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Nuclear Reprogramming of Human Somatic Cells by *Xenopus* Egg Extract Requires BRG1

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Summary

Animal cloning by nuclear transplantation in amphibia was demonstrated almost half a century ago [1] and raised the question of the mechanisms and genes involved in nuclear reprogramming. Here, we demonstrate nuclear reprogramming of permeabilized human cells using extracts from *Xenopus laevis* eggs and early embryos. We show upregulation of pluripotency markers *Oct-4* and *germ cell alkaline phosphatase* (*GCAP*) in 293T cells and human primary leukocytes. Reprogrammed leukocytes had a limited life span and did not express surface antigens characteristic of pluripotent cells, indicating that reprogramming was incomplete. Reprogramming activity was detected in egg and early embryo extracts until early blastula stage. Late blastula-stage extracts were not only inactive but also inhibitory to reprogramming. Screening for factors required for reprogramming identified the chromatin remodeling ATPase BRG1. Antibody depletion of BRG1 protein or expression of dominant-negative BRG1 abolished the reprogramming ability of amphibian extracts. Conversely, overexpression of BRG1 in *Xenopus* animal caps extended their competence from blastula to gastrula stage to respond to basic fibroblast growth factor (bFGF) treatment with induction of the mesodermal marker *Xbra*. Dissection of the molecular machinery using a simplified assay system may aid in achieving complete nuclear reprogramming of somatic cells for regenerative medicine.

Results and Discussion

Nuclear Reprogramming of Human Cells

Since its initial demonstration in frogs [1], nuclear reprogramming by animal cloning has been demonstrated in sheep [2] as well as in numerous other vertebrates, proving the reversibility of cell differentiation [3, 4]. Cell reprogramming has also been achieved by fusing adult thymocytes with murine embryonic stem (ES) cells, resulting in induction of an *Oct4-GFP* transgene in the heterokaryons [5]. Byrne et al. [6] recently showed that *Oct-4* was upregulated in somatic nuclei injected into *Xenopus* oocytes. These experiments demonstrated that an adult nucleus can be reprogrammed to induce or upregulate embryonic stem cell markers. We wished to develop a nuclear reprogramming assay that permits manipulation of the reprogramming agent and allows for cultivation of intact reprogrammed cells to eventually establish undifferentiated cell lines. To this end, we have developed a mild and reversible permeabilization protocol with digitonin to deliver *Xenopus* egg extract to mammalian cells. Reversible cell permeabilization protocols have been used to reprogram somatic cells by exposure to cell extracts from T cells and neuronal cells, which induced hematopoietic and neural specific genes, respectively [7]. Such protocols were also recently applied to identify a nucleolar disassembly activity in *Xenopus* egg extract [8].

In dishes of human 293T kidney cells subjected to the reprogramming protocol (Figure 1A), spheroid cell aggregates became visible at day 4 (Figures 1B–1D), which formed even in the absence of egg extract and digitonin. 293T spheres were initially nonadherent and grew rapidly. Spheres could be maintained as large aggregates for weeks, and some would eventually attach.

To test for nuclear reprogramming, mRNA expression of *Oct-4* and *GCAP*, which are pluripotency markers in human and mouse [9–13], was analyzed by RT-PCR (Figure 1E). 293T cells express low levels of endogenous *Oct-4* and *GCAP* mRNAs, which are detectable by extensive PCR amplification (Figure 1E, lane 5). However, *Oct-4* and *GCAP* expression was strongly upregulated in 293T spheres treated with egg extract (Figure 1E, lanes 1 and 3; summarized in Table S1). In egg extract itself, no *Oct-4* or *GCAP* mRNA was detectable, excluding amplification of *Xenopus* orthologs (Figure 1E, lane 6). Surprisingly, while digitonin permeabilization slightly enhanced *Oct-4* upregulation by egg extract, it was not essential (Figure 1E, lane 3). Concomitant to upregulation of *Oct-4* and *GCAP*, expression of the kidney cell marker *natriuretic peptide receptor type A* (*NPR-A*) [14] was downregulated. Occasionally, samples treated without egg extract showed upregulation of one pluripotency marker (data not shown). *Oct-4* mRNA expression levels were significantly lower in reprogrammed 293T spheres compared to endogenous levels in the human embryonal carcinoma cell line TERA-1, indicating that reprogramming was incomplete, but *GCAP* mRNA levels were higher (Figure 1F). However, the upregulation of pluripotency markers suggests that some degree of nuclear reprogramming toward dedifferentiation has taken place.

