Reprogramming of gene expression following nuclear transfer to the *Xenopus* oocyte

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Abstract – Transplantation of *Xenopus laevis* cell nucleus to enucleated *Xenopus* egg leads to the generation of cloned animal. This exemplifies the process of nuclear reprogramming by which the nucleus of a specialized cell is reset to an embryonic state from which it can generate all the cells of an organism. Using the precursor of the egg, the oocyte, it is also possible to reprogram somatic cell. The advantage of this approach is the direct reprogramming of gene expression in the absence of cell division. Using this strategy it is possible to investigate the mechanism leading to transcriptional reprogramming of somatic nuclei. By combining real time monitoring of chromatin protein exchange and gene expression analysis, we have observed that a simultaneous loss of somatic H1 linker histone and incorporation of the oocyte-specific linker histone B4 precede transcriptional reprogramming. The loss of H1 is not required for gene reprogramming. We have demonstrated both by antibody injection experiments and by dominant negative interference that the incorporation of B4 linker histone is required for pluripotency gene reactivation during nuclear reprogramming. We suggest that the binding of oocyte specific B4 linker histone to chromatin is a key primary event in the reprogramming of somatic nuclei transplanted to amphibian oocytes.

Key words: *Xenopus* / reprogramming / nuclear transfer / real time monitoring

Résumé – Reprogrammation nucléaire par transfert de noyau dans l'ovocyte de Xénope.

La transplantation d'un noyau cellulaire de *Xenopus laevis* dans un œuf énucléé de xénope conduit à la création d'un animal cloné. C'est l'exemple type du processus de reprogrammation nucléaire, par lequel le noyau d'une cellule spécialisée est ramené vers un état embryonnaire, capable de générer toutes les cellules d'un organisme. En utilisant l'ovocyte, précurseur de l'œuf, il est également possible de reprogrammer le noyau d'une cellule somatique. L'intérêt de cette approche est la reprogrammation directe de l'expression génique en l'absence de division cellulaire. Grâce à cette stratégie, il est possible d'analyser le mécanisme de la reprogrammation transcriptionnelle des noyaux somatiques. En combinant le suivi en temps réel du flux des protéines entrant et sortant du noyau et l'analyse de l'expression génique, nous avons observé que la perte de l'histone somatique linker H1 et l'incorporation simultanée de l'histone ovocyte-spécifique linker B4 précèdent la reprogrammation transcriptionnelle. Cependant même si H1 persiste, la reprogrammation peut avoir lieu. Nous avons démontré, d'une part par des expériences d'injection d'anticorps, d'autre part par interférence dominante négative, que l'incorporation d'histone linker B4 est requise pour la réactivation des gènes de pluripotence. Nous suggérons que la liaison de l'histone B4 à la chromatine est un événement primaire, clé de la reprogrammation des noyaux somatiques transplantés dans l'ovocyte d'amphibien.

Mots clés : *Xenopus* / reprogrammation / transfert nucléaire / monitoring en temps réel

The process of differentiation leads to the production of cells with extremely stable state. One can distinguish different levels of differentiation as development of an organism proceeds. Cells become specified when they are able to differentiate autonomously into a cell type even when explanted into a neutral environment. This state of specialization is reversible. By contrast, cells that are determined will irreversibly differentiate into a given pathway even when exposed to adverse differentiation cues. For example, muscle cells of the early frog embryo are determined since they will maintain a muscle differentiation program when transplanted into the gut region of the embryo (Kato & Gurdon, 1993). So, terminally differentiated cells hardly ever change cell fate when exposed, as whole cells, to various experimental conditions. This is in contrast to what happens when the nucleus of a differentiated cell is exposed to a new environment. In that case a reversal of the differentiated cell is observed. This reversal has been obtained using various experimental strategies. In nuclear transfer experiments, the nucleus of a differentiated cell is transferred to an enucleated egg (Fig. 1A). The resulting reconstitued embryo, or cloned embryo, can develop into a whole organism (Gurdon *et al.*, 1958; Campbell *et al.*, 1996). This demonstrates that the nucleus of a specialized cell can be reprogrammed, so that it will allow development into all the specialized cell types of an adult organism. Shift in gene expression can also be obtained by fusing a differentiated cell to an embryonic cell (Fig. 1B). In that condition, one can observe the expression of embryonic genes from the differentiated cell nucleus as well as the silencing of differentiation specific genes (Pereira *et al.*, 2008). More recently, Takahashi & Yamanaka (2006) have demonstrated that exposing a nucleus to embryonic transcription factors by retroviral infection drives reprogramming of the differentiated cell towards an embryonic cell state very similar to that of an ES cell derived from an embryo (Fig. 1C).

