



Ectopic expression of *Cvh* (Chicken *Vasa* homologue) mediates the reprogramming of chicken embryonic stem cells to a germ cell fate

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ABSTRACT

When they are derived from blastodermal cells of the pre-primitive streak *in vitro*, the pluripotency of Chicken Embryonic Stem Cells (cESC) can be controlled by the *cPouV* and *Nanog* genes. These cESC can differentiate into derivatives of the three germ layers both *in vitro* and *in vivo*, but they only weakly colonize the gonads of host embryos. By contrast, non-cultured blastodermal cells and long-term cultured chicken primordial germ cells maintain full germline competence. This restriction in the germline potential of the cESC may result from either early germline determination in the donor embryos or it may occur as a result of *in vitro* culture. We are interested in understanding the genetic determinants of germline programming. The RNA binding protein *Cvh* (Chicken *Vasa* Homologue) is considered as one such determinant, although its role in germ cell physiology is still unclear. Here we show that the exogenous expression of *Cvh*, combined with appropriate culture conditions, induces cESC reprogramming towards a germ cell fate. Indeed, these cells express the *Dazl*, *Tudor* and *Sycp3* germline markers, and they display improved germline colonization and adopt a germ cell fate when injected into recipient embryos. Thus, our results demonstrate that *Vasa* can drive ES cell differentiation towards the germ cell lineage, both *in vitro* and *in vivo*.

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Introduction

Chicken Embryonic Stem Cells (cESC) can be derived *in vitro* from pre-primitive streak (stages IX–XII, EG and K, Eyal-Giladi and Kochav, 1976) blastodermal cells under specific culture conditions. They are characterized by the presence of typical ESC markers such as alkaline phosphatase, telomerase activity, *cENS-1/ERN1* expression and reactivity toward different specific antibodies including ECMA-7, SSEA-1 and SSEA-3 (Pain et al., 1996, Acloque et al., 2001; Petitte et al., 2004). Like their mammalian counterparts, these cells are pluripotent and can give rise to differentiated derivatives of the three germ layers, both *in vitro* and *in vivo* (Pain et al., 1996; van de Lavoie et al., 2006b). We recently demonstrated that the maintenance of their pluripotency was due to the expression of the *cPouV* and *cNanog* genes (Laval et al., 2007), whose mammalian homologues *Oct4* and *Nanog* have been shown to be key components of gene networks associated with pluri-

potency in both mouse and human ESC (Chambers et al., 2003; Mitsui et al., 2003; Ginis et al., 2004; Kehler et al., 2004; Niwa et al., 2005; Chambers et al., 2007).

Pre-primitive streak chicken embryos contain germline competent cells, as demonstrated by the direct injection of Stage X embryo (Eg and K) blastodermal cells (cBC) into recipient embryos (Carscience et al., 1993; Thoraval et al., 1994). At Stage X, 25 to 45 cells express SSEA1 and EMA1, suggesting that they are already differentiated primordial germ cells (PGCs) at this developmental stage (Karagenç et al., 1996). In addition, migrating PGC can be isolated from embryonic blood (Vick et al., 1993; Naito et al., 1994) and maintained under specific culture conditions to give rise to long-term *in vitro* cultures of germline competent cells (van de Lavoie et al., 2006a). However, embryonic germinal cells (EG) derived from these cultured PGC are morphologically distinct, and they adopt a more pronounced adherent phenotype with less potential for germline colonization (Park et al., 2003; van de Lavoie et al., 2006a). Indeed, the potential of long-term cultured cESC to colonize the germline is less than that of cBC, although the molecular events associated with this reduction remain unknown (Pain et al., 1996, Petitte et al., 2004; van de Lavoie et al., 2006b).

The *Vasa* and *Dazl* genes are among the most specific markers for germ cells (Tanaka et al., 2000; Extavour and Akam, 2003; Cauffman et al., 2005). Initially identified in *Drosophila*, the *Vasa* gene encodes a DEAD box RNA binding protein with ATP-dependent RNA helicase

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activity that is homologous to the *eIF4* transcription factor (Lasko and Ashburner, 1988), and which is highly conserved in all metazoans (Mochizuki et al., 2001). A Chicken *Vasa* homologue (*Cvh*) has been cloned (Tsunekawa et al., 2000) and cells expressing *Cvh* are detected as early as the first cleavage stages. Around 30 *Cvh* positive cells are detected in stage X embryos, reinforcing the hypothesis that PGC are present at this stage (Tsunekawa et al., 2000). The *Dazl* (Deleted in azoospermia) gene has also been identified in *Xenopus* (Houston et al., 1998), zebrafish (Maegawa et al., 2002) and mammals (Reijo et al., 2000; Xu et al., 2001). *Dazl* appears to be expressed exclusively in germ cells (Reijo et al., 2000; Cauffman et al., 2005) and it is involved in germ cell proliferation and differentiation (Eberhart et al., 1996; Xu et al., 2001; Yen, 2004).

Chicken homologues of other germline associated genes have also been reported, including *Deadend* (Weidinger et al., 2003; Aramaki et al., 2007), *Piwi* (Cox et al., 2000), *Plfz* (Cook et al., 1995; Costoya et al., 2004), *Tudor* (Thomson and Lasko, 2005; Chuma et al., 2006; Hosokawa et al., 2007), the *Sdf1-Cxcr4* couple (Molyneaux et al., 2003; Stebler et al., 2004), *Sycp3* (Dyce et al., 2006) and *Stra8* (Oulad-Abdelghani et al., 1996). However, the function and regulation of these different genes is still poorly understood, although there is some evidence that *Dazl* is involved in the translational control of the *Mvh* and *Sycp3* proteins (Reynolds et al., 2005, 2007). The limited capacity of cESC to colonize germlines might result from either early germline determination in chicken donor embryos or from the reduction of germline competence *in vitro*. Here we show that the exogenous expression of the *Cvh* gene in cESC, combined with appropriate culture conditions, can trigger differentiation into cells that express specific germline and meiotic markers. This stable genetic modification impairs *in vitro* differentiation potential and greatly improves the gonad colonization ability, also enabling cESC to adopt a germ cell fate *in vivo*.

Materials and methods

Eggs and cells

Fertilized eggs from White Leghorn and Red Naked Neck hens were purchased from a local breeder and incubated in a humidified incubator at 37.5 °C. Chicken blastodermal cells (cBC) were obtained directly from StageX embryos as described previously (Pain et al., 1996; Laval et al., 2007), and they were maintained and transfected in proliferative medium (PM) containing: 10% FBS, 1 mM sodium pyruvate, 1% non-essential amino acids, 0.16 mM β -mercaptoethanol, 0.1 ng/ml of hrIL-6, 0.1 ng/ml hrIL-6Rs, 0.1 ng/ml rhSCF, 1 ng/ml bFGF and 1 ng/ml IGF-1. All factors and cytokines were purchased from R and D systems or Peprotech. Stable clones were obtained after transfection of the expression vectors as described (Pain et al., 1999, Laval et al., 2007). Differentiation was accomplished by incubating cells in differentiation medium (DM) containing 5% FBS, 1% non-essential amino acids, and no growth factors or cytokines. Chicken embryoid body (cEB) formation was induced from 500 to 1000 dissociated cells in 100 μ l DM medium using the hanging drop procedure described previously (Laval et al., 2007).

