Epigenetic memory of active gene transcription is inherited through somatic cell nuclear transfer

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The transplantation of somatic cell nuclei to enucleated eggs has shown that genes can be reprogrammed to an embryonic pattern of expression, thereby indicating a reversal of their epigenetic state. However, in *Xenopus* nuclear transfer experiments using both endoderm and neuroectoderm donor cells, we have observed substantial overexpression of donor cell type-specific genes, both spatially and temporally, in the wrong cell type in some nuclear transplant embryos. For example, more than half of the embryos prepared from transplanted neuroectoderm nuclei overexpressed the neuroectodermal marker gene *Sox2* to an excessive level in their endoderm cells. Because, in *Xenopus*, there is no transcription for the first 12 cell cycles, some somatic cell nuclei must remember a developmentally activated gene state and transmit this to their mitotic progeny in the absence of the conditions that induced that state. We also find that donor cell-specific genes are transcribed at an earlier stage than normal in an inappropriate cell type. This phenomenon of epigenetic memory applies to genes that are transcribed in donor nuclei; it does not influence those genes that are competent to be transcribed in nuclear transplant embryo tissue, but were not actually transcribed in donor nuclei at the time of nuclear transfer. We conclude that an epigenetic memory is established in differentiating somatic cells and applies to genes that are in a transcriptionally active state.

Xenopus | cloning

In normal embryonic development, cells lose their pluripotent status and become restricted to a particular differentiation pathway by receiving signals from other cells. Once on the pathway to one kind of differentiation, cells do not redifferentiate in other directions and their commitment to differentiation is very stable. However, this does not mean that their nuclei have stably lost their earlier developmental plasticity. By nuclear transplantation, which involves the transfer of nuclei from partially or fully differentiated cells into enucleated eggs of Amphibia or mammals, various stages of larval or fetal development, and sometimes, adult animals, can be obtained (1–6). These results demonstrate that the epigenetic program of nuclei of differentiated cells can be reversed by appropriate reprogramming in recipient egg cytoplasm and that the pattern of gene expression established during development is rapidly reversed.

On the other hand, cloning by the use of adult somatic cell nuclei is inefficient. The ability of transplanted nuclei to promote normal development declines as development progresses in both amphibian and mammalian nuclear transfer experiments (2, 3, 6). Typically ≈1% of the nuclei of differentiated amphibian cells yield feeding tadpoles (4, 7) and <3% of cloned mammalian embryos develop into live births (8, 9). In Amphibia, a substantial proportion of nuclear transplant (NT) embryos cleave abnormally as partially cleaved blastulae, which die within 24 h (2, 3). Moreover, a majority of cloned embryos show a variety of abnormalities during development, and these abnormalities often cause lethality before adulthood. Because successful cloning involves efficient DNA replication, appropriate DNA methylation/demethylation, chromatin remodeling, X-chromosome reactivation, etc. (8, 10, 11), the low success rate could be caused by an incomplete reprogramming of the epigenetic state of nuclei from differentiated donor cells.

These considerations led us to explore further the nature of the constraints that limit the efficiency with which somatic cell nuclei can be reprogrammed by egg cytoplasm. It is easy to understand that genes permanently switched off in the course of cell differentiation may be hard to reactivate efficiently. An example of such a gene is *oct4*, whose product is required for embryonic development and the maintenance of stem cell status (12–14). The incomplete reactivation of *oct4* may account for some failures of NT embryo development (15, 16). Methylation of promoter DNA in such genes provides a ready explanation for incomplete reprogramming, because demethylation of DNA, although able to occur after nuclear transfer (17), seems not to be fully efficient. Thus persistence of the "off-state" of a gene may contribute to failures of nuclear transfer.

We now ask whether a reciprocal process might exist, namely a persistent "on-state" of a gene. If there is such a mechanism, transplanted nuclei from differentiating or differentiated cells might overexpress developmentally activated genes inappropriately and so interfere with normal development. By relating patterns of gene expression in NT embryos to donor cells of defined cell type, we conclude that cells can propagate, for many cell divisions, an on-state of gene expression in the absence of the conditions that induce these genes. We describe this process as epigenetic memory. This characteristic may be of developmental significance in normal development by facilitating the inheritance of a differentiated state in cells of the same lineage and may also account for some of the abnormalities observed after somatic cell nuclear transfer.