Interest in nuclear reprogramming comes from the prospect of therapeutic application of that technology. Using that procedure, it should be possible to derive embryonic stem cells from the somatic cells of any individual and redifferentiate them into any desired differentiated cell type. This would open the way for cell replacement therapy without the need for immunosuppression (Fig. 2).

The nuclear reprogramming strategies available at the moment are very inefficient. For example induced pluripotent cells are generated at a frequency of about 0.1% from embryonic fibroblasts (Stadtfeld & Hochedliger, 2010; Pasque *et al.*, 2010). To improve the efficiency of nuclear reprogramming, a better understanding of this process is needed. For that purpose it is important to better define the mode of action of the reprogramming factor. We focus on the natural components of egg and oocyte that represent the more efficient reprogramming activity available. It is also desirable to define what restricts the reprogramming of a differentiated cell nucleus. This analysis will better define the basis for the differentiated state stability and could be relevant to diseases such as cancer, in which such cell identity is perturbed.

In the next sections we will describe the various nuclear transfer strategies available to us as well as new experimental approaches we have developed in order to investigate the mechanism of nuclear reprogramming. We will discuss the kinetics and efficiency of reprogramming observed using this system. Lastly, we will summarize experiments demonstrating the effects of exchange of somatic nuclei and oocyte components in the reprogramming process.