Expression vector construction

Total RNA was isolated from the gonads of 17 day old male embryos using the RNA easy mini extraction kit (Qiagen 74104) and it was reverse transcribed using SuperscriptII (Invitrogen). The *Cvh* and *Dazl* cDNAs were amplified by PCR with the appropriate primers: *Cvh*-XhoI (5'-tatctcgagaggaggactgggacacg-3'), *Cvh*-BamHIAS (5'-gcgatccttactccatgacttaaatg-3'), *Dazl*-KpnI (5'-tttggtaccatgtctgcaaa-tgcggaagc-3') and *Dazl*-SmaIAS (5'-tttcccgggtcaaacacttttgagcact-3'), and they were cloned in frame into the same sites of pEGFP-C1 plasmid (Clontech). For experiments *in situ*, *Nanog* cDNA was amplified with *Nanog*-EcoRI (5'-atgaattcatgagcgtcactctggcc-3') and

Nanog-EcoRIAS (5'-atgaattcctaagtctcataaccatt-3') primers, and *Nanog* and *Dazl* cDNAs was cloned into pGEMTeasy (Promega).

Antibody staining

The cESC were washed with PBS and fixed for 15 min in 1.5% formaldehyde and 0.5% glutaraldehyde. Staining with SSEA1 (diluted 1/500, MC480 from DSHB, Iowa) and *Dazl* antibodies (diluted 1:100, kindly provided by Dr. J. Petite, North Carolina University) was detected with PE-IgM (Jackson Laboratories) and observed on an Axioplan 2 Imaging system (Zeiss) using CoolSnap software.

Flow cytometry analysis

Analysis and sorting was performed on a dual-laser FACSDiVa Vantage[®] flow cytometer (Becton Dickinson) equipped with an Enterprise (Coherent) argon UV laser (351 to 364 nm) and a visible laser at 488 nm. Cells expressing GFP were sorted, washed immediately, and centrifuged, before being plated directly in fresh medium or frozen for RNA extraction and gene expression analysis.

Total RNA, genomic DNA extraction and QRT-PCR analysis

Total mRNA was extracted from cESC using the RNA easy mini extraction kit (Qiagen 74104) and the quality of the RNA was assessed using a BioAnalyser 2100 (Agilent Biotechnologies). Quantitative RT-PCR was performed with the oligonucleotide sequences listed in Supplementary Table 1 (Laval et al., 2007) and the levels of expression were calculated using the $\Delta\Delta$ Ct method (<http://www.gene-quantification.info>) in duplicate samples. The ribosomal gene *RPS17* (X07257) was used as a reference and in differentiation experiments, a Ct value of 45 was arbitrarily attributed to undetected genes such as *Dazl*. Each experiment was repeated at least twice as indicated in the figures.

In chimera experiments, the heads were removed from the embryos and the tissues required were rinsed in PBS before being placed in the lysis buffer provided with the kit. Genomic DNA was extracted with the AllPrep DNA/RNA kit (Qiagen 80204), quantified using a Nanodrop device and 100 ng was used for genomic PCR. PCR conditions were optimized with the GFP forward and *Cvh* reverse primers using GFP::Cvh cESC genomic DNA as the template and SYBR green (Qiagen). PCR was carried out over 45 cycles consisting of 1 min denaturation at 95 °C, 30 s hybridization at 55 °C, and 30 s elongation at 72 °C. Each sample was analyzed three times and chimerism was validated if amplification occurred in each well of the triplicate. The nature of the amplicon was validated by analyzing its melting curve. No *Cvh* amplicons were detected from control embryos, even after 50 PCR cycles.

Chicken embryo electroporation

Chicken embryo electroporations were performed as described in Morales et al. (2007). Briefly stage X–XII (EG) chicken embryos were explanted using a sterile paper ring and placed on the platinum electrode in the electroporation chamber (Nepagen, Japan) that was connected to the negative pole. The pEGFP-C1 or pEGFP::Cvh plasmid (2 mg/ml in PBS with 0.1% Fastgreen) was injected between the vitelline membrane and the epiblast and a positive electrode was placed over the embryo before making contact with PBS. Five 50 ms pulses of 4 V were applied at 1 s intervals using an Intracell TSS10 pulse stimulator (Intracell). The embryos were then placed in culture at 37 °C as described previously (Chapman et al., 2001) and allowed to develop for 40 h. Finally, they were photographed with a Leica MZFLIII microscope to assess the expression of GFP and fixed overnight in 4% paraformaldehyde at 4 °C for further analysis.

Whole mount *in situ* hybridization and immunohistochemistry

Chicken embryos were staged according to [Hamburger and Hamilton \(1951\)](#) and whole mount *in situ* hybridization was performed using digoxigenin-labeled riboprobes to detect *Dazl*, *Cvh* and *Nanog* transcripts as described previously ([Acloque et al., 2008](#)). The embryos were then subjected to immunohistochemistry with an antibody against GFP (1:500, Molecular Probes).

Immunohistochemistry of the gonads and the entire urogenital tract of injected embryos was carried out having fixed these tissues in methanol. The tissues were then rehydrated in 0.5% PBS-Triton (PBT) and blocked overnight in PBT with 1% BSA. The gonads were then incubated overnight at 4 °C with primary antibodies against GFP (1:500) and SSEA-1 (1:500) in blocking solution, which were then detected with Alexa 488 conjugated anti-rabbit IgG (1:1000, Molecular probe) or a biotinylated anti-mouse IgM (1:500) coupled with Alexa 568 conjugated Streptavidin (1:1000, Molecular Probe), respectively. The gonads were then embedded in gelatin, sectioned at 40 μm and photographed using a Leica DMR microscope with Nomarski optics and with an Olympus DP70 digital camera.

Injection of cESC into stage X embryos

Stage X recipient embryos were irradiated at 6 Gy (Cobalt source) as described previously ([Pain et al., 1996](#)). A window was drilled in the lateral shell of the egg and the shell membrane was removed. Five hundred to 2000 dissociated cells were injected into the subgerminal cavity in 1 to 3 μl using a 20 μl borosilicate micropipette. The window was resealed with two layers of shell membrane that were reinforced with a piece of Visulin (Hartmann, UK) and the eggs were incubated for a further 6–7 or 12–15 days before the embryos were analyzed.

Results

Expression of *Cvh* and *Dazl* decreases during *in vitro* generation of chicken embryonic stem cells

Since chicken embryonic stem cells lose germline competence compared to that in chicken blastodermal cells, it seems likely that the expression of genes related to pluripotency and germline competence decrease during this differentiation process. Thus, we decided to analyze the expression of candidate genes in these two populations and in 17 day-old male embryonic gonads as a control tissue containing differentiated germ cells.