Materials and Methods

Donor Cell Preparation. In endoderm NT experiments, stage 21 (18) tail-bud embryos were dissected, and the endoderm tissue was isolated. Endoderm cells were dissociated in calcium- and magnesium-free modified Barth saline (MBS) with 1 mM EDTA, pH 8.2. In neuroectoderm NT experiments, stage-26 (18) tail-bud embryos were dissected and the upper dorsal region was isolated. Neuroectoderm tissue was isolated by removal of the dorsal endoderm, notochord, and somites in MBS with 1 μg/ml collagenase (Sigma-Aldrich). Neuroectoderm cells were dissociated in calcium- and magnesium-free MBS with 1 mM EDTA, pH 8.2.

Nuclear Transplantation. The procedure was carried out as described (19). In brief, streptolysin-O (SLO) (Sigma-Aldrich) was mixed with a suspension of dissociated endoderm or neuroectoderm cells in calcium- and magnesium-free MBS to give a concentration of 5,000 units/ml, and the suspension was kept at 37°C for 15 min to permeabilize cells. SLO activity was arrested

Abbreviations: NT, nuclear transplant; *edd*, endodermin; *Eomes*, eomesodermin; MBT, midblastula transition; BMP4, bone morphogenetic protein 4; MBS, modified Barth saline; NCAM, neural cell adhesion molecule; EF1α, elongation factor 1 α.

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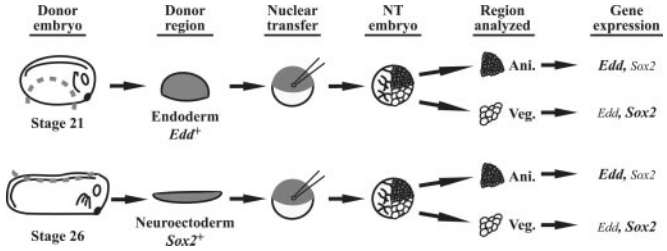


Fig. 1. Design of experiments. After nuclear transfer of a donor nucleus to an enucleated egg, most embryos developed as partially cleaved blastulae. These were divided into an animal (Ani.) region (future neuroectoderm) or a vegetal (Veg.) region (future endoderm) and analyzed for gene expression by RT-PCR. The gene expressions in bold in the last column provide the most appropriate tests of donor cell memory.

by the addition of BSA to 0.5%. Nuclear transplantation was performed by injection of a whole permeabilized cell into an egg enucleated by UV irradiation. The NT embryos were cultured in MBS containing 0.5% BSA at 14°C.

RT–PCR Analysis. This procedure was carried out as described (20). RT-PCRs were performed with the relevant primers: endodermin (*edd*), forward 5′-GTGCAGTGTTTCACTACTTGG-3′, backward 5′-TAAAGCGCACAGAGACGCACG-3′; *Sox2*, forward 5′-GAGGATGGACACTTATGCCCAC-3′, backward 5′-GGACATG CTGTAGGTAGGCGA-3′; neural cell adhesion molecule (*NCAM*): forward 5′-CACAGTTCCACCAAATGC-3′, backward 5′-GGAATCAAGCGGTACAGA-3′; *XAG*, forward 5′-CTGACTGTCCGATCAGAC-3′, backward 5′-GAGTTGCTTCTCTGGCAT-3′; *Xk81*, forward 5′-CACCAG AACACAGAGTAC-3′, backward 5′-CAACCTTCCCATCAACCA-3′; eomesodermin (*Eomes*), forward 5′-CCATCCAAACCTCCCACC-3′, backward 5′-CTTCTCTTACATGCACCCG-3′; and elongation factor 1 α (*EF1α*): forward 5′-CAGATTGGTGCTGGATATGC-3′, backward 5′-ACTGCCTTGAGACTCCTAG-3′. PCR products were then run on a 6% polyacrylamide gel and quantified by a PhosophorImager. To ensure that the PCR was in the quantitative linear range, a dilution series of samples was performed.