We next tested whether egg extract was able to reprogram human primary cells. We therefore treated blood leukocytes in an analogous fashion. Rapidly proliferating cell clusters became visible at day 2 regardless of the presence of egg extract and digitonin, and these developed into spheres (Figures 1G and 1H). Similar to 293T cells, leukocyte spheres expressed low levels of endogenous *Oct-4* and *GCAP* mRNAs, which were significantly upregulated in egg extract-treated leukocyte spheres (Figure 1I and Table S1). Again, *Oct-4* mRNA upregulation by egg extract was enhanced by digitonin treatment (Figure 1I, compare lanes 1 and 3), and few samples treated without egg extract showed upregula-

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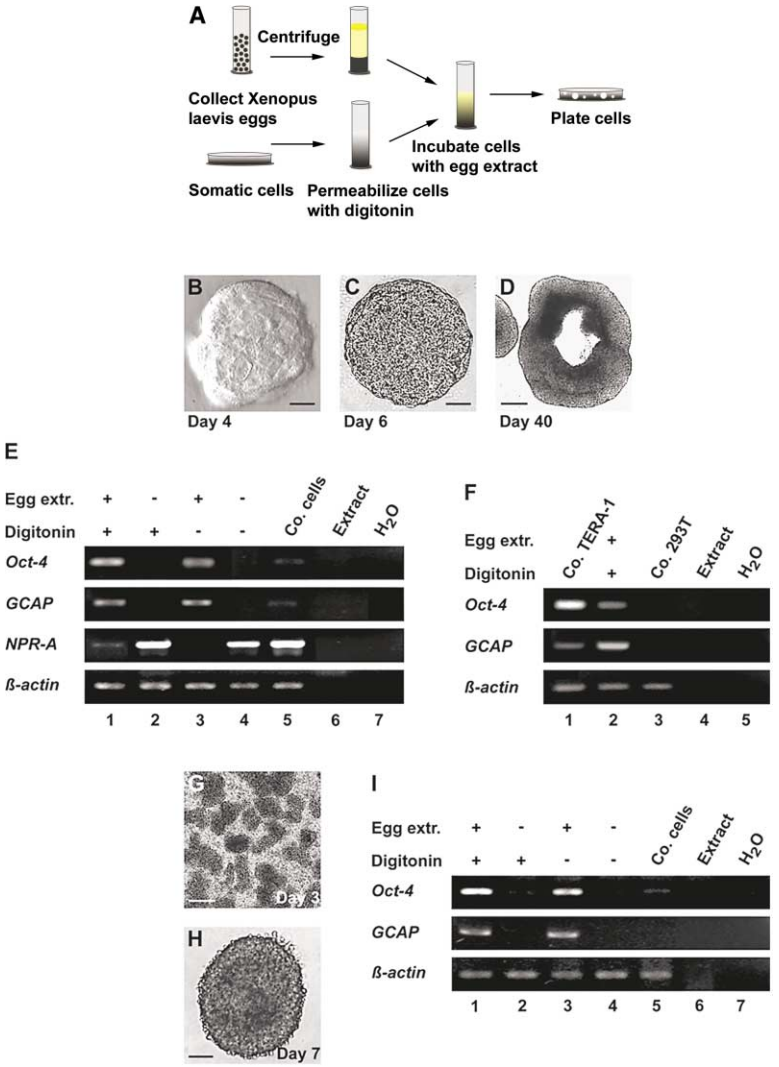

Figure 1. Reprogramming of Human Cells by *Xenopus* Egg Extract

(A) Diagram of the experiment. Top, preparation of egg extract; bottom, preparation of human cells.

(B–D) Morphology of 293T cell spheres developing after treatment with egg extract and digitonin. Bars indicate 20 μm, 75 μm, and 500 μm, respectively.