The two main candidate genes considered to be associated with pluripotency are *cPouV* and *cNanog* as they were recently shown to maintain cESC pluripotency ([Laval et al., 2007](#)). Germline associated genes were selected based on their known and identified function in other model systems, and on the availability of homologous chicken sequences. Accordingly, we selected specific germ cell markers such as *Deadend*, *Piwi*, *Plzf*, *Tudor*, *Sdf1*, *Cxcr4*, *Sycp3* and *Stra8*. Following QRT-PCR analysis of the different cell types, we found that the expression of *Deadend*, *Piwi* and *Plzf* did not change significantly (data not shown) in contrast to that of genes associated with pluripotency which expression was stronger in cESC than in cBC (e.g. the *cPouV* and *Nanog* genes, [Fig. 1A](#)). We also found that the expression of some germline genes were downregulated or even absent in cESC when compared to cBC. Indeed, *Cvh*, *Dazl*, *Sycp3* and *Stra8* transcripts were significantly downregulated in cESC ([Fig. 1B](#)). Since these genes are involved in germ cell physiology, it is likely that their absence in cESC might account for the reduction in their germline competence.

Based on these results, we focused on the two RNA binding proteins *Cvh* and *Dazl*, known to be indispensable for germ cell formation and proliferation in various vertebrate and invertebrate species. During early chicken development, *Cvh* and *Dazl* expression is rapidly restricted to a few cells of the germinal crescent in Stage 5 embryos (H and H table, [Supplementary Fig. 1](#), [Tsunekawa et al., 2000](#); [Song, 2003](#)).

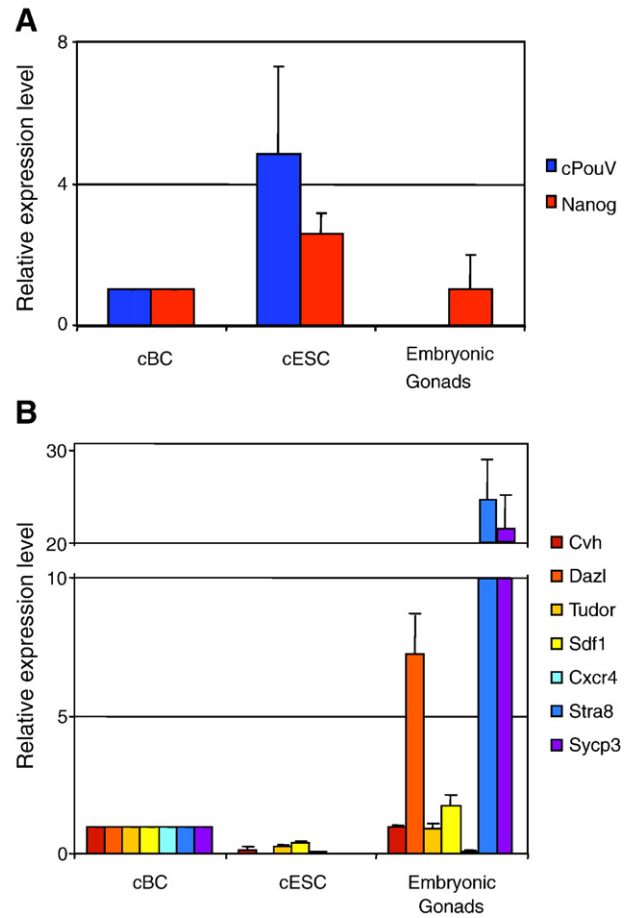


Fig. 1. Expression profile of pluripotency and germ cell associated genes. A, B, expression profile of several pluripotencies (A) and germ cell (B) associated genes in cBC, cESC and 17 day old embryo gonads. A Q-RT-PCR level of 1 was arbitrarily attributed to the level of expression in cBC and each sample was run in duplicate. Error bars represent standard errors (s.e.m) from three experiments carried out on three independent samples.

Molecular characterization of GFP::*Cvh* expressing cESC

In order to investigate whether the loss of endogenous *Cvh* expression in cESC might be linked to poor germline competence, we over-expressed a GFP::*Cvh* fusion protein in cESC. Stable transfected clones expressing the GFP::*Cvh* construct were obtained after drug selection, pooled and cells expressing GFP were FACS cell sorted. About 0.5% of the resistant cells strongly expressed GFP::*Cvh* protein (hereafter referred as GFP::*Cvh* high cESC) after a first round of FACS enrichment, and they were again plated *in vitro* for further amplification under proliferative conditions. After a few days, the amplified cells were again FACS cell sorted to obtain further enrichment ([Figs. 2A, B](#)). These purified GFP::*Cvh* high cESC proliferated at the same rate as the GFP::*Cvh* negative cells (data not shown). However, while they displayed a 50% decrease in the expression of the *cPouV* gene associated with pluripotency, there was a slight increase in the expression of the endogenous *Cvh* and *Stra8* genes but not of other germ cell markers ([Fig. 2C](#)). Such modifications were not observed in control cells over-expressing GFP alone and more surprisingly, cells expressing the GFP::*Dazl* protein and subjected to a similar selection procedure did not display any significant changes in gene expression ([Supplementary Fig. 2](#)). Thus, overexpression of *Cvh* alone did not appear to induce a significant change in cESC phenotype when they were maintained in the proliferation medium (PM). Indeed, this modification neither changed their proliferation rate nor the expression of germ cell