Bone Morphogenetic Protein 4 (BMP4) or Activin Treatment of Animal Cap Cells. Stage-8 embryos were dissected and the animal cap region was isolated. Animal cap cells were dissociated in calcium- and magnesium-free MBS with 1 mM EDTA, pH 8.2. BMP4 (R & D Systems) was mixed with a suspension of dissociated animal cap cells in calcium- and magnesium-free MBS at the appropriate concentrations. The mixture was incubated at room temperature for 20 min. BMP4 was removed by three washes of calcium- and magnesium-free MBS. Cell reaggregation was carried out in calcium- and magnesium-containing MBS and cultured to stage 17 for RT-PCR analysis. Activin protein (R & D Systems) (10 ng/ml) was added to dissociated animal cap cells from each single embryo at stage 9 and cultured to stage 14 for RT-PCR analysis.

Results

Donor Cells and NT Embryo Development. Nuclei from stage 21 (endoderm) or stage 26 (neuroectoderm) were transplanted singly to enucleated eggs (Fig. 1). At these stages, cells express the cell type-specific marker *edd* (endoderm cells) and *Sox2* or *NCAM* (neuroectoderm). These cells are committed to their cell fates (either endoderm or neuroectoderm derivatives) and do not change during normal development or if grafted to ectopic sites (21). Consistent with our previous reports (3, 22), ≈15% of these NT embryos developed as partially cleaved blastulae, which died within 24 h of culture, and ≈10% formed completely cleaved blastulae. The remaining 75% were abortively cleaved and soon died (Table 1).

Epigenetic Memory in NT Embryos. First, we wanted to know whether epigenetic memory is retained in donor nuclei after somatic cell nuclear transfer. We determined the extent of epigenetic memory by testing the expression of donor cell marker genes in NT embryos (Fig. 1). If there is overexpression of donor marker genes in NT embryo tissues unrelated to the donor cell type, it would indicate a retention of memory in the donor nucleus by incomplete epigenetic reprogramming. Therefore, we dissected both the partially and completely cleaved NT blastulae into animal and vegetal regions (future neuroectoderm and endoderm regions, respectively) and assayed donor gene expression in these two regions. For control samples, we used either completely cleaved embryos (from fertilized eggs) or dorsal/ventral half embryos generated by injection of an excess amount of salmon sperm DNA into two blastomeres at the four-cell stage (20). For endoderm nuclear transfer experiments, we tested the expression level of an endoderm marker gene, *edd*, in each single NT embryo. *Edd* is well documented for high expression in the vegetal region of normal embryos (23). In normal embryos reared from fertilized eggs, we noticed that the vegetal region was 10 times higher in *edd* expression (relative to the housekeeping gene *EF1α*) than the background level in the animal region (Fig. 2 *A* and *B*, blue bars; 2.0 vs. 0.2 on the vertical scale). In the animal region (neuroectoderm lineage) of endoderm-derived NT samples, some embryos (Fig. 2*A*, red bars) showed a background level of *edd* expression comparable to that of normally developing controls from fertilized eggs (Fig. 2*A*, blue bars). However, 9 of 20 (45%) of these endoderm-derived NT embryos showed a >2-fold enhancement of *edd* expression above background (Fig. 2*A*, dark and light red bars above the red dotted line). In contrast, the vegetal region (future endoderm) of endoderm-derived NT embryos expressed *edd* almost entirely within the normal range ≈10 times above background; only 2 of 16 were significantly above that of controls (Fig. 2*B*, light red bars above the red dotted line). This excessive expression of an endoderm-specific gene in a nonendoderm region of endoderm-derived NT embryos is consistent with transplanted nuclei having a memory of their donor cell origin. We know that this effect is not connected with the fact that most of the NT embryos tested were partially cleaved blastulae, because experimental half embryos derived from fertilized eggs showed no such effect (0 of 8 embryos in Fig. 2*A*, light and dark yellow bars).

We now ask whether this apparent memory is also true for NT embryos of nonendoderm origin. For neuroectoderm nuclear transfer experiments, we used *Sox2*, which is a pan-neural marker gene (24), to test its expression in NT embryos. *Sox2* was found to be at least 25 times higher in the animal region than in the

Table 1. Results of *Xenopus* somatic cell nuclear transfer experiments

Donor cell	No. of total transfers	No. of uncleaved (%)	No. of partial blastula (%)	No. of complete blastula (%)
Stage-21 endoderm	125	95 (76.0)	17 (13.6)	13 (10.4)
Stage-26 neuroectoderm	116	86 (74.1)	19 (16.4)	11 (9.5)