(E) RT-PCR analysis of 293T cell spheres harvested at day 6. Co. cells, untreated 293T cells; extract, cell-free *Xenopus* egg extract; H_2O, water control. RT-minus controls were all negative (data not shown).

(F) Comparative RT-PCR analysis of reprogrammed 293T cell spheres harvested at day 5 and the human embryonal carcinoma cell line TERA-1. Details are as in (E).

(G and H) Morphology of leukocyte clusters and spheres after treatment. Bars indicate 200 μm and 100 μm, respectively.

(I) RT-PCR analysis of human leukocyte spheres harvested at day 3. Details are as in (E).

tion of one pluripotency marker (data not shown). Leukocyte spheres could be cultured in DMEM for up to 42 days, but they had lost *Oct-4* and *GCAP* upregulation (data not shown). Since leukocytes are a heterogeneous population, we cannot exclude the possibility that the results are due to selection of *Oct-4*/*GCAP*-positive clones. However, together with the effect on 293T cells, the data suggest that transient nuclear reprogramming occurred.

Characterization of Reprogramming by Embryo Extract

We tested the competence of extracts prepared from different stages of *Xenopus* embryogenesis to reprogram 293T cells. Upregulation of *Oct-4* mRNA expression was detected with extracts prepared from eggs and fertilized embryos at stage 4 (8 cell) and stage 5 (16 cell) but was lost at late blastula stage 9 (Figure 2A, lanes 2–4, and Table S1). Furthermore, when stage 9 embryo and egg extracts were mixed 1:4, *Oct-4* upregulation was inhibited (Figure 2A, lane 5). Since early blastula stage 8 extracts showed only slight *Oct-4* upregulation (not shown), we conclude that reprogramming activity is lost at midblastula transition (MBT), when zygotic transcription begins in *Xenopus* embryos.

To identify factors required for reprogramming, we carried out an immunodepletion screen. Egg extracts were immunodepleted with commercially available antibodies against a panel of chromatin regulators and transcription factors which appeared as possible candidates involved in reprogramming. Immunodepletion of the chromatin-remodeling ATPase *b*rahma-*r*elated *g*ene 1 (BRG1) [15, 16] reproducibly inhibited *Oct-4* upregulation by egg extract in 293T spheres (Figure 2B, lanes 1 and 2, and Table S1). Employing histone core or Oct-4 antibody led to the same result (data not shown), but they were not followed up because the specificity of the antibody was too broad (histone core), or because the endogenous protein in *Xenopus* is unknown (Oct-4). Immunodepletions of other components of chromatin-remodeling complexes were inconclusive (Ini1, BAF 170, CAF-1 p60, CAF-1 p150, p300) or showed no effect (BAF 155) (data not shown).

Endogenous *Xenopus* BRG1 protein was detectable by anti-BRG1 antibody in *Xenopus* egg extracts, where

