenic differentially methylated region (IG DMR) analyzed by bisulfite sequencing (CpGs indicated by circles) and pyrosequencing (indicated by black bar). Numbers indicate position relative to the *Gtl2* transcription start site; (B) Percentage of DNA methylation of CpG-sites within IG DMR in *tef4*, *taf2*, *tef9*, and *taf5* hybrid clones, as well as parental tauGFP embryonic stem (ES) cells (ESC) and fibroblasts (MEFs), based on the data of pyrosequencing.

cell fusion reprogramming system that were previously described in iPS cells. Quantitative estimation of *Gtl2*, *Rian*, and *Mirg* gene expression located within the imprinted *Dlk1-Dio3* gene cluster in hybrid cell clones, *tef4*, *taf2*, *tef9* and *taf5*, is shown in Figure 2. Among hybrid clones, expression of *Gtl2*, *Rian* and *Mirg* genes was highest in the *tef4* clone, while the *tef9* clone had the lowest expression. Levels of *Gtl2*, *Rian*, and *Mirg* expression in *tef4*, *taf5* and *taf2* hybrid clones (but not in *tef9*) were comparable with those found in MA01 and MEFs, but somewhat lower than in tauGFP ES cells (Figure 2). It should be noted that expression of these three genes was higher in tauGFP ES cells than in MA01 cells (Figure 2).