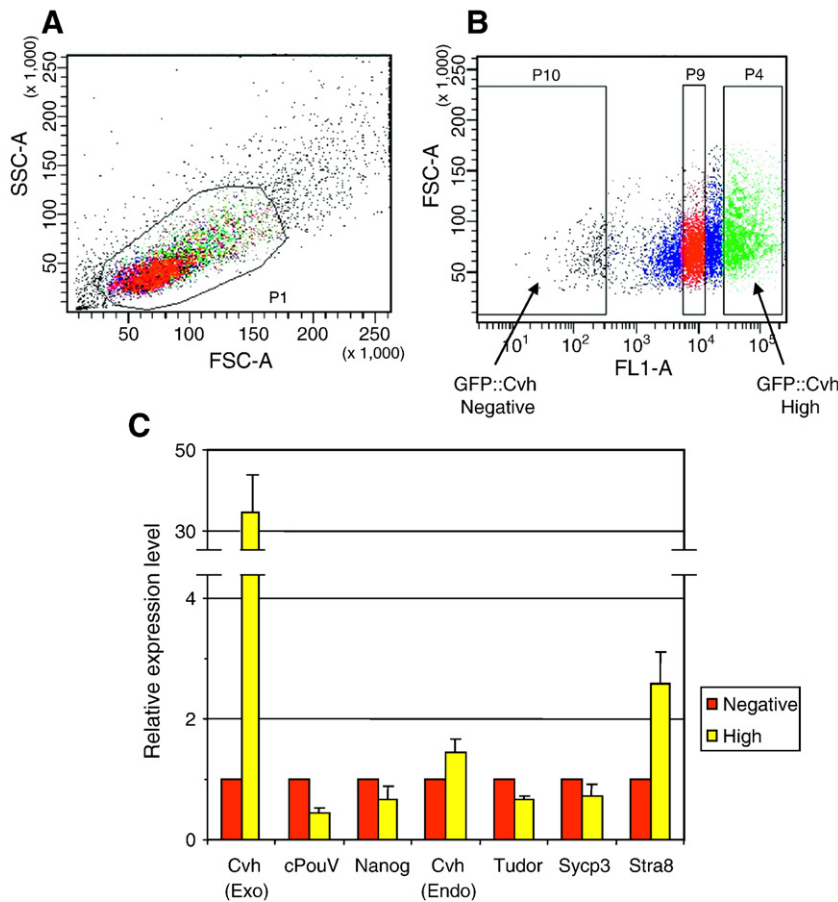


Fig. 2. Molecular characterization of GFP::Cvh expressing cESC. (A, B) FACS profile of GFP::Cvh stably transfected cESC. After drug selection, clones were pooled and amplified, and their size and morphology was analyzed by FACS (A) before they were sorted for GFP levels (B). GFP::Cvh negative and GFP::Cvh high cESC were again plated in culture and amplified for 5 days in Proliferation medium before analyzing by Q-RT-PCR (C). A Q-RT-PCR level of 1 was arbitrarily attributed to the gene expression level in the GFP::Cvh negative cESC. Each sample was run in duplicate and three independent samples were used to calculate the mean and standard error (error bars).

associated genes, and *Dazl* expression could still not be detected. However, the overexpression of *Cvh* did affect the levels of *cPouV* expression, which must be precisely regulated to maintain cESC in the undifferentiated state (Laval et al., 2007).

Cvh over-expression in cESC impairs their ability to form mesendodermal derivatives

The differentiation potential of GFP::Cvh high cESC was assessed by their capacity to form embryoid bodies (EBs). Dissociated GFP::Cvh high cESC were plated for 9 days in hanging drops of differentiation medium (DM), devoid of growth factors and cytokines. On the whole, GFP::Cvh high cESC were unable to form cystic EBs and instead, they generated small 3D structures in 2/3 of the drops, unlike the typical EBs generated by control GFP::Cvh negative cells in more than 70% of the cases ($n = 100$ in each case, Figs. 3A, B). The expression of *cPouV* and *Nanog* decreased in both cESC populations during the differentiation process (Data not shown) while as expected, the expression of mesodermal (*Gooseoid* and *Brachyury*, Kaji et al., 2006), neurectodermal (*Sox2*) and endodermal markers (*Hnf3 β* , *Sox7* and *Gata6*, Kaji et al., 2006) was strongly up-regulated during EB formation in GFP::Cvh negative cells. By contrast, these markers were not strongly induced in GFP::Cvh high cESC, which only slightly increased the expression of *Sox2* and *Brachyury* (Figs. 3C, D). Therefore, the sustained expression of *Cvh* in cESC modified their differentiation potential and especially, their ability to form mesendoderm derivatives in non-adherent differentiation culture conditions.

Cvh over-expression in cESC induces a germ cell phenotype

As GFP::Cvh high cESC only form poor 3D differentiated structures under non-adherent conditions, we better characterized the differentiation potential by studying their behavior in adherent culture conditions (Fig. 4A). When plated for 5 days under adherent conditions in DM medium, GFP::Cvh high cESC maintained high levels of *cPouV* and *Nanog* expression (Fig. 4B). Moreover, the expression of germ cell markers was induced, including *Tudor*, *Sdf1* and *Cxcr4*, notably involved in the guidance of chicken PGCs, as well as the expression of the premeiotic marker *Sycp3*. Endogenous *Cvh* was also strongly induced and more importantly, *Dazl* mRNA was also highly expressed for the first time in culture. This up-regulation is very similar to the expression detected recently when chicken PGC are amplified *in vitro* (van de Lavoie et al., 2006a). After 5 days in the same culture conditions, there was a 50% decrease in *cPouV* and *Nanog* expression in GFP::Cvh negative cells (Fig. 4C), coupled to a slight increase in germ cell markers and a constant level of endogenous *Cvh* expression, while *Dazl* expression remained undetectable (Fig. 4C). After this 5 day differentiation period, clusters of GFP::Cvh high cESC can be detected by direct immunofluorescence. These cells had a round morphology typical of PGCs and they exhibited a strong reactivity to the specific SSEA-1 antigen associated with strong GFP::Cvh expression (Figs. 4F, G). Some GFP::Cvh high cESC also expressed the *Dazl* protein, as detected with the chicken *Dazl* antibody (Figs. 4J, K). However, no SSEA-1 expression or *Dazl* positive cells were detected in the differentiated GFP::Cvh negative cESC cultures (data not shown). Thus, the exogenous expression of *Cvh* can drive the cESC towards a