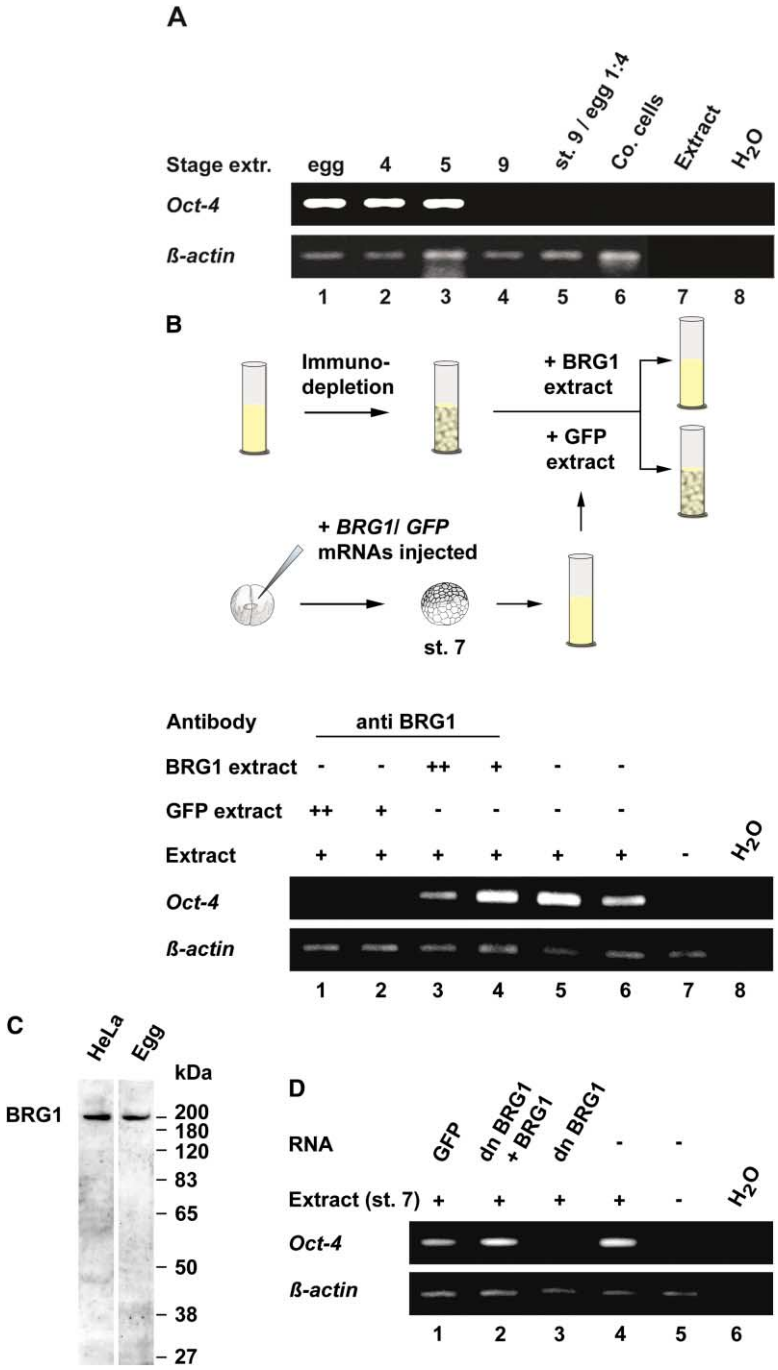

Figure 2. Properties of *Xenopus* Reprogramming Extracts

(A) Dependency of embryo extract reprogramming capacity on developmental stage. Eggs or embryos of the indicated stages were harvested, and extract was prepared and tested for reprogramming as described in Figure 1. A RT-PCR analysis of 293T cell spheres, harvested at day 5 and treated as indicated, was performed.

(B) BRG1 is required for reprogramming. Schematic diagram of the experiment is shown. Egg extract ("Extract") was immunodepleted with anti-BRG1 antibody and supplemented with 5% or 10% rescue extract as indicated (+, ++). Rescue extracts were prepared from stage 7 embryos that had been injected with 1 ng of either *GFP* or *BRG1* mRNA into both blastomeres at the two-cell stage ("BRG1 extract," "GFP extract"). The reconstituted extracts were tested in a standard reprogramming experiment with digitonin in 293T cells. Lane 5, cells treated with egg extract only; lane 6, cells treated with egg extract that had been mock depleted by uncoated beads.

(C) Western blot of HeLa nuclear extract (10 μg) and *Xenopus* egg extract (26 μg) probed with anti-BRG1 (1:200 dilution).

(D) Blocked reprogramming by dominant-negative *BRG1* can be rescued with wild-type *BRG1*. Extracts were prepared as described in (B) from embryos injected with 2 ng of dn *BRG1*, *BRG1*, or *GFP* mRNA. Embryo extracts were tested in a standard reprogramming experiment with digitonin in 293T cells.

it recognizes an expected 200 kDa band, as it does in human HeLa cells (Figure 2C). This confirms an earlier report showing that *Xenopus* BRG1 protein is present in oocytes, eggs, and embryos at various stages [17].