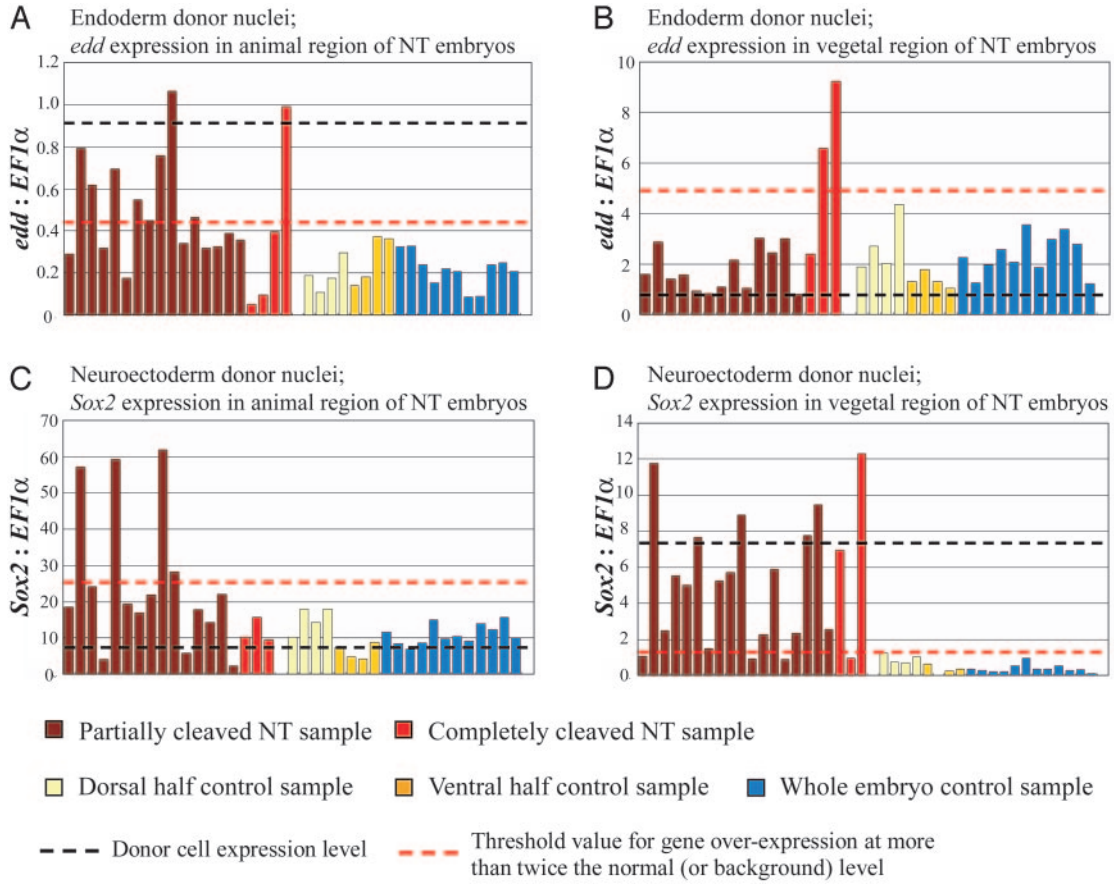


Fig. 2. Expression of donor cell marker genes in the animal and vegetal regions of NT embryos at the early gastrula stage (stage 10.5) as revealed by RT-PCR analysis. (*A* and *B*) *edd* expression, relative to *EF1α*, in the animal region or vegetal region of partially, or completely, cleaved endoderm NT embryos is shown. The black dotted line represents the level of *edd* expression in the stage-21 endoderm donor cells. (*C* and *D*) *Sox2* expression, relative to *EF1α*, in the animal region or vegetal region of partially, or completely cleaved neuroectoderm NT embryos. The black dotted line represents the level of *Sox2* expression in the stage-26 neuroectoderm donor cells. Control samples include dorsal/ventral half or whole *in vitro*-fertilized control embryos. The red dotted line represents the threshold value (two times or more above the average expression level in normal control embryos) for gene overexpression. Note: The vertical scales in each graph are not the same. *edd*, 30 cycles; *Sox2*, 32 cycles. The term control sample refers to embryos obtained from fertilized eggs.