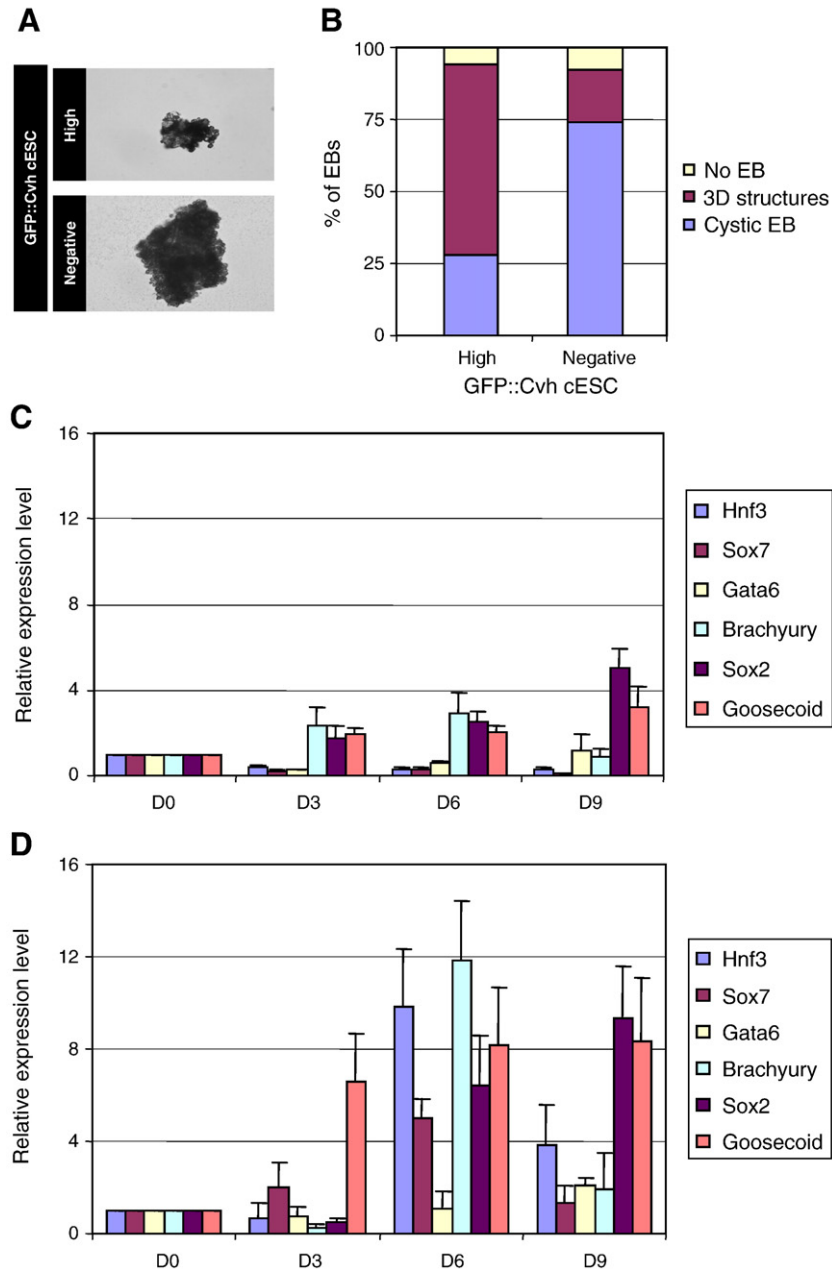


Fig. 3. Cvh Over-expression alters the *in vitro* differentiation properties of cESC. GFP::Cvh high cESC and control GFP::Cvh negative cESC were induced to form embryoid bodies (EB) in hanging drops. Microscopic observation of a 9 day-old EB derived from GFP::Cvh high cESC (A, upper panel) and from GFP::Cvh negative cESC (A, lower panel) clearly shows that GFP::Cvh high cESC form small poorly developed 3D structures whereas GFP::Cvh negative cells form typical EBs. (B). Each structure was counted and GFP::Cvh high cESC formed poorly developed 3D structures in more than 2/3 of the drops, whereas GFP::Cvh negative cESC gave rise to typical EBs in more than 70% of the drops. (C, D) The expression of differentiation associated genes *cPouV* and *Nanog* decreases in both GFP::Cvh high (C) and GFP::Cvh negative (D) derived structures, as analyzed by QRT-PCR throughout the process of EB formation: on day 0 (D0), when plated, on day 3 (D3), 6 (D6) and 9 (D9). GFP::Cvh high cESC are unable to induce strong expression of endodermal, mesodermal and neuroectodermal markers (C) after 9 days of differentiation, unlike the GFP::Cvh negative cells (D). Q-RT-PCR was performed in duplicate and a value of 1 was attributed to the level of gene expression at the beginning of the differentiation process (D0). The results and standard errors (s.e.m.) presented correspond to the means from two independent experiments.

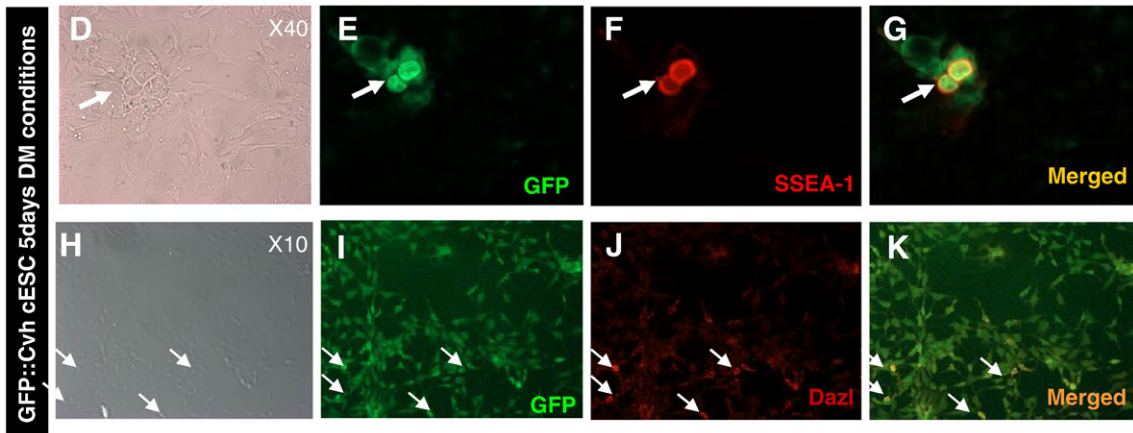
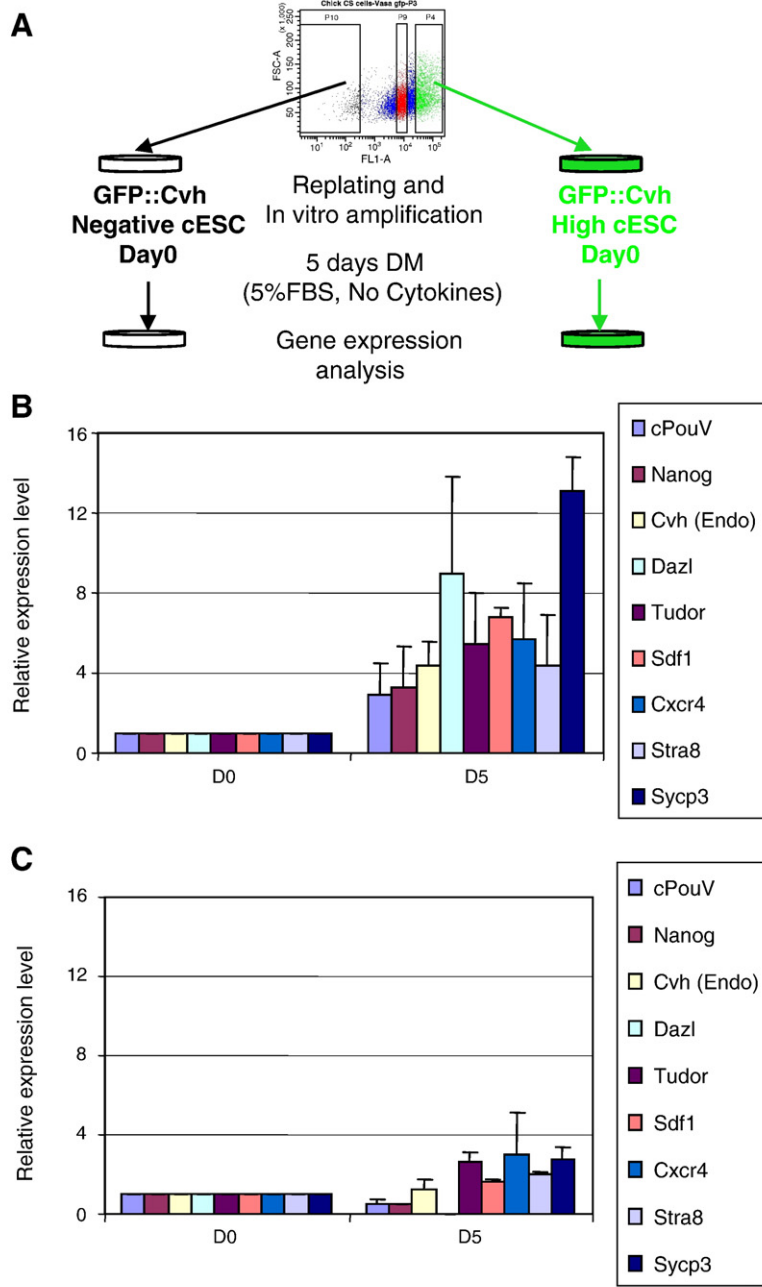
germline fate when they are maintained in differentiation culture conditions.

