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Subsets of cloned mouse embryos and their non-random relationship to development and nuclear reprogramming

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ABSTRACT

An important question in oocyte-mediated nuclear reprogramming is whether gene expression of the donor nucleus changes randomly or follows a pattern. Since cloned embryos are very heterogenous and arrest frequently during preimplantation development, a random scenario is generally accepted. In the present study, we resolve the heterogeneity of cumulus cell-derived mouse clones by recognizing structured subsets, and we analyze their relationship to reprogramming of donor nuclei. We utilize live cell imaging of the *Oct4* promoter-driven *GFP* transgene to resolve the populations of cloned and ICSI-fertilized morulae, and we sort them both into three subsets based on different *GFP* expression. Functionally, subsets of cloned but not ICSI morulae form blastocysts and ES cells proportional to *Oct4-GFP* expression. Regulatively, the subsets of cloned morulae are characterized by small differences of transcript level for the pluripotency-associated genes *Oct4*, *Nanog* and *Sox2*. Small differences of the level of select mRNAs across subsets suggest a uniform rather than random course of reprogramming from the morula stage on. Since these small differences correspond with substantial differences in developmental competence, we propose that developmental potential of clones relates to levels of gene expression in a different way than fertilized embryos.

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[3] Performed the live imaging of morulae and the comparative analysis of aneuploidy in cloned and fertilized embryos.
[4] Performed the real-time PCR analysis of transcripts in morulae and blastocysts.
[5] These authors performed the M-FISH of chromosomes from blastomeres.
[6] Performed the DNA methylation analysis.
[7] Provided intellectual input and the research infrastructure.
[8] Performed the mouse cloning and ICSI experiments.

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1. Introduction

Nuclei of unipotent somatic cells may become toti- or pluripotent after transfer into the metaphase cytoplasm of oocytes (2nd meiotic metaphase) or zygotes (1st mitotic metaphase) as shown by full-term development of the constructs or derivation of pluripotent embryonic stem (ES) cells (Egli et al., 2007). The gene expression pattern of the donor nucleus undergoes extensive change within the recipient cytoplasm (reprogramming); presumably it must resemble that of a zygotic embryo in order for the clone to succeed. The prevalent view is that nuclear reprogramming does not follow a scheme and therefore is error-prone; but, in fact, the recipient cytoplasm might process the somatic nucleus as if it was a gametic nucleus. Therefore, it has been proposed that the limited developmental potential of cloned embryos is a result of skipping gametogenesis, which is necessary for complete programming and then reprogramming of key genes in the ooplasm (Fulka et al., 2004).

Key embryonic genes include *Oct4*, which encodes a POU-domain transcription factor that is regarded as a 'gatekeeper' of pluripotency (Pesce and Schöler, 2001). Homozygous *Oct4* mutant mouse embryos form morphologically normal blastocysts, but their inner cell mass (ICM) is not pluripotent (Nichols et al., 1998). Experimental manipulation of Oct4 protein levels below 50% or above 150% of the physiologic level in mouse ES cells leads to in vitro differentiation along different lineages (Niwa et al., 2000). During embryogenesis, ubiquitous *Oct4* expression driven by the chicken β-actin promoter and cytomegalovirus enhancer affects mid-hindbrain patterning (Ramos-Mejia et al., 2005), whereas Oct4 knock-down in the ICM by RNA interference impairs cardiogenesis (Zeineddine et al., 2006). In the adult mouse, epithelial cells respond to a bulk increase in Oct4 level and become neoplastic (Hochedlinger et al., 2005). Thus, faithful reprogramming of the *Oct4* gene is necessary for cloned embryo development beyond the blastocyst stage, but we should not discount an earlier role of maternal Oct4 product in the ooplasm. In cumulus cell-derived mouse blastocysts, both the level and distribution of *Oct4* expression is aberrant (Boiani et al., 2002). Bortvin and colleagues found that only 62% of such cloned blastocysts expressed *Oct4* and ten *Oct4*-related genes; the same genes were expressed in all ES cell-derived clones and fertilized embryos (Bortvin et al., 2003). Nevertheless, ES cell-derived clones fail in large numbers at gastrulation (Jouneau et al., 2006) even though the donor nuclei may need less reprogramming than somatic nuclei (Blelloch et al., 2006) and may be more compatible with the recipient ooplasm (ES cells as 'earliest germ cells'; Zwaka and Thomson, 2005).

An important question regarding nuclear reprogramming is whether changes of gene expression, in particular those concerned with pluripotency, occur randomly or follow a pattern. Investigating patterns of gene expression, however, is difficult. Gene expression studies based on pools of embryos are not suitable to address the question of random vs patterned change, as diversities are evened out. Studies of individual cloned embryos, on the other hand, produce too much detail to recognize patterns. Moreover, examination of transcript levels of a single gene in individual embryos could not distinguish a clone from a control embryo (Somers et al., 2006). Cloned fetuses or newborns are even less suitable for gene expression studies as they already survived many critical phases of embryogenesis. However, because culture conditions can consistently change the level and distribution of Oct4 transcript in cumulus cell-derived mouse blastocysts (Boiani et al., 2005), subsets of clones are produced, regardless of whether the cues from the culture medium affect the reprogramming process or simply select for competent clones. Therefore, gene analysis of defined subsets of the cloned embryo population might facilitate understanding of their gene regulation and help to revise the analytical approach that has been used so far.

In this study, we expose the structured diversity existing among cloned mouse morulae using *Oct4-GFP* transgenic nuclei (Boiani et al., 2002; Szabó et al., 2002), live-cell confocal microscopy and basic image analysis. Morulae were viably sorted into three subsets according to mean GFP intensity and analyzed for GFP distribution, cell number, gene expression, ES cell and fetal potential. Functionally, developmental heterogeneity of clones can be reconciled with subsets of morulae that have different developmental competence within the embryo population. Although changes in gene expression may still be random until the morula stage, our results clearly preclude ongoing randomness with regard to blastocyst, ES cell and fetus formation. Regulatively, subsets of cloned morulae are characterized by small differences of transcripts levels of the pluripotency-associated genes *Oct4*, *Nanog* and *Sox2*. Whether there are other genes that show greater differences and are responsible for the effect, or clones indeed respond to small differences of gene expression unlike ICSI counterparts, these two possibilities point in the same direction: developmental potential of clones relates to levels of gene expression in a different way than fertilized embryos, and brings yet another level of complexity to nuclear reprogramming.

2. Results

2.1. *Subsets of embryos defined by intervals of Oct4-GFP intensity provide a new system to study nuclear reprogramming*

Patterns of GFP in blastocysts predict ES cell formation in cloned mouse embryos derived from *Oct4-GFP* transgenic cumulus cells (Boiani et al., 2002). Transcription from the mouse *Oct4* promoter begins at the 8-cell stage (Yoshimizu et al., 1999), which suggests that the morula stage (≈16-cell) clones may already harbor a bias towards developmental success and may thus reveal earlier aspects of nuclear reprogramming than previously suspected.

We devised an imaging protocol to measure GFP intensity without compromising embryo viability (Section 4). Seventy-eight hours after transfer of *Oct4-GFP* cumulus cell nuclei into mouse ooplasts, more than 70% of the reconstructed oocytes attained the morula stage and variably expressed GFP under our experimental conditions. GFP intensity correlated to both the Oct4 mRNA level ($r^2 = 0.943$; *Spearman test*) and anti-Oct4 immunofluorescence signal ($r^2 = 0.923$; *Spearman test*; Table 1). After chromosome multicolor FISH, we could detect only