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Conversion of mouse fibroblasts into cardiomyocytes using a direct reprogramming strategy

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Here we show that conventional reprogramming towards pluripotency through overexpression of Oct4, Sox2, Klf4 and c-Myc can be shortcut and directed towards cardiogenesis in a fast and efficient manner. With as little as 4 days of transgenic expression of these factors, mouse embryonic fibroblasts (MEFs) can be directly reprogrammed to spontaneously contracting patches of differentiated cardiomyocytes over a period of 11–12 days. Several lines of evidence suggest that a pluripotent intermediate is not involved. Our method represents a unique strategy that allows a transient, plastic developmental state established early in reprogramming to effectively function as a cellular transdifferentiation platform, the use of which could extend beyond cardiogenesis. Our study has potentially wide-ranging implications for induced pluripotent stem cell (iPSC)-factor-based reprogramming and broadens the existing paradigm.

The mammalian heart lacks significant regenerative capacity. As such, *in vitro* generation of autologous cardiac cells for transplantation and the effective treatment of heart disease is a key area of study. A recent report describing the successful transdifferentiation of somatic cells to a cardiac fate *in vitro*[1] has raised the possibility that this process might eventually be used for cell-based cardiac therapy. However, speed and efficiency must first be improved, especially if the ultimate goal is to provide a faster and safer alternative to the re-differentiation of autologous induced iPSCs[2].

In the reprogramming field the very rare but generally reproducible appearance of various terminally differentiated cell types—including spontaneously contracting cardiac colonies—during the later stages of iPSC formation has often been noted. From these observations, and because induced pluripotency is established in a step-wise and stochastic manner[3,4], we hypothesized that it might be possible to modify the reprogramming process to favour such alternative outcomes. We reasoned that providing the appropriate developmental cues after an initial epigenetic 'activation phase' might allow us to hijack conventional reprogramming at this early unstable stage, and specifically shift the outcome towards cardiogenesis.

RESULTS

Three-factor reprogramming is sufficient to activate the early cardiac programme

To test the validity of our hypothesis and gauge technical feasibility, we virally transduced MEFs harbouring a Nebulette–LacZ reporter expressed only in nascent myocardium[5] with genes encoding the four transcription factors Oct4, Sox2, Klf4 and c-Myc[6], and colorimetrically monitored early cardiogenesis. We modified standard reprogramming medium by removing leukaemia inhibitory factor (LIF) to avoid the generation and/or maintenance of pluripotent cells. We also tried different basement membranes (gelatin, fibronectin, Matrigel or Geltrex) and added fetal bovine serum (FBS) at concentrations of 1–15% to increase cell viability and/or the cardioinductive properties of the medium. When cells were cultured on Matrigel or Geltrex in LIF-free medium containing 5% FBS, we observed transient (≤ 2 days) but widespread (approximately 50%) β-galactosidase expression in nascent colonies after a week. Importantly, systematic omission of factors from the viral cocktail revealed that c-Myc was dispensable, and that the transduction of just three factors worked equally well (Fig. 1a).

Induction of spontaneous contraction requires growth factor signalling and is enhanced by a small molecule

Having successfully initiated the early cardiogenic programme in MEFs, we next sought to induce robust activation of mid-stage cardiac gene expression and to ultimately generate spontaneously contracting patches of cardiac cells, a hallmark of terminal differentiation that is easily observed and quantified. MEFs transduced with three factors were treated with chemically defined media (CDM) as well as cytokines and small molecules that might enhance cardiogenesis by regulating TGFβ, bone morphogenetic protein (BMP), Hedgehog, Wnt or Notch pathway activity. Treatments were done individually and in combination, and at different times during the reprogramming process. Despite the general unsuitability of the resulting protocol for iPSC generation, we also explored whether small-molecule inhibition of JAK-STAT (Janus kinase-signal transducer