In vivo overexpression of GFP::Cvh converts epiblast cells into germ cells that preferentially colonize the embryonic gonads

In order to test whether the behavior observed *in vitro* was applicable to *in vivo* differentiation, the GFP::Cvh construct was electroporated into explanted stage X embryos maintained for 40 h in culture until stage HH9 (Hamburger and Hamilton, 1951). At this stage of development, endogenous PGC are localized in the germinal

crest as assessed by the expression of *Nanog* and *Dazl*. In 40% (4/10) and 64% (9/14) of electroporated embryos there was an ectopic induction of *Dazl* and *Nanog* expression, respectively (black arrows on Figs. 5C, F). More interestingly, *Dazl* was induced in 18% and *Nanog* in 56% of GFP::Cvh expressing cells (Fig. 5M). In control embryos, the over-expression of GFP alone ($n = 14$) did not induce the ectopic expression of either *Dazl* or *Nanog* (Figs. 5I–L), indicating that the ectopic expression of *Cvh* in pluripotent epiblast cells can also drive embryonic cells towards a germ cell fate.

To assess the colonizing potential of the modified cESC *in vivo*, FACS cell-sorted GFP::Cvh negative and GFP::Cvh high cESC were



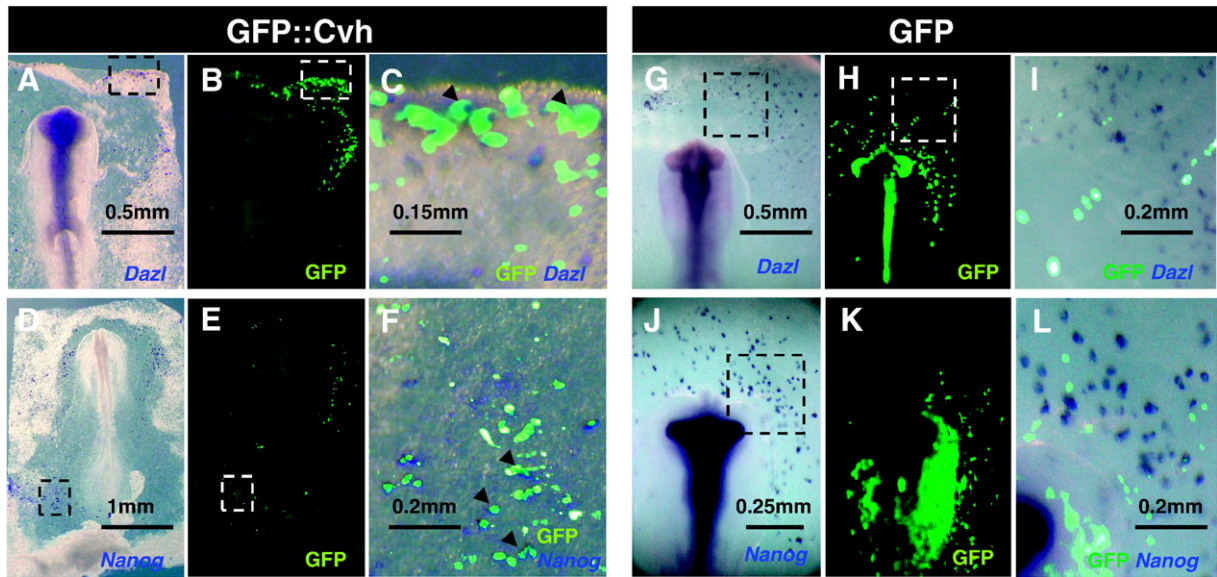


Fig. 5. Cvh over-expression drives avian embryonic cells to the germ fate *in vivo*. A–F, induction of germ cell markers in embryonic cells electroporated with the GFP::Cvh construct. Stage X (EG) embryos were electroporated and maintained in culture for 40 h. The ectopic expression of GFP::Cvh (detected by immunohistochemistry against GFP, green) induces *Dazl* (C) and *Nanog* (F) expression in HH embryos (both detected by whole mount *in situ* hybridization, blue) when compared to control embryos (I and L). Black arrows in C and F indicate cells co-expressing the fusion protein Vasa-GFP (green) and *Dazl* or *Nanog* transcripts (blue), respectively.

maintained in either proliferative (PM) or differentiation (DM) culture conditions for 5 days. Subsequently, the different cell types were injected into StageX recipient embryos as described previously (Pain et al., 1996). Embryos were incubated for 12–15 days and the presence of GFP::Cvh cells in somatic tissues (heart and kidney), and in the gonads, was assessed by genomic PCR. The presence of GFP::Cvh cells was detected in 60% of the embryos injected with the GFP::Cvh high cESC and maintained in PM ($n = 15/25$) and in 50% of those maintained in DM conditions ($n = 9/18$; Fig. 6A). Similarly, 33% of the GFP::Cvh negative cESC embryos maintained in PM were chimeric ($n = 6/18$) and 50% were chimeric in DM conditions ($n = 7/14$; Fig. 6A), like those cESCs expressing GFP alone (data not shown). No GFP was detected in control embryos.

Three categories of chimeric embryos could be distinguished by genomic PCR analysis: somatic chimera embryos (SCE) with GFP::Cvh cells in somatic tissue(s) alone; mixed chimeric embryos (MCE) with injected cells in both somatic tissue(s) and gonads; and germline chimeric embryos (GCE) only containing GFP::Cvh cells in the gonads. The GFP::Cvh high cESC maintained under DM conditions for 5 days exhibited strong gonad colonization, and 67% of the chimeras were considered GCE ($n = 6/9$), 11% SCE ($n = 1/9$) and 22% were MCE ($n = 2/9$). By contrast, when the GFP::Cvh high cESC were maintained under PM conditions before being injected, only 20% were GCE ($n = 3/15$), 40% were MCE ($n = 6/15$) and 40% were SCE ($n = 6/15$; Fig. 6B). Interestingly, control GFP::Cvh negative cESC also maintained under PM conditions, showed a similar colonization potential (16% GC, $n = 1/6$; 33% SCE, $n = 2/6$; and 50% MCE, $n = 3/6$). When these GFP::Cvh negative cESC were cultured for 5 days under DM conditions prior to injection, they produced only 14% GCE ($n = 1/7$), with 28% SCE ($n = 2/7$) and 57% MCE ($n = 4/7$; Fig. 6B). cESC expressing GFP alone behaved similarly to these GFP::Cvh negative cells, producing 12.5% and 11% GCE, 37.5% and 33.3% SCE, and 50% and 55.5% MCE under PM and DM culture conditions, respectively (data not shown). These data

show that there is strong germinal tropism, with preferential colonization of the host gonads by the GFP::Cvh high cESC maintained under DM culture conditions (Supplementary Table 2), suggesting that sustained expression of Cvh is required to modify the colonization potential of cESC.