vegetal region (background) of normal embryos (Fig. 2 *C* and *D*, blue bars; 10 vs. 0.4 on the vertical scale). In the animal region of neuroectoderm NT embryos, only 4 of 18 samples showed abnormally high *Sox2* expression compared with normal embryo controls, and the majority of NT samples have a normal level of *Sox2* expression (Fig. 2*C*, dark and light red bars below red dotted line). However, in the vegetal region of neuroectoderm NT samples, 17 of 21 (81%) NT embryos showed a significantly higher *Sox2* expression than normal embryo control samples (Fig. 2*D*, dark and light red bars above the red dotted line). Again, this effect is not seen in experimental partial embryos (Fig. 2*D*, 0 of 8 embryos, light and dark yellow bars). In Fig. 2, the black dotted lines represent the levels of *edd* or *Sox2* expression in the donor tissues of normal embryos, namely in stage-21 endoderm or stage-26 neuroectoderm. Therefore, in some NT embryos, the excessive level of donor-specific gene expression is as high in the inappropriate germ layer as it is in the proper germ layer of normal control embryos, that is ≈10–25 times higher than expected if nuclei had been completely reprogrammed. In summary, 45% of the endoderm NT embryos showed *edd* overexpression in the animal region, whereas the majority had normal *edd* expression in the vegetal region, and 81% of the neuroectoderm NT embryos demonstrated *Sox2* overexpression in the vegetal region, whereas the majority had normal *Sox2* expression in the animal region (Table 2).

We have also analyzed the expression level of donor nonexpressed genes in these NT embryos and ask whether the neuroectoderm marker *Sox2* is overexpressed in endoderm-derived NT embryos and whether the endoderm marker *edd* is overexpressed in neuroectoderm NT embryos. By this approach, we can find out whether the overexpression of donor-specific genes in NT embryos is caused by a global deregulation of gene transcription by the NT procedure or whether the excess gene expression we see is restricted to genes that are transcriptionally activated in a cell type-specific way. Our results show that in both types of NT embryos there were only one or two individual NT samples with such overexpression of the donor nonexpressed gene in the irrelevant region (Table 2). Almost all of them had a normal expression level of the donor nonexpressed gene in the appropriate regions. The level of *EF1α* transcripts was the same in nuclear transfer embryos as in fertilized egg controls (data not shown). Therefore the excessive level of overexpression that we see applies only to those genes that have undergone developmental activation.

In conclusion, the overexpression of *edd* in the animal region of endoderm NT embryos, and of *Sox2* in the vegetal region of neuroectoderm NT embryos, shows that some transplanted nuclei retain an epigenetic memory of their developmental history. Two characteristics are of special note. One is that the memory is very variable; some transplanted nuclei are com-

Table 2. Summary of the number of NT embryos showing overexpression at stage 10.5 of donor-expressed genes and donor-nonexpressed genes in their animal and vegetal regions

	Endoderm donor nuclear transfers, %		Neuroectoderm donor nuclear transfers, %	
Marker gene	Animal region	Vegetal region	Animal region	Vegetal region
edd	45 (9/20)	12.5 (2/16)	5.5 (1/18)	0 (0/21)
Sox2	5 (1/20)	0 (0/16)	22.2 (4/18)	81 (17/21)

The values show only those cases where the level of gene expression was two or more times greater than the normal (or background) level. For example, the vegetal region of neuroectoderm-derived NT embryos expressed *edd* to a normal level in all 21 cases.

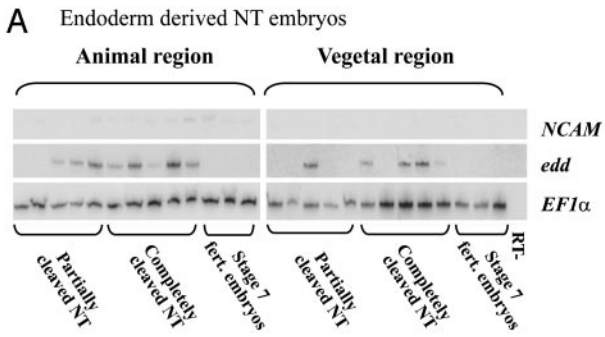
pletely reprogrammed, and others hardly at all. Second, this memory can persist, in some cases with almost no loss, through >12 mitotic cell divisions (from nuclear transfer to stage 10.5).