To test if inhibition of reprogramming was indeed due to BRG1 depletion, a rescue experiment was carried out (Figure 2B, top). *Oct-4* upregulation was reconstituted in BRG1-immunodepleted extracts only when they were supplemented with extracts from early embryos injected with *BRG1* mRNA but not with *GFP* mRNA (Figure 2B, lanes 1–4, and Table S1). Since nonreprogrammed 293T cell nuclei already harbor endogenous BRG1 protein [7], reprogramming may require supraphysiological amounts of BRG1. However, the rescue effect was more pronounced when less *BRG1*-injected embryo extract was used (compare lanes 3 and 4), suggesting that too-high amounts of *BRG1* may also be inhibitory. Alternatively, *Xenopus*- and 293T-BRG1 may differ in their functional states.

To corroborate the role of *BRG1*, reprogramming of 293T cells was carried out with extracts from early embryos injected with a dominant-negative (dn) form of *BRG1* [18] or coinjected with wild-type and dn *BRG1* mRNA. While dn *BRG1* inhibited *Oct-4* upregulation,

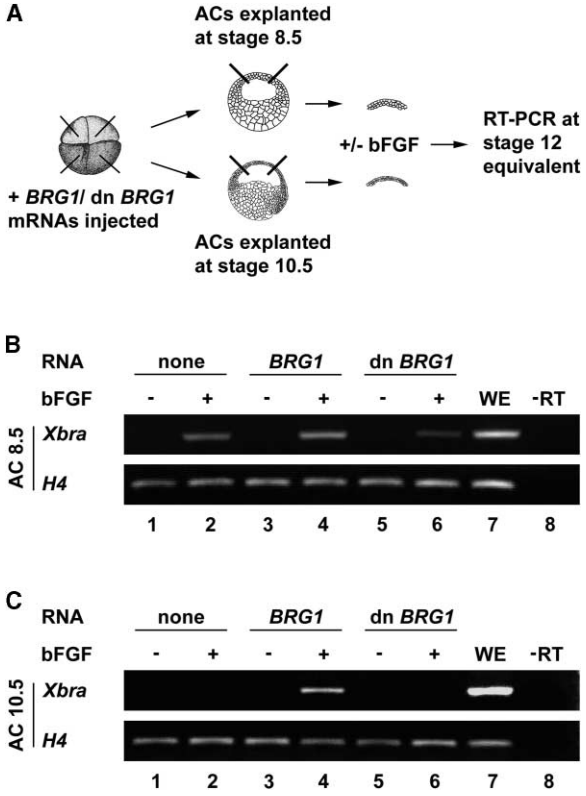

Figure 3. *BRG1* Extends the Competence for Mesoderm Induction

(A) shows a schematic representation of animal cap (AC) assay. Four-cell *Xenopus* embryos were injected or noninjected into the animal pole of each blastomere with 0.75 ng *BRG1* mRNA per blastomere or 0.25 ng dn *BRG1* mRNA per blastomere. ACs were explanted at stage 8.5 (B) or at stage 10.5 (C) and treated or nontreated with 100 ng/ml (stage 8.5) or 200 ng/ml bFGF (stage 10.5) for 3 hr. At the equivalent of stage 12, animal caps were harvested and analyzed by RT-PCR. None, noninjected ACs; WE, whole embryo.

coexpression with wild-type *BRG1* rescued this effect (Figure 2D, lanes 2 and 3, and Table S1). However, injection of wild-type or dn mRNA of the related chromatin-remodeling ATPase *brahma* (*BRM*) [16, 19, 20] showed no effect on *Oct-4* expression (data not shown). Taken together, the results indicate that the chromatin remodeler BRG1 is required during reprogramming of 293T cells by egg extract.