Nuclear transfer strategies using *Xenopus* eggs and oocytes

Eggs of the African clawed frog *Xenopus laevis* are abundant ($\sim$10 000/female) and large cells, making them a very suitable source as recipients for nuclear transfer. When using donor nuclei from endoderm cells of the early embryo, nuclear transfer to egg leads to high rate of development to the blastula stage and up to 1% of reconstructed embryo reaches the adult stage (Gurdon, 1960). In such experiments, a large number of cloned embryos give rise to partial blastulae embryos that cannot achieve proper development. When grafting cells from such a partial embryo to another obtained by normal fertilization, it was demonstrated that transplanted nuclei were able to shift from their original differentiated state to an unrelated differentiation path. For example 30% of cells isolated from a partial embryo generated by nuclear transfer from an endodermal donor cell were redirected towards muscle differentiation following graft to the prospective mesoderm of a gastrula stage embryo (Byrne *et al.*, 2002). Altogether these experiments demonstrate the remarkable reprogramming activity of the natural component of the eggs. As exemplified by the grafting experiment, it is very difficult to judge the efficiency of nuclear reprogramming. A majority of cells from a cloned embryo could be reprogrammed and nonetheless the development of the embryo could fail due to the stringency of embryo development. Understanding the mechanism of nuclear reprogramming following nuclear transfer to an egg is also made difficult by the high number of cell divisions the embryo needs to go through before it reaches the midblastula transition where zygotic gene are eventually expressed. These numerous cell divisions before any
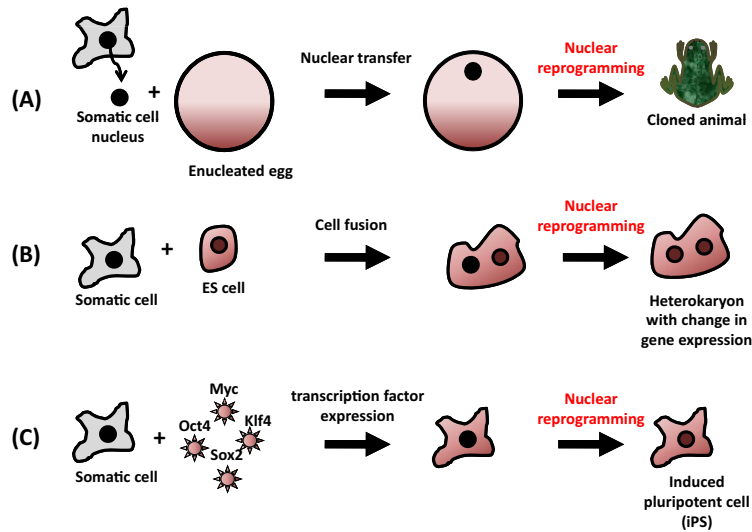
Fig. 1. The different experimental approaches to nuclear reprogramming. (A) Nuclear transfer: the nucleus of a somatic cell is transplanted to the cytoplasm of an enucleated egg. The resulting reconstituted embryo can give rise to a cloned animal. (B) Cell fusion: a differentiated cell is fused to an ES cell. In the resulting heterokaryon, the nucleus of the differentiated cell exposed to embryonic factors is reprogrammed to express stem cell specific genes. (C) Induced pluripotency: expression of four transcription factors (Myc; Klf4; Sox2 and Oct4) reprograms the somatic cell to an induced pluripotent state (iPS) very similar to that of embryonic stem cell.
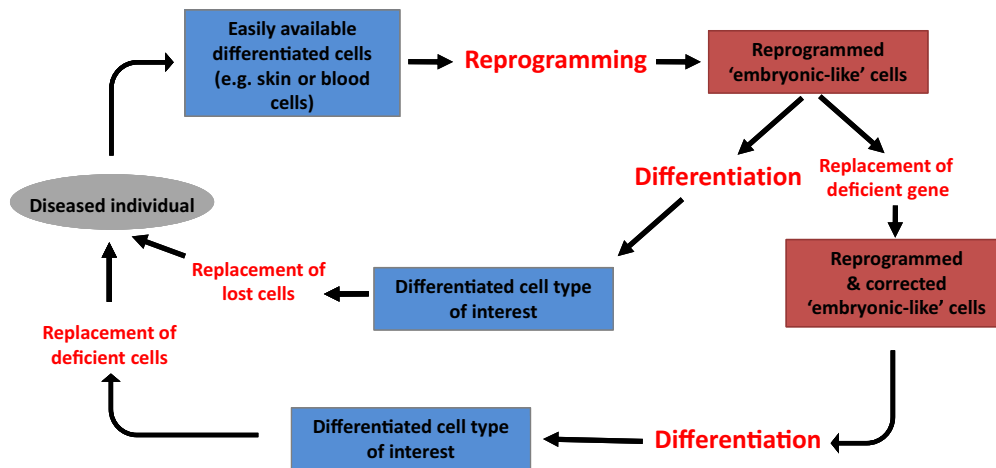

Fig. 2. Potential therapeutic applications of nuclear reprogramming.

molecular marker can be measured obscured the analysis of such reprogramming experiments.

To overcome these difficulties, we have performed nuclear transfers using the immediate precursor of the egg, the oocyte. The egg is blocked in metaphase II of meiosis and resumes development as soon as a nucleus is transplanted into it. By contrast, the oocyte is in prophase I of meiosis. This cell is highly transcriptionally active and, upon nuclear transplantation, no cell division is initiated. When incubated in such