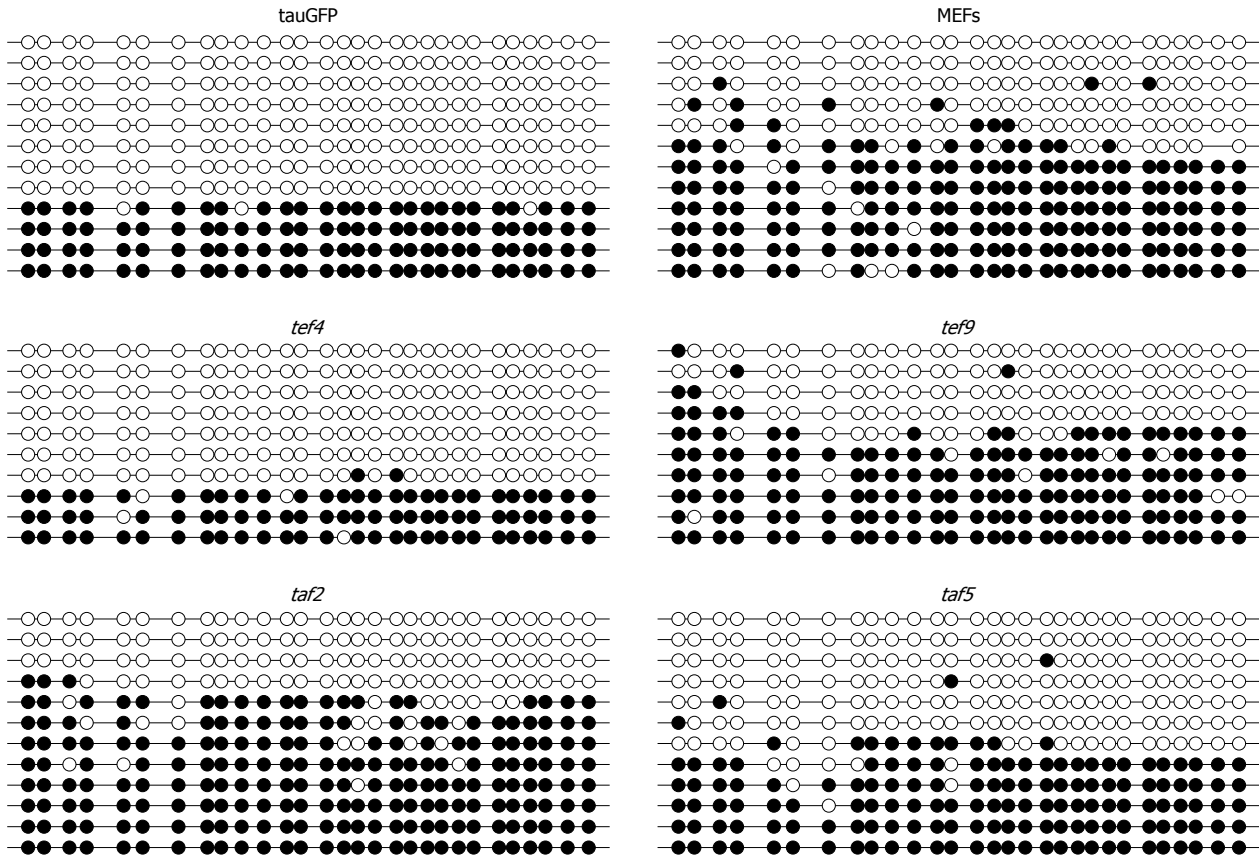


Figure 4 Representations of the DNA methylation profiles of the intergenic differentially methylated region in embryonic stem cells (tauGFP), MEFs, and four hybrid clones, based on the data of bisulfite analysis. Methylation status of CpG-sites is depicted by open circles (unmethylated) and closed circles (methylated).

#### DNA methylation of imprinting control region within *Dlk1-Dio3* gene cluster

Imprinting of the *Dlk1-Dio3* cluster is regulated by differentially methylated regions (DMRs) that become epigenetically modified in the germ line. One of these regions, the intergenic DMR (IG DMR), is located between the *Dlk1* and *Gtl2* genes and is considered to be a master regulator of the entire locus (Figure 3A)<sup>[25]</sup>. To determine whether DNA methylation of this region is altered during reprogramming, we examined the methylation status of IG DMR in four hybrid cell clones, in parental tauGFP ES cells, and in MEFs (Figure 3B). Pyrosequencing analysis revealed that the level of DNA methylation of IG DMR in the parental tauGFP ES cells was about 50%, which is expected for germline-imprinted regions. Similar methylation profiles were observed in all four hybrid clones (Figure 3B).

These data were supported by bisulfite sequencing analysis of the IG DMR (Figure 3). As shown in Figure 3, the percentage of CpG-site methylation of IG DMR in *taf4*, *taf2*, *taf9*, and *taf5* hybrid clones was similar to that of tauGFP ES cells and MEFs. Approximately half of the alleles at the IG DMR were hypermethylated, whereas the other half were unmethylated (Figure 4). Both approaches suggest that the hybrid clone methylation pattern of IG DMR remains stable compared to parental cells, tauGFP ES cells, and MEFs.

#### DISCUSSION

To determine whether *Dlk1-Dio3* locus status is altered in hybrid clones we analyzed expression of several genes within that locus. It should be noted, that hybrid clones used in this study are intraspecific and have near-tetraploid karyotype. Thus it was not possible to perform a detailed analysis of the activity of each of the four alleles. Instead we used indirect methods such as quantitative evaluation of imprinted gene allele expression and assessment of the methylation status of the IG DMR which is a master regulator of the entire locus.

Our data suggest that the genes encoding *Gtl2*, *Rian*, and *Mirg* within the *Dlk1-Dio3* cluster are active in all examined ES cell/fibroblast hybrid clones. Regardless of interclonal variability, expression of *Gtl2*, *Rian*, and *Mirg* in the ES cell/fibroblast hybrid clones is comparable to that in MA01 ES cells, but somewhat lower than in tauGFP ES cells. Interclonal variability is not specific to the imprinted genes because it was also observed in *Oct4* and *Nanog* gene expression. We cannot exclude the possibility of aberrant silencing of some of the four alleles in hybrid clones. On the other hand, our data allow us to suggest that at least one allele remains active as there was no dramatic decrease of the expression level of *Gtl2*, *Rian*, and *Mirg* genes in iPS cells.