To further study the role of *BRG1* in the maintenance of pluripotency, we investigated the process of *Xenopus* mesoderm induction. Dissected *Xenopus* animal caps can be induced by growth factors such as bFGF to form mesoderm up until the late blastula stage [21]. Afterwards, the competence of animal caps for mesoderm induction gradually decreases because cells loose their pluripotency and differentiate into ectoderm [22]. We therefore tested if forced *BRG1* expression by mRNA injection might promote pluripotency in animal cap cells (Figure 3A). As expected, animal caps induced with bFGF expressed the mesodermal marker *Xenopus brachyury* (*Xbra*) only when they were treated at blastula (stage 8.5) but not at gastrula stage (stage 10.5) (Figures 3B and 3C, lanes 1 and 2). However, expression of *BRG1* but not dn *BRG1* rendered animal caps permissive for *Xbra* induction even at gastrula stage (Figure 3C, lanes 3–6, and Table S1). *Xbra* mRNA induction at gastrula stage still required bFGF treatment, and *BRG1* overexpression alone was ineffective. In animal caps that were bFGF treated at blastula stage, *BRG1* and dn *BRG1* had minor enhancing and inhibiting effects on *Xbra* induction, respectively. Morphology of *BRG1*- and dn *BRG1*-injected embryos was normal in all cases at stages 8.5 and 10.5, and no delay of development was visible. We conclude that *BRG1* extends the competence period of animal cap cells to differentiate into mesoderm. This suggests that *BRG1* overexpression can prolong the pluripotency of embryonic cells in *Xenopus*.

In this study, we have demonstrated that intact adult human cells can be reprogrammed to upregulate embryonic stem cell markers. The in vitro system we developed allows for the study of reprogrammed cells as well as the reprogramming extract. The fact that digitonin treatment is in principle dispensable during reprogramming suggests that the necessary factors can (also) enter the cells by an endocytic route. However, the leukocyte reprogramming achieved by our protocol was clearly incomplete: upregulation of *Oct-4* and *GCAP* was transient and lost after prolonged culture, characteristic pluripotency-associated cell surface markers such as SSEA-3, -4, Tra-1-60, and Tra-1-81 were not detectable, and permanent cell lines could not be established (data not shown). Furthermore, leukocyte clusters and spheres failed to grow when transplanted in nude mice (data not shown). General intra- and interexperimental variability of reprogramming success as reported by Byrne and colleagues [6] was confirmed in our system (see Table S1). More refined reprogramming protocols may allow the full equivalent of ES cells to be obtained in the future. Nevertheless, the protocol allowed us to characterize cell extracts, and we showed that reprogramming capacity is not limited to eggs but extends to fertilized embryos until the MBT. The fact that post-MBT extract dominantly inhibited reprogramming by egg extract suggests that zygotic transcription may activate genes which repress the pluripotent cell state. This is consistent with the gradual loss of competence of *Xenopus* cells to contribute to all three germ layers [23].

Importantly, our protocol allowed us to identify BRG1 as a component required for reprogramming by *Xenopus* egg extracts. *BRG1* is a homolog of the yeast *SWI2/SNF2* and the *Drosophila brahma* genes [15]. Together with BRM, it constitutes the two ATPase subunits of the human SWI-SNF chromatin-remodeling complex [16]. SWI-SNF is involved in remodeling of nucleosomes as well as in transfer of histones and acts by increasing DNA access (for review see [24]). We show that BRG1 is not only required for upregulation of *Oct-4*, but that it can extend the competence period to form mesoderm in *Xenopus* animal cap explants. This finding points to an important role of chromatin remodeling for both induction and maintenance of pluripotency during reprogramming and early embryogensis. Indeed, homozygous *BRG1* mutant mice die during the periimplantation stage [25].

Interestingly, another chromatin-remodeling ATPase, ISWI-D, has also been implicated in nuclear reprogramming [26]. ISWI-D is a member of the ISWI remodeling

complex, which is distinct from the SWI-SNF complex. ISWI-D is required for the specific release of TATA binding factor from adult nuclei incubated with *Xenopus* oocyte extracts, which accompanies chromatin decondensation and protein exchange. ATPase-dependent remodeling may thus act in concert with traditional transcriptional regulators such as *Oct-4* [9] and *Nanog* [27, 28] to achieve pluripotency in mammals.