Premature Gene Transcription in NT Embryos. Normal zygotic gene transcription starts at a developmental stage called the mid-blastula transition (MBT) at stage 8 in *Xenopus laevis*. Before MBT, only maternal transcripts can be found in the developing embryos (25). As we have shown above that there is a quantitative overexpression of genes in NT embryos, we now ask whether there is also temporal overexpression in NT embryos affecting the time of onset of gene expression. Therefore, we generated NT embryos with stage-21 endoderm nuclei as donors and assayed gene expression at stage 7, which is 3 h (at 23°C) before zygotic gene transcription is readily detected at stage 9. NT embryos were divided, as above, into animal and vegetal regions at stage 7. Remarkably, it is observed that *edd* (donor marker) is expressed in most of the animal regions (8 of 10) and in some of the vegetal regions (5 of 10) of these stage-7 NT embryos, but in none of the control stage-7 embryos (Fig. 3*A*). To determine whether this pre-MBT gene expression is specific to the donor cell marker gene, we assayed the expression of a neural marker gene, *NCAM*, which is not expressed in these endoderm donor cells. In both NT and WT samples, there is only a faint expression of *NCAM* and this weak expression is attributable to maternal transcripts in the embryos (26) (Fig. 3*A*). For this analysis, *NCAM* is a more suitable neuroectoderm marker than *Sox2* because the maternal content of *NCAM* can be used for quantitative comparison of our PCR results. We conclude that there is no *NCAM* transcription in any of the endoderm-derived NT embryos before MBT. Consequently, this finding demonstrates that only a donor cell-expressed gene is transcribed prematurely in the NT embryos before MBT.

As an explanation for our detection of pre-MBT gene transcription before the normal timing of zygotic gene transcription, it is possible that the *edd* transcripts detected in the NT embryos were not newly synthesized, but were present in the donor cells and then carried over with donor nuclei into the recipient eggs during nuclear transfer. To check this possibility, we determined the presence of *edd* transcripts in NT embryos shortly after nuclear transfer. Two hours (at 14°C) after nuclear transfer (before the two-cell stage), no *edd* transcripts were detected in any of the NT embryos ($n = 5$) (Fig. 3*B*). Furthermore, by coinjecting donor nuclei and α-amanitin (1 or 10 μg/ml), a transcriptional inhibitor, it is found there are no inherited donor cell-specific gene transcripts in stage-7 NT embryos (Fig. 3*B*). This finding clearly shows that the premature donor gene transcripts are newly synthesized in the NT embryos, and that they depend on the transcriptional activities of the embryo.

Response to Signal Factors. We have seen that NT embryos can show inappropriate overexpression of donor-specific genes, but not of genes that are permanently inactive in the same lineage. We now ask about genes that are competent to be transcribed in NT embryo tissue, but not actually transcribed in donor nuclei at the time of nuclear transfer. Early developmental decisions are much influenced by members of the TGF-β class of signal factors such as BMP (27, 28) and activin (29).

To test the response of NT embryo cells to BMP4, we dissociated animal cap cells from both partially and completely cleaved NT blastulae and treated them with different concentrations of exogenous BMP4 for 20 min (Fig. 4*A*). Then we assayed the expression levels of different BMP-responsive genes, including epidermal keratin *Xk81* (an epidermal marker), *XAG* (a cement gland marker), and *NCAM* (a pan-neural marker). In animal cap cells derived from fertilized eggs, 20 min of treatment in a high BMP4 concentration (1,000 ng/ml) caused high expression of *Xk81*, whereas middle (50 ng/ml) and nil (0 ng/ml) concentrations of BMP4 induced *XAG* and *NCAM* expression, respectively (Fig. 4*B*, fertilized



B

Sample region	Donor gene	Numbers of embryos			
		NT (stage 1)	NT (stage 7)	NT (stage 7 + 1 μg/ml α-aminatin)	NT (stage 7 + 10 μg/ml α-aminatin)
Animal	*edd*	0 (n=5)	8 (n=10)	0 (n=2)	0 (n=9)
Vegetal	*edd*	0 (n=5)	5 (n=10)	0 (n=2)	0 (n=9)

Fig. 3. Premature expression of donor-specific genes in NT embryos before MBT. (*A*) Expression of the donor marker gene, *edd*, and donor nonexpressed gene, *NCAM*, in stage-7 partially or completely cleaved endoderm NT embryos and whole control embryos from fertilized eggs. (*B*) Summary of the number of endoderm NT embryos showing *edd* expression at different developmental periods (stage 1 or 7) and after treatment with different concentrations of α-amanitin (1 or 10 μg/ml). The numbers in parentheses represent the total numbers of embryos analyzed.