Stadtfield *et al.*<sup>[21]</sup> found that hypermethylation at the DMRs occurs within the *Dlk1-Dio3* cluster in all iPS cell clones with a silent *Gtl2* gene<sup>[21]</sup>. These findings prompted us to perform quantitative analysis of DNA methylation status of the IG DMR region within the *Dlk1-Dio3* locus. Both quantitative methylation analyses by pyrosequencing and bisulfite sequencing clearly showed that the DNA methylation status of the imprinted region in *tef4*, *taf2*, *tef9*, and *taf5* hybrid clones was comparable to that of tauGFP ES cells and MEFs. Since tauGFP cells and MEFs served as parental cell partners in the generation of the hybrid cells used in our study, we conclude that methylation status of the IG DMR region within the *Dlk1-Dio3* locus was not altered.

Two cell lines, tauGFP and MA01 cells, were used as controls in the analysis of gene expression by qPCR. Expression of the *Oct4* and *Nanog* genes and the imprinted *Gtl2*, *Rian*, and *Mirg* genes was higher in the tauGFP ES cells than other ES cells or the MA01 cell line. However, these lines showed no differences in expression levels of the *Gapdh* housekeeping gene (data not shown). The cause of these differences is unclear. However, long-term *in vitro* cultivation of tauGFP cells (over 40 passages) could lead to the appearance of undefined characteristic(s) *in vitro* conditions, whereas MA01 cells have only been passaged five to eleven times following isolation from blastocysts. In general, the findings agree with observation of other researchers that no abnormalities at the *Dlk1-Dio3* cluster are evident in ES cells derived from normal blastocysts or even blastocysts developed after somatic cell nuclear transfer<sup>[20,21]</sup>.

As mentioned above, the examined hybrid clones *tef4*, *taf2*, *tef9* and *taf5* had robust levels of pluripotency judging from the generation of embryonic and adult chimeras<sup>[9]</sup>. However, adult chimera yield varied significantly between the clones. For example, injection of *tef4* cells into C57BL blastocysts generated 15 adult chimeras with seven of them showing more than 50% visible color chimerism. Injection of *taf2* and *tef9* cells resulted in the birth of six and one adult chimeras, respectively, with moderate color chimerism. However, it is important to note that the development of tetraploid mice has generally not been observed beyond mid-gestation<sup>[26]</sup>. We have not excluded the possibility that high levels of chimerism based on excessive contribution of near-tetraploid hybrid cells could potentially halt embryonic development before birth, thereby decreasing the rate of chimera births. Of note, 30% to 65% of chimeric embryos with high contribution from *tef4* and *taf5* died between embryonic days 11-13<sup>[9]</sup>. Nevertheless, capacity of the tested hybrid clones to produce adult chimeras and contribute to coat color can tentatively be ranked as *tef4* > *taf2* > *tef9*. From data in Figure 3 it follows that expression levels of the maternally imprinted *Gtl2*, *Rian*, and *Mirg* genes can tentatively be ranked as *tef4* > *taf2* > *tef9*. Comparison of adult chimera yield and expression levels of the maternally imprinted genes suggests that there is a positive correlation. Interestingly, Liu *et al.*<sup>[20]</sup> observed a similar

phenomenon when comparing two partially pluripotent iPS cell lines with different levels of pluripotency: one cell line was germline transmittable whereas the other was not. Expression levels of all *Dlk1-Dio3* miRNAs were consistently higher in the germline-transmittable cells than in the non-transmittable cells. Thus, a high level of pluripotency could be associated with precise regulation but not simply with the “switch on” state of the locus.

Our data suggest that, in contrast to iPS cells, the reprogramming process in hybrid cell systems is achieved without marked alteration of the major imprinted *Dlk1-Dio3* locus, its methylation status, and *Gtl2*, *Rian* and *Mirg* gene expression.

## COMMENTS

### Background

Currently there are several methods of somatic cell genome reprogramming to a pluripotent state. The most promising for practical usage is induced pluripotent stem (iPS) cell derivation. The drawback of this approach is the possibility of reprogramming errors. It was shown that aberrant silencing of the *Dlk1-Dio3* locus is observed in most iPS cell lines and can cause restriction of iPS cell differentiation potential. The mechanisms of this abnormality are unknown.

### Research frontiers

Cell fusion is another approach for somatic cell genome reprogramming. It was reported that cell fusion mediated reprogramming is fast, effective and relatively correct. Thus, this model is convenient for studying the basic principles of reprogramming. In this research article the authors have examined whether there is iPS cell characteristic aberrant silencing of the *Dlk1-Dio3* locus in a cell fusion reprogramming system.