Experimental Procedures

Xenopus laevis Egg Extract

Xenopus laevis egg and embryo extracts were essentially prepared as described [29]. Eggs were obtained by priming females with 600 IU human chorionic gonadotropin and were dejellied with 2% cysteine HCl. In vitro fertilizations were carried out as described [30]. Eggs and embryos were washed two times with ice-cold extraction buffer (50 mM HEPES/KOH [pH 7.4], 50 mM KCl, 5 mM $MgCl_2$, 2 mM β-mercaptoethanol, 5 mM EGTA, second wash including 10 μg/ml each cytochalasin B [Sigma, Munich, Germany], leupeptin, aprotinin, pepstatin A [all Calbiochem Biosciences, La Jolla, CA]). Eggs or embryos were centrifuged at 1000 rpm for 1 min and transferred to 2 ml Eppendorf tubes after excessive buffer was carefully removed. Eggs and embryos were crushed by centrifugation at 10,000 × g for 15 min at 4°C. The middle layer was collected and recentrifuged at 16,000 × g to clear the extract. The extract was substituted with 2% glycerol, and aliquots were snap frozen in liquid nitrogen and stored at −80°C.

RNA Injection and Animal Cap Assay

Microinjection of mRNAs as well as *Xenopus* embryo and explant culture were performed as previously described [30]. Synthetic mRNA of human *BRG1*, dn *BRG1* [18], and *enhanced green fluorescent protein* (*eGFP*; Clontech, Palo Alto, CA) were in vitro transcribed using the MegaScript kit (Ambion, Austin, TX) according to the manufacturer's instructions. Animal caps were dissected at stages 8.5 or 10.5 and incubated with 100 or 200 ng/ml bFGF (Sigma) for 2–3 hr and harvested at stage 12 for RT-PCR analysis.

Immunodepletion

Protein A- or protein G-coupled Dynabeads in 10 μl suspension (Dynal, Hamburg, Germany) were washed four times with 1× TBS + 0.1% BSA (washing solution) in 0.2 ml Eppendorf tubes using a magnet. Beads were precharged with 25 μg anti-BRG1 N-15 antibody (Santa Cruz Biotechnology, Heidelberg, Germany) by incubating for 2 hr at room temperature with agitation. Unbound antibody was washed away with washing solution, and beads were resuspended in 3 μl washing solution and stored at 4°C. For immunodepletion, 1 μl beads was washed once again in washing solution, and 10 μl egg extract was added and incubated for 1 hr at 4°C with agitation. Beads were removed by magnet, and the supernatant was used for reprogramming without delay. For control experiments, uncoupled beads were used. For rescue experiments, 9 or 9.5 μl immunodepleted extract was supplemented with 0.5 or 1 μl of *GFP* or *BRG1* mRNA-injected embryo (stage 7) extract. For Western blot analysis, anti-BRG1 P18 antibody (Santa Cruz Biotechnology) was used.

Reprogramming with *Xenopus* Extracts

293T cells were grown to subconfluency in cell culture medium (DMEM) (Cambrex Bio Sciences Verviers, Verviers, Belgium), 10% FCS (PAA Laboratories, Linz, Austria), 1% glutamine (Cambrex Bio Sciences Verviers) and incubated in 10% CO_2. Cells (4×10^6 per reprogramming experiment) were harvested by flushing with PBS and transferred to 15 ml Falcon tubes. Cells were pelleted and resuspended in 0.5 ml ice-cold transport buffer (based on [31]; 20 mM HEPES [pH 7.3], 110 mM KAc, 5 mM NaAc, 2 mM MgAc, 1 mM EGTA, 2 mM DTT, 1 μg/ml each of aprotinin, pepstatin A, and leupeptin). To obtain human leukocytes, buffy coat from HIV-, HBV-, and HCV-negative donors collected at a blood bank was diluted 1:1 with PBS, and 25 ml was laid over 16 ml Ficoll (Amersham Biosciences, Freiburg, Germany) in a 50 ml Falcon tube. After centrifugation for 20 min at 2000 rpm, the purified buffy coat layer containing leukocytes was harvested, transferred to a new 50 ml Falcon tube, diluted with PBS up to 50 ml, and recentrifuged for 10 min at 1500 rpm. Cells were resuspended in 1 ml ice-cold transport buffer, and 0.5 ml was used per reprogramming experiment.