We then analyzed the localization and the fate of injected cells in embryonic gonads by *in situ* hybridization for GFP. Indeed, given the proportion of mixed chimeric embryos obtained with control cells, it was necessary to characterize the fate of the GFP::Cvh high cESC once in the gonads. Clumps of injected cells were found in day 12 male embryonic gonads (Figs. 6C, C') and cells were also detected in the cortical zone of a day 12 female gonad (Figs. 6D, D') where *Dazl* expressing germ cells are located (see Supplementary Figs. 1e, e'). In order to characterize the fate of the GFP::Cvh expressing cells detected in the embryonic gonads, immunohistochemistry against GFP and SSEA1 was performed on 6 day-old embryonic gonads taken from embryos injected with GFP::Cvh high cESC maintained under DM conditions. Accordingly, we detected groups of cells expressing GFP::Cvh and SSEA-1 surrounded by host germ cells expressing only SSEA-1 (Figs. 6F, F', G, G'). Thus, when maintained under DM conditions, GFP::Cvh high cESCs colonize preferentially and follow a germ cell fate in host embryo gonads.

Discussion

Chicken Embryonic Stem cells (cESC) are non-mammalian embryonic stem cells with strong similarity to their murine counterparts in terms of growth factor requirements (Pain et al., 1996; Petitte et al., 2004) and the control of pluripotency exerted by *cPouV* and *cNanog* (recently identified avian homologues of the mammalian *Oct4* and *Nanog* genes; Laval et al., 2007). However, it appears that their germline competence is much more sensitive to culture conditions, and it rapidly and dramatically diminishes after long-

Fig. 4. GFP::Cvh high cESC differentiate into germ cells *in vitro*. (A) schematic representation of the process used to differentiate the transfected cells *in vitro*. GFP::Cvh high and negative cESC were FACS sorted, plated, amplified and induced to differentiate for 5 days under DM conditions (see Materials and methods). (B, C) expression profile of GFP::Cvh high and negative cESC after 5 days (D5) differentiation *in vitro*. (B) GFP::Cvh high cESC maintain high levels of pluripotency associated gene expression and germline marker expression is strongly induced when compared to control GFP::Cvh negative cells (C). Q-RT-PCR was performed in duplicate and a value of 1 was arbitrarily attributed to the level at the beginning of the differentiation process (D0). The means and standard errors (s.e.m.) of three independent experiments are shown. (D–K) Immunofluorescence of GFP::Cvh high cESC after 5 days in DM conditions. GFP::Cvh high cESC plated under these conditions (D, H: brightfield) co-expressed GFP (E, I: GFP) and either the specific SSEA1 antigen (F, G) or *Dazl* protein (J, K).