### Innovations and breakthroughs

This report is the first study of the aberrant *Dlk1-Dio3* locus reprogramming in hybrid cells. The qPCR analysis of *Dlk1-Dio3* locus gene expression and its DNA methylation level in hybrids cell allowed the conclusion that, in contrast to iPS cells the reprogramming process in a hybrid cell system is achieved without marked alteration of the imprinted *Dlk1-Dio3* locus.

### Applications

The understanding of the reprogramming mechanisms will provide clues to development of improved protocols for the derivation of reprogrammed cells.

### Terminology

Reprogramming: a process of somatic cell transition from one differentiation state to another, including pluripotency; *Dlk1-Dio3* locus-a large region located on mouse chromosome 12. It has important functions in the regulation of the development. Aberrant expression of its genes results in the termination of pre-natal development.

### Peer review

In general, the manuscript is interesting and well written and the authors provide convincing evidences suggesting that the reprogramming process in a hybrid cells did not alter the imprinted *Dlk1-Dio3* locus (its methylation status, or the expression of *Gtl2*, *Rian* and *Mirg* genes).

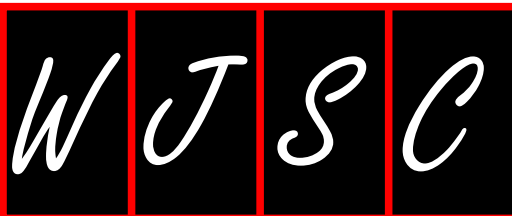
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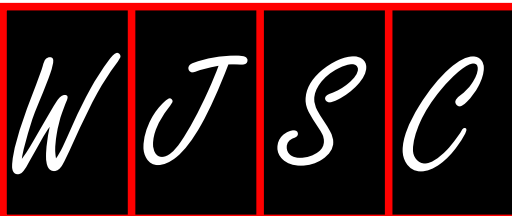
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**Andre Van Wijnen, PhD**, Department of Cell Biology, Rm S3-322, University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, MA 01655, United States



## Events Calendar 2012

January 22-27, 2012

Keystone Symposia: Cardiovascular  
Development and Regeneration  
Taos, NM, United States

February 2-3, 2012

Stem Cells 2012  
San Diego, CA, United States

February 16, 2012

The 2012 London Regenerative  
Medicine Event  
London, United Kingdom

February 23, 2012

CiRA Symposium: Advances in  
Nuclear Reprogramming and Stem  
Cell Research  
Kyoto, Japan

February 26 - March 2, 2012

Gordon Research Conference:  
Reprogramming Cell Fate  
Galveston, TX, United States

March 9, 2012

Cell Culture Technology: Recent  
Advances, Future Prospects  
London, United Kingdom

March 11-16, 2012

Keystone Symposia: The Life of a  
Stem Cell: From Birth to Death  
Olympic Valley (Lake Tahoe),  
CA, United States

March 25-30, 2012

Keystone Symposia: Advances in  
Islet Biology  
Monterey, CA, United States

March 28-29, 2012

Single Cell Analysis Europe  
Edinburgh, United Kingdom

April 1 - 6, 2012

Keystone Symposia: Mechanisms  
of Whole Organ Regeneration,  
joint with Regenerative Tissue  
Engineering and Transplantation  
Breckenridge, CO, United States

April 25-28, 2012

3rd International Congress on  
Responsible Stem Cell Research  
Aula Nuova del Sinodo  
Vatican City, Vatican City

April 27-29, 2012

2nd Institute of Advanced Dental  
Sciences & Research International  
Conference 2012: Fundamentals of  
Conducting and Reporting Research-  
Biological, Pharmaceutical, Medical  
& Dental Sciences  
University of the Punjab, Lahore,  
Pakistan

April 29 - May 2, 2012

3rd International Conference on  
Stem Cell Engineering (ICSCE)  
Co-organized by the Society for  
Biological Engineering (SBE) and the  
ISSCR  
Seattle, WA, United States