For reprogramming, cells were pelleted and resuspended in 0.5 ml transport buffer ± 35 μg/ml digitonin (Calbiochem) and incubated for 5 min on ice. Cells were washed in transport buffer, resuspended in 20 μl reprogramming mix (1 μl 20 mg/ml BSA [Roche, Mannheim, Germany], 1 μl 20 mM ATP, 1 μl 100 mM phosphocreatine, 1 μl 400 U/ml creatine kinase [all Sigma], 1 μl RNAsin [40 U/μl; Promega, Madison, WI], 5 μl transport buffer, 10 μl egg or embryo extract) and incubated for 30 min at 37°C. Incubation was terminated by addition of 0.5 ml ice-cold transport buffer. 293T cells and leukocytes were centrifuged, resuspended in cell culture medium, and cultivated in 4 cm petri dishes (Renner, Dannstadt, Germany) at 37°C with 10% CO_2. For the standard assay, spheres were harvested at day 6 (day 1 = experiment).

RT-PCR

RT-PCR for *Xenopus* animal cap assays was carried out as described [30]. RT-PCR of human cells was carried out as follows. RNA was essentially prepared as described [32]. Spheres, clusters, and cells were harvested by glass needle aspiration and transferred to 100 μl denaturing solution (4 M GnSCN, 0.5% sarcosinate, 1% β-mercaptoethanol, 20 μg rRNA), laid over 100 μl 5.7 M CsCl, and spun for 3.5 hr at 174,000 × g in a TL-100 Tabletop Ultracentrifuge (Beckman Coulter, Fullerton, CA) using a TLA 100.2 rotor. The supernatant was discarded in several steps with new pipette tips to carefully avoid DNA contamination. The RNA pellet was washed in 70% EtOH, dried, resuspended in 20 μl DEPC-H_2O, and reprecipitated with sodium acetate. The washed and dried pellet was resuspended in 1.3 μl DEPC-H_2O, and 0.18 μl 10× reaction buffer and 0.3 μl DNase I (Invitrogen Life Technologies, Carlsbad, CA) were added and incubated for 15 min at room temperature. DNase digestion was stopped by addition of 0.18 μl 25 mM EDTA and heat inactivation at 65°C for 15 min.

For reverse transcription, 4 μl random hexamers (50 ng/μl; Invitrogen) was added to the DNase-treated RNA solution, incubated at 70°C for 10 min, and placed on ice. 1 μl 10× PCR buffer, 1 μl 25 mM $MgCl_2$, 0.5 μl 10 mM dNTP, 1 μl 0.1 M DTT, and 0.1 μl RNAsin (Promega) were added and incubated at 25°C for 5 min. 1.9 μl of the sample was removed for RT-negative reactions; to the rest, 0.4 μl (80 U) Superscript II RT (Invitrogen) was added and incubated at 25°C for 10 min and at 42°C for 50 min. The reaction was terminated by heating to 95°C for 5 min.

PCR reactions for human cells were carried out with 0.1μl (1:10 diluted) to 3 μl (undiluted) RT reactions. Primers were *Oct-4* (based on [33]; 5′-GACAACAATGAAAATCTTCAGGAGA, 3′-TTCTGGCGCC GGTTACAGAACCA; 218 bp product) and β-*actin* (5′-CGTGGGGCG CCCCAGGCACCA, 3′-TTGGCCTTGGGGTTCAGGGGGG; 243 bp product); other primers were described: *GCAP* [34] (484 bp product), *NPR-A* [35] (491 bp product), *Xbra* [30] (324 bp product), *histone 4* (*H4*) [30] (188 bp product). For all RT-PCR experiments, the samples were first analyzed for β-*actin* or *H4* mRNA expression, and template concentrations were then adjusted accordingly to obtain normalized samples. In the final PCRs, the same number of cycles for all samples was used for each gene and experiment so that results are comparable. PCR cycles for *Oct-4* were 36–42; *GCAP*, 44; *NPR*-A, 41; *Xbra*, 26–36; β-*actin*, 24–30; and *H4*, 24. Control reactions included RT-negative, H_2O, media, and egg extract samples.

Supplemental Data

Supplemental Table S1 is available at http://www.current-biology.com/cgi/content/full/14/16/1475/DC1.

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