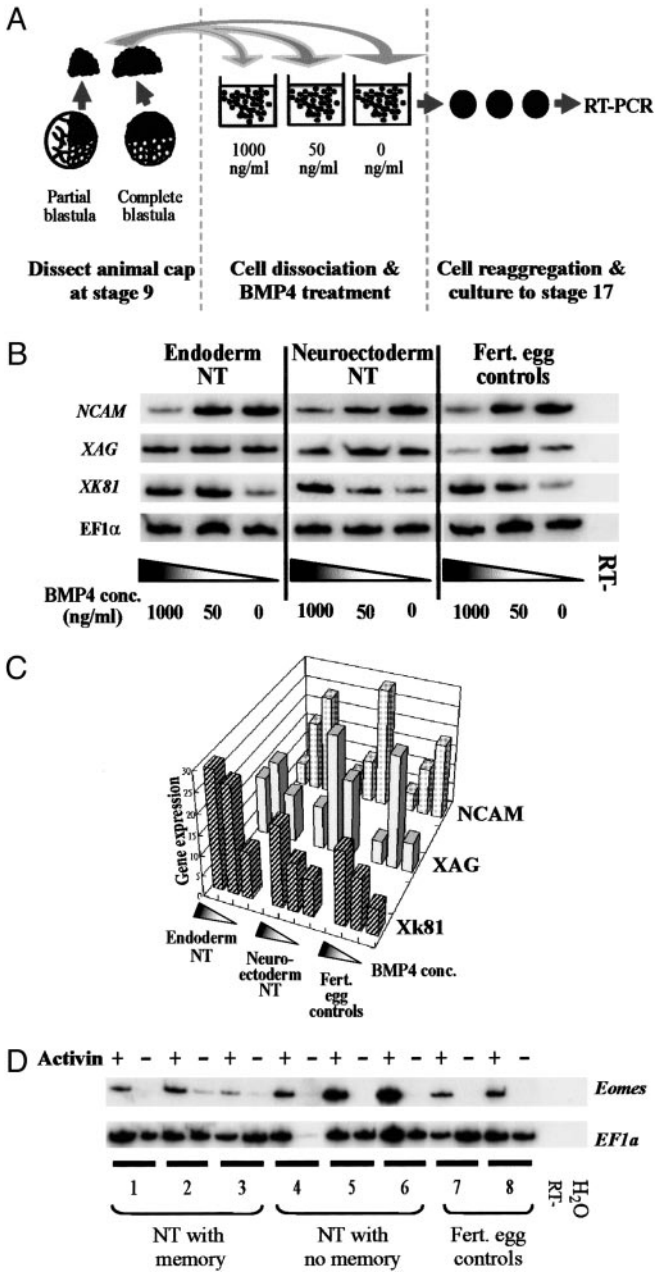


Fig. 4. BMP4 responses in animal cap cells of NT embryos. (*A*) Design of the BMP4 response experiment, using donor nuclei from stage-21 endoderm. (*B*) BMP4 induction of different cell marker genes at stage 17 by RT-PCR analysis. Epidermal keratin *Xk81*, an epidermal marker; *XAG*, a cement gland marker; and *NCAM*, a pan-neural marker. A high concentration of BMP4 (1,000 ng/ml) induced *Xk81* expression; a middle concentration (50 ng/ml) induced *XAG*; nil concentration (0 ng/ml) induced *NCAM* in both NT and *in vitro*-fertilized animal cap cells. (*C*) Quantitation of marker gene expression from the RT-PCR results shown in *B*. (*D*) A similar design of experiment as *A*. Donor nuclei were from stage-21 endoderm. A small piece of each individual NT embryo animal region was tested for *edd* expression. Embryos overexpressing *edd* in this region (lanes 1–3) or not (lanes 4–6) were selected, and the remaining part of each embryo was incubated with (+) or without (−) activin (10 ng/ml) at the equivalent of stage 9 to stage 14. Each analysis is of a single NT embryo. Overexpressing, nonoverexpressing, and control embryos all respond similarly to activin by expressing the gene *Eomes*.

egg controls). In the absence of added BMP, animal cap cells of neither endoderm- nor neuroectoderm-derived NT embryos expressed any of these three markers at the blastula stage. Later, at stage 17, these embryo cells demonstrated similar responses to BMP4 in respect of marker gene induction as did control samples from fertilized eggs (Fig. 4 *B* and *C*).