an oocyte environment, the transplanted nuclei reactivate embryonic genes. This system provides us with an assay in which a direct reprogramming of gene expression can be precisely measured in the absence of cell division (Byrne *et al.*, 2003; Jullien *et al.*, 2010; Halley-Stott *et al.*, 2010) For this experiment, an interspecies nuclear transfer is performed. In such a heterologous system, it is possible to distinguish, by qRT-PCR assay, between the transcript produced from transplanted mouse nuclei and the large amount
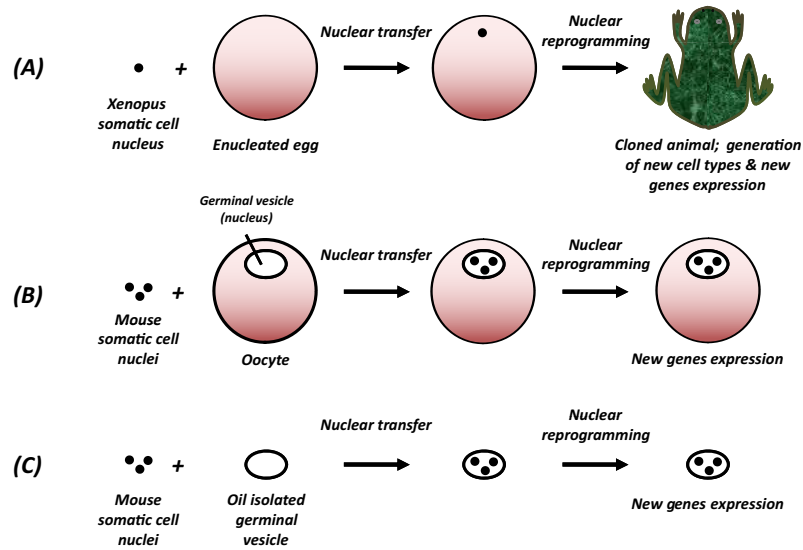
Fig. 3. Nuclear reprogramming strategies using *Xenopus* oocytes and eggs. (A) Cloning experiment: a single *Xenopus* nucleus is transplanted to an enucleated *Xenopus* egg. In that experimental setting a large number of cell divisions is required before new gene transcription is initiated. Eventually new cell types and even new organisms are generated. (B) Nuclear transfer to *Xenopus* oocyte: up to several hundreds mammalian nuclei are transplanted to the nucleus (germinal vesicle) of a *Xenopus* oocyte. In that experimental setting, nuclei are not undergoing cell division and new cell types are not generated. Instead, a direct reprogramming of gene expression is triggered by exposure to the oocyte components. (C) Nuclear transfer to *Xenopus* oocyte germinal vesicle: in this experiment the germinal vesicle of the oocyte is taken out of the oocyte prior to transplantation of mammalian nuclei. Reprogramming of gene expression happens in a similar way to that in the nuclear transplantation to whole oocyte (as in B). By contrast to the procedure described in (B), transplantation to isolated oil-GV allows real time monitoring of nuclear reprogramming by confocal microscopy.

of *Xenopus* transcripts stored in the oocyte. Using this system, it was shown that promoter DNA demethylation is part of the process leading to transcriptional reprogramming of the pluripotency gene *Oct4* (Barreto *et al.*, 2007; Simonsson & Gurdon, 2004). As mentioned before, one other advantage of the *Xenopus* system is the large amount of material available, opening the way for biochemical analysis of oocyte/egg components. By combining oocyte extract analysis and nuclear transfer, it was shown that *Oct4* reactivation required the activity of several components of the oocyte such as the chromatin remodelling factor Brg1 (Hansis *et al.*, 2004) and the transcription factor Tpt1 (Koziol *et al.*, 2007).

The egg and oocyte of *Xenopus laevis* are characterized by accumulation of pigment and yolk granules that largely precludes the use of fluorescence microscopy to monitor the behavior of nuclei undergoing reprogramming. To circumvent this problem, we have modified the oocyte nuclear transfer protocol (Fig. 3). It is known that the reprogramming activity

of the oocyte, in which nuclei are transplanted in standard nuclear transfer experiments, is localized in its large nucleus, the germinal vesicle (Fig. 3B). Previous studies indicated that isolation of the oocyte germinal vesicle in oil prevents the loss of nuclear content. Such a germinal vesicle isolated in oil (oil-GV) can perform most of its function up to 24 h after it has been taken out of the oocyte (Lund & Paine, 1990). When used as recipient for nuclear transfer experiments, these oil-GV exhibit remarkable transcriptional reprogramming activity (Fig. 3C) (Jullien *et al.*, 2010). In particular, we have demonstrated that oil-GV monitored in real time by confocal analysis is able to trigger transcription factor dependent induction of a reporter gene from transplanted nuclei (Jullien *et al.*, 2010). This type of analysis paves the way for real time monitoring of the reprogramming process (see last section).

In order to precisely quantify the transcriptional reprogramming observed following nuclear transfer to *Xenopus* oocyte, we carried out RT-PCR analysis of gene expression from transplanted ES cells *versus*

differentiated cell nuclei. In that case we can directly compare the transcription, in the oocyte environment, of a pluripotency gene that is not repressed (ES nuclei) to one that is getting reactivated (Jullien *et al.*, 2010; Halley-Stott *et al.*, 2010). Using that method we observed that pluripotency genes are reactivated with various efficiencies with *Sox2*, better reactivated than *Oct4*, and *nanog*. Importantly, gene reactivation from nuclei transplanted into oocytes occurs with the same trend in efficiency as that of nuclear transfer to eggs: the more differentiated a cell, the more resistant to reprogramming.