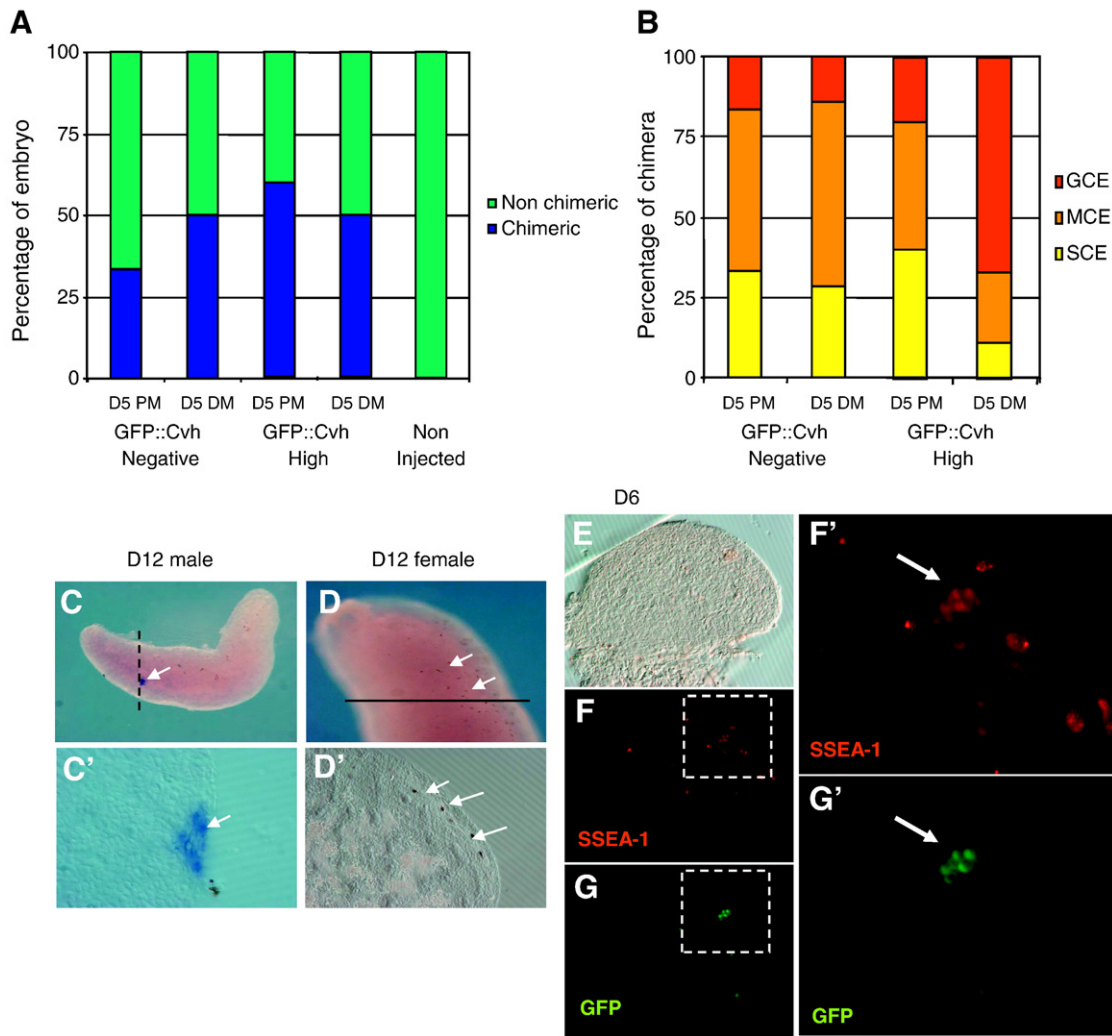


Fig. 6. Differentiated GFP::Cvh cESC preferentially colonize the gonads of host embryos and adopt a germ cell fate. (A) genomic DNA of injected or uninjected embryos was analyzed for the presence of the GFP::Cvh transgene. The percentage of chimeric embryos with GFP::Cvh negative or GFP::Cvh high cESC maintained under either PM or DM conditions for 5 days, was represented as a function of total number of embryos analyzed. (B) chimeric embryos were classified according to the presence or absence of transgenes in somatic cells and/or gonads: Somatic Chimeric Embryos (SCE) were those where the injected cells were detected only in somatic tissue(s); in Mixed Chimeric Embryos (MCE) injected cells were detected in both the gonads and somatic tissue(s); and in Germline Chimeric embryo (GCE) injected cells were exclusively found in the gonads. (A, B) PCR was performed on genomic DNA from somatic tissues (heart and kidney) and from the gonads of live embryos 12–15 days old. The total number of embryos analyzed was indicated in Supplementary Table 2. Each sample was run in triplicate and the nature of the fragment amplified was validated by the melting curve. C, D: localization of GFP::Cvh positive cells in the embryonic gonads of host 12–15 day embryos injected with GFP::Cvh high cESC. GFP expression was detected by *in situ* hybridization. Positive cells (white arrows) are detected in a 12 day-old male gonad (C, C') but also in the cortical area of a 12 day-old female gonad (D, D'), both sexes were determined by macroscopic observation. E–G: immunohistochemistry against GFP and SSEA1 in embryonic gonads of 6 day-old embryos injected with GFP::Cvh high cESC that were maintained for 5 days under DM conditions. GFP::Cvh positive cells expressing SSEA1 are detected in the host gonad (white arrow in F, F') surrounded by endogenous germ cells expressing only SSEA-1 (broken white line in G, G').

term culture (Pain et al., 1996; Petite et al., 2004; van de Lavoie et al., 2006b). The decrease of germline competence of cESC can be explained if germline determination in chicken occurs prior to cESC derivation. Indeed, several studies have suggested that the chicken germ lineage is maternally predetermined, notably since the sub-cellular distribution of the Cvh protein in the cytoplasm of chicken oocytes appears to be equivalent to that described in the germplasm of *Xenopus* oocytes. Germline precursors can be detected in stage X chicken embryos and they consist of approximately 30 Cvh positive cells, supporting previous observations with two other germ cell markers, SSEA1 and EMA1.

Among the candidates to regulate germline competence, *Cvh* (Chicken *Vasa* homologue) and *cDazl* (chicken Deleted in Azoospermia) are two genes that encode RNA binding proteins and whose expression is strongly down-regulated (*Cvh*) or even abolished (*Dazl*) in cESC. Our data indicate that the decrease in *Cvh* is likely to be responsible for the decreased germline competence of the cESC. Indeed, *Cvh* ectopic expression in cESC modifies their differentiation

potential, accompanied by a change in gene expression profile. Under similar conditions, the *Dazl* gene is insufficient to modify the cESC phenotype, although it cannot be excluded that under other culture conditions, the expression of the *Dazl* gene may influence the cESC phenotype. Moreover, the exogenous levels of GFP::Dazl tolerated by the cESC are lower than those of GFP::Cvh suggesting that cESC expressing high levels of cDazl cannot be maintained. Importantly, the presence of the *Cvh* RNA binding protein alters the phenotype of these stem cells, favoring their differentiation into germ cells. In mESC cells, the upregulation of *Dazl* is part of an hierarchical translational network involved in mESC proper differentiation process (Sampath et al., 2008) and *Esg1*, another RNA binding protein, has also been shown to bind a broad range of targets and is expressed exclusively in mESC cells and germline (Tanaka et al., 2006).

In cESC, *Cvh* expression may be a preliminary signal necessary to down-regulate some genes linked to pluripotency, such as *cPouV* and *Nanog*. However, this may not be sufficient to induce complete *in vitro* germ cell differentiation under proliferative culture conditions.

Therefore, it appears that the modified cESC cells require a further step to pursue their differentiation, which can be achieved by decreasing their capacity to proliferate by serum and growth factor depletion. This two-step scenario probably could reflect the gradual induction towards the germ cell lineage by first down-regulating pluripotency-associated gene expression and by then inducing a specific germ cell program. Our results also suggest that a high level of *Cvh* expression may be incompatible with a stem cell phenotype of high proliferative ability and large differentiation potential. This could be also one of the main differences between the cESC and the PGC maintained under non adherent conditions as these cells express a high level of *Cvh* (van de Lavoie et al., 2006a). Based on these observations, it may be now interesting to compare the culture conditions previously used to obtain germ cells *in vitro* from mouse ESC with our chicken conditions (Hübner et al., 2003; Geijsen et al., 2004). In particular, the role of BMP4 and BMP8 could be tested in terms of their influence on *in vivo* germ cell development (Lawson et al., 1999; Saitou et al., 2002).

Our data also demonstrate that after a long-term culture *in vitro*, cESC derived from cBC cells retain the ability to respond to the exogenous expression of *Cvh*, and are able to become germ cells with *in vivo* colonization potential. Undoubtedly, the *in vivo* environment encountered by the injected cells may help them to finalize their differentiation by enhancing their germinal program. Although we cannot formally exclude that any of these cells enter a non-germ cell lineage, this seems unlikely due to their poor differentiation potential. Long-term analysis of injected embryos allowed to hatch as live animals in order to study their progeny, would also help to define this *in vitro* cESC reprogramming as well as the *in vivo* germ cell terminal differentiation.

Regarding *Vasa* and *Dazl* RNA binding proteins, it is also worth noting that the recently described iPS cells (Okita et al., 2007; Wernig et al., 2007) express high levels of *Mvh* and *Dazl*, and are germline competent when compared to the parental fibroblasts (MEF; Maherali et al., 2007). Moreover, murine EpiSC express reduced *Dazl* levels when compared to mESC cells and they seem to show a different colonization capacity (Supplementary Table 3: Brons et al., 2007; Tesar et al., 2007). Thus, it would be interesting to test whether modulation of *Vasa* expression in murine EpiSC could change their behavior.

In summary, we show that *Cvh* expression allows cESC to differentiate into the germ fate, suggesting that its absence in cESC is probably responsible for their very low germline competency. As *Cvh* is expressed very early in chicken embryos, these results are in favor of the predetermination model supported by Tsunekawa et al. (2000) even if *Cvh* expression could also be induced later in hypoblastic or epiblastic responsive cells. Further experiments to track cells expressing GFP under the *Cvh* promoter for example would help answer the question of which model of the predetermination or the inductive one is valid in the chick. We finally propose that differences in competency of embryonic stem cells to differentiate into the germinal layer may rely upon levels of *Vasa* expression that could therefore be used as a marker of germline competency in newly derived pluripotent cells.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.03.012.

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