For BMP assays, we analyzed groups of four NT embryos, some of which, judging from Fig. 2 *A* and *C*, would not have overexpressed genes. We cannot be sure that the BMP responses described apply to those particular embryos that demonstrate epigenetic memory. We therefore repeated, with activin, this design of experiment on single NT embryos of endoderm derivation, part of each embryo being assayed in the animal region for overexpression of *edd*. Having therefore identified individual overexpressing NT embryos, the remaining part of each was treated with activin. We found that, in each case, the NT embryo cells responded to activin, by induction of *Eomes*, to a similar extent as fertilized egg controls (Fig. 4*D*).

In conclusion, we see no inappropriate expression of signal factor responsive genes by NT embryos even though these embryo cells have retained the ability to respond correctly to the supply of such factors. Therefore epigenetic memory applies to genes that are in a transcriptionally active state and does not influence the correct responsiveness of genes, which are inactive at the time of nuclear transfer, but able to be activated by signal factors.

Discussion

Our results provide evidence that cell type-specific gene expression can be inherited after nuclear transfer through many cell divisions. This effect is very variable, ranging from complete reprogramming to almost perfect persistence of gene expression. In previous work (20), we did not see in NT embryos any persistent expression of *IFABP*, an intestinal marker gene, possibly because it is a late expressing gene that might depend on several earlier genes, not all of which would be expressed in one NT embryo. It is possible that persistent gene expression may have been seen in past work. Briggs and King (2) described a tendency for endoderm-derived NT embryos to suffer morphological defects in nonendoderm tissues. It was pointed out (30) that this effect could be because the endoderm differentiates later than other tissues and may therefore survive longer than these. Indeed, in support of this interpretation, Simnett (31) could see no morphological differences between NT embryos of neuroectoderm or endoderm origin. Another conclusion from our work concerns premature gene transcription and this result is in agreement with previous findings. It has been shown that pre-MBT egg cytoplasm can have transcriptional activity (32–34) and an experimental slowing of cell division can induce premature transcription (35). As a general conclusion, the results reported here suggest that the failure to suppress previously active gene transcription, as well as failure to activate previously inactive genes (15, 16), may both contribute to abnormalities that follow somatic cell nuclear transfer.

A further interest in our results attaches to our finding that cells can remember an active gene state for many cell divisions in the absence of conditions that induce this state. In a few cases gene expression was as strong after at least 12 cell cycles as it was in the donor nucleus. A previous example of a persistent state of a gene also comes from a nuclear transfer experiment. Mouse embryos obtained by transplanting a nucleus with a repressed X chromosome undergo random X-chromosome inactivation in the inner cell mass but, in the trophectoderm, the repressed X is always the same one as in the donor nucleus (36). In this case it is the off-state of gene expression that persists. The propagation of an off-state could possibly explain some examples of genes imprinted for persistent expression (37). Thus the imprinted inactivation of a regulatory component could cause continuing expression (the

on-state) of its target gene. For all cases of a persistent off-state the simplest mechanism is DNA methylation, known to result in transcriptional repression (38). It is possible that methylation of the promoter of a repressor could account for the overexpressing on-state that we see in the NT experiments described here. However, it would then be necessary to suppose that the early developmental expression of all genes studied here, namely *edd*, *Sox2*, and *NCAM*, is in each case achieved by inhibition of a repressor for which there is no evidence. Otherwise a persistent on-state might be explained if histone modifications associated with gene activity (e.g., histone 3 Lys-9 acetylation) can be inherited through mitosis in the absence of DNA methylation (39, 40). The persistent epigenetic state described here may reflect a mechanism of cell differentiation that contributes to the stability of the differentiated state in normal development.

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