Exchange of nuclear component during reprogramming

When transplanted into an egg or oocyte, a nucleus is exposed to the relatively large amount of maternal components stored in the female germ cell. We have investigated to what extent nuclear reprogramming relies on chromatin protein exchange between the transplanted nuclei and the oocyte. For that purpose we have monitored the movement of fluorescent proteins in and out of transplanted nuclei. In these experiments we used fluorescent chromatin proteins that are expressed prior to nuclear transfer either into the somatic nuclei or in the recipient oocyte. We have focused our analysis on chromatin proteins that bind on large parts of the genome such as the major chromatin component (core/linker histone) as well as chromatin proteins involved in gene repression such as members of the HP1 family as well as the polycomb protein (Bmi1). The real time monitoring of such exchanges using the oil-GV approach shows that all the highly mobile chromatin proteins (linker histone, HP1, Bmi1) are replaced within the first few hours following nuclear transfer, prior to any detectable reprogramming of transcription. By contrast, core histones are replaced to a much lesser extent and over a much longer time scale. Based on the observed kinetics, we have focused our investigations on the loss of somatic linker histone (H1) from transplanted nuclei and the concomitant incorporation of an oocyte specific linker histone (B4) in these nuclei. By protein overexpression, protein knockdown and dominant negative approach we have demonstrated that transcriptional reprogramming following nuclear transfer to oocyte does not require loss of somatic linker histone but is dependent on incorporation of the oocyte specific linker histone (Jullien *et al.*, 2010). This analysis also suggests that, in the oocyte environment at least, somatic and oocyte linker histones are not competing for the same chromatin binding site on a genome wide scale. The oil-GV based nuclear transfer permits analysis of protein mobility by Fluorescence Recovery After Photobleaching (FRAP). FRAP analysis indicates that nuclear reprogramming is associated with increased protein mobility. This observation, although not yet explained, could be relevant to the process of nuclear reprogramming, since the measured increase in mobility following nuclear transfer mirrors the decrease in chromatin protein mobility happening during differentiation of pluripotent cells (Meshorer *et al.*, 2006).

Conclusions and perspectives

The direct reprogramming of gene expression observed following nuclear transplantation of mammalian nuclei to *Xenopus* oocyte is well suited for the analysis of the mechanisms underlying transcriptional reprogramming. Using this approach, we have identified genome wide exchange of chromatin components that are necessary for the resetting of gene expression in nuclei undergoing nuclear reprogramming.

Several questions remained unanswered. For example it would be important to identify the component of oocyte transcription machinery involved in the specific reactivation of embryonic genes. Of particular interest is the question as to whether oocyte reactivates embryonic genes using a similar transcription factor set than those used in induced pluripotency. Indeed the oocyte contains large amounts of such transcription factors of the POU (Whitfield *et al.*, 1993) and SRY-box (Sox) family (El Jamil *et al.*, 2008) that could participate in gene reactivation. Alternatively gene reactivation could rely mainly on the oocyte specific components of the basal transcription machinery (TBP2, ALF) (D'Alessio *et al.*, 2009). Answering these questions will tell us the fundamental differences between the different reprogramming routes (see Fig. 1). In particular we would be able to determine if the oocyte contains specific reprogramming activity acting upstream of the transcription factor mediated gene reactivation at work in other reprogramming situations.

Lastly, one shared characteristic of all reprogramming avenues is the increased resistance of nuclei to reprogramming with the increased level of cell differentiation. Understanding the basis for this restriction to gene reactivation will undoubtedly provide a way to improve the efficiency of nuclear reprogramming. The epigenetic processes preventing gene reactivation are described in more and more details. These includes the methylation of DNA, the incorporation of histone variants as well as the post-translational modifications of histone tails, on the regulatory regions of repressed genes (Koche *et al.*, 2011; Pasque *et al.*, 2011). Nuclear transfer and induced pluripotency both show that some epigenetic marks on chromatin are efficiently modified during reprogramming (Koche *et al.*,

2011; Murata *et al.*, 2010). One major challenge is now to identify which epigenetic mark or combination of marks constitute the major hurdle to the reprogramming process.

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