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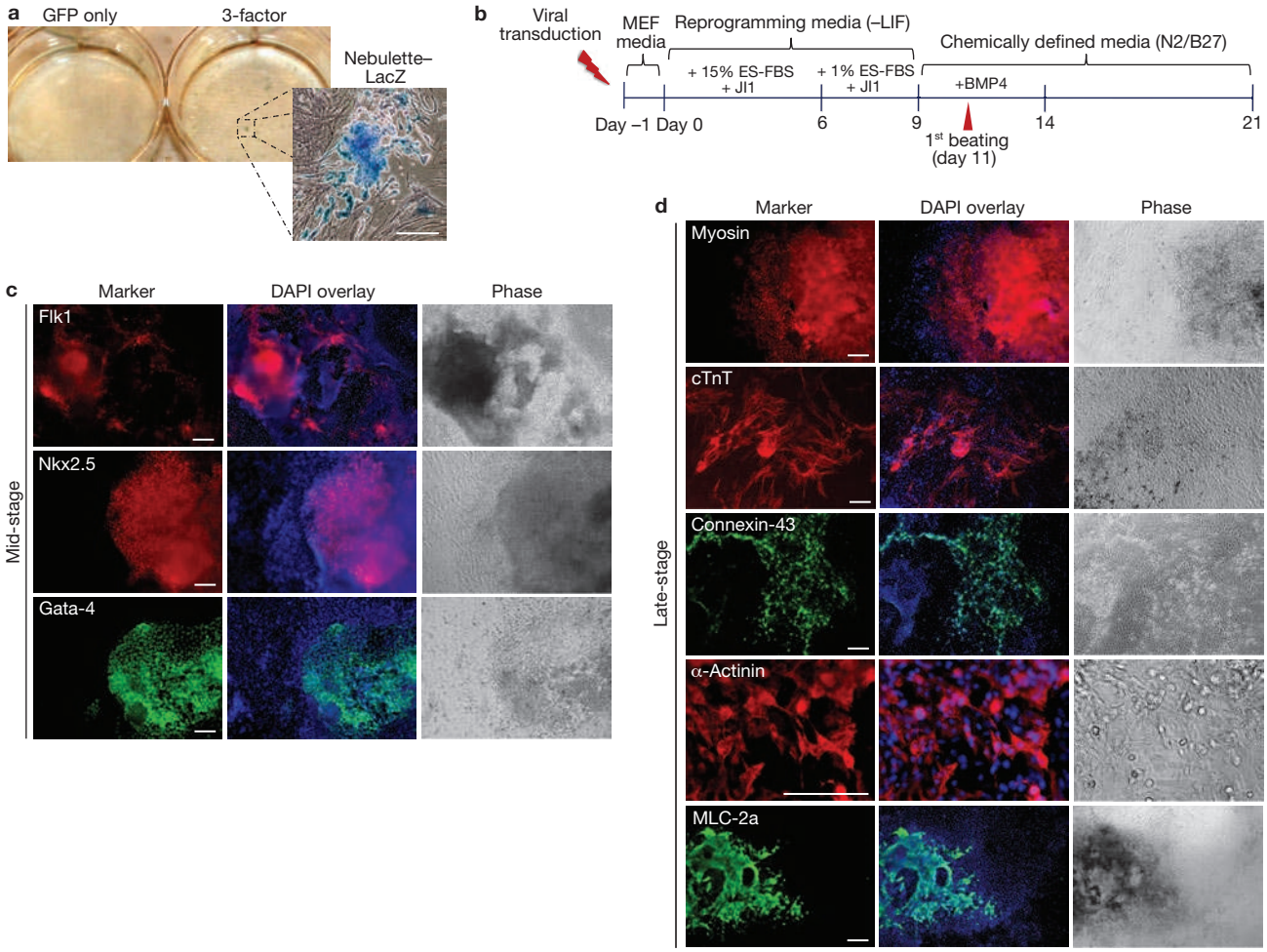