April 30, 2012

Stem Cells to Tissues  
Boston, MA, United States

April 30, 2012

Regenerative Biology: From Stem  
Cells to Tissues  
The Joseph B Martin Conference  
Center, Harvard Medical School  
Boston, MA, United States

April 30-May 2, 2012

Till & McCulloch Meetings  
Montreal, QC, Canada

May 18, 2012

The 2012 Stem Cell Discussion  
Forum  
London, United Kingdom

May 21-22, 2012

Driving Stem Cell Research Towards  
Therapy.  
Edinburgh, United Kingdom

June 5-8, 2012

18th Annual International Society  
for Cellular Therapy Meeting  
Washington, DC, United States

June 13-16, 2012

International Society for Stem Cell  
Research 10th Annual Meeting  
Yokohama, Japan

June 25-27, 2012

The Stem Cell Niche - development  
and disease  
Copenhagen, Denmark

June 27-28, 2012

Bioprocessing & Stem Cells Europe  
London, United Kingdom

June 28-29, 2012

Origins of Tissue Stem Cells  
Edinburgh, United Kingdom

July 9-11, 2012

Stem Cells in Cancer - 2nd annual  
Cambridge Stem Cell International  
Symposium  
Cambridge, United Kingdom

July 15-18, 2012

39th Annual Meeting & Exposition  
of the Controlled Release Society  
Smart Materials - From Innovation  
to Translation  
Centre des Congrès de Québec,  
Québec City, Canada

August 29 - September 1, 2012

EMBL Conference: Stem Cells in  
Cancer and Regenerative Medicine  
Heidelberg, Germany

September 5-8, 2012

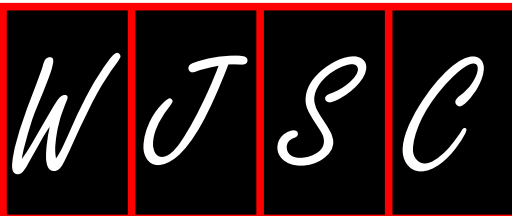
TERMIS World Congress 2012  
Vienna, Austria

October 15-17 2012

13th World Congress of the  
International Society for Diseases of  
the Esophagus  
Venice, Italy

November 6, 2012

Stem Cells and Metabolism  
The Salk Institute of Biological  
Studies La Jolla,  
CA, United States



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**Acknowledgments**

Brief acknowledgments of persons who have made genuine con-

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### Format

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- 1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarhoea. *Shijie Huaren Xiaobua Zazhi* 1999; **7**: 285-287

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]

Both personal authors and an organization as author

- 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]

No author given

- 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

- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

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

### Books

Personal author(s)

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary 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. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

- 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

### Statistical data

Write as mean  $\pm$  SD or mean  $\pm$  SE.

### Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as  $\chi^2$  (in Greek), related coefficient as *r* (in italics), degree of freedom as *v* (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

### Units

Use SI units. For example: body mass, *m* (B) = 78 kg; blood pres-



sure,  $p$  (B) = 16.2/12.3 kPa; incubation time,  $t$  (incubation) = 96 h, blood glucose concentration,  $c$  (glucose)  $6.4 \pm 2.1$  mmol/L; blood CEA mass concentration,  $p$  (CEA) = 8.6 24.5  $\mu$ g/L; CO<sub>2</sub> volume fraction, 50 mL/L CO<sub>2</sub>, not 5% CO<sub>2</sub>; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

The format for how to accurately write common units and quantums can be found at: [http://www.wjgnet.com/1948-0210/g\\_info\\_20100313172144.htm](http://www.wjgnet.com/1948-0210/g_info_20100313172144.htm).

### Abbreviations

Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

### Italics

Quantities:  $t$  time or temperature,  $c$  concentration,  $A$  area,  $l$  length,  $m$  mass,  $V$  volume.

Genotypes: *gyrA*, *arg 1*, *c myc*, *c fos*, *etc.*

Restriction enzymes: *EcoRI*, *HindII*, *BamHI*, *Kho I*, *Kpn I*, *etc.*

Biology: *H. pylori*, *E. coli*, *etc.*

### Examples for paper writing

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