Figure 1 Development of robust conditions for direct reprogramming of fibroblasts to a mature cardiac fate. (**a**) X-gal staining of Nebulette–LacZ$^+$ colonies 7 days after three-factor transduction (cells transfected with GFP only used as a control). Images show culture wells and inset is an enlarged phase-contrast microscopy image of a LacZ$^+$ colony. (**b**) Schematic representation of the final direct cardiac reprogramming conditions, with the onset of spontaneous contraction indicated. (**c**, **d**) Immunostaining and microscopy of mid- and late-stage cardiac lineage markers on days 11–15 (**c**) and 18–21 (**d**) from MEFs treated as indicated in **b**. Scale bars; 100 μm.

and activator of transcription), PI(3)K (phosphatidylinositol-3-kinase) or Wnt signalling during the early stages of the protocol might boost the effectiveness of cardiomyogenesis by preventing even a small number of iPSC intermediates from forming. Finally, we experimented with a more gradual transition between different media by lowering FBS concentration step-wise in increments of 1–4% over 3–6 days (immediately before switching to CDM). After several rounds of experimentation employing many different combinations, we determined the following conditions to be by far the most effective: cells are first exposed to reprogramming media containing 15% FBS and 5% knockout serum replacer (KSR) for 6 days, followed by a switch to 1% FBS and 14% KSR for 3 days. During this initial 9-day period, the small-molecule JAK inhibitor JI1 is continuously kept in the media at a concentration of 0.5 μM. From day 9 onwards, the cells are cultured in CDM, with the cardioinductive growth factor BMP4 (ref. 7) added at 20 ng ml^{-1} for the first 5 days (Fig. 1b).

Using three-factor transduction with the above conditions, we observed robust expression of the mid-stage cardiac markers[8] Flk1, Nkx2.5 and Gata-4 starting on days 9–10 (Fig. 1c). Late-stage markers including cardiac troponin T (cTnT), sarcomeric myosin heavy chain and α-actinin were observed from day 11 onwards (Fig. 1d) . Simultaneously, Connexin-43 staining became apparent along the periphery of many cells. Interestingly, regardless of the time at which immunocytochemistry was performed, we only detected the atrial isoform of myosin light chain (MLC-2a), indicating that the cardiomyocytes being generated were mostly, if not exclusively, of the atrial subtype (Fig. 1d).

The earliest wave-like spontaneous contractions also began on day 11, and many colonies were seen forcefully contracting in their entirety by day 15 (Supplementary Movie S1). Although not absolutely required, BMP4 seems to be the major driving force behind the robust development of beating; on average, its addition increased the formation of contracting patches nearly 150-fold (149 ± 13 patches per 100,000 MEFs plated, $n = 6$). Strikingly, sequential application of the JAK inhibitor JI1 and BMP4 can even further increase the number of contracting patches; we have obtained as many as 317 independently contracting patches per 100,000 MEFs plated by day 21 (mean = 257 ± 17, $n = 6$; Fig. 2a). Under these conditions, the incidence of beating in terms of total colony number could be as high as 90% (mean = 79 ± 4%, $n = 5$; Fig. 2b and Supplementary Movie S2). The marked nature of this shift and

the pervasiveness of the cardiogenic outcome were especially evident in whole-well microscopic analyses, in which all large colonies in a well could be seen expressing high levels of cTnT and spontaneously contracting by day 18. Conversely, they did not express above-background levels of Nanog–GFP (green fluorescent protein) at the same time (Fig. 2c and Supplementary Movie S3).

JI1 was most effective when applied continuously for the first 9 days—the period during which there might be a remote possibility of generating iPSC intermediates owing to the use of Oct4, Sox2, Klf4 and c-Myc (Fig. 1b). Accordingly, the effectiveness of JI1 declined with shorter treatment periods; however, extending JI1 treatment beyond 9 days to overlap with CDM and BMP4 application also seemed to be counterproductive (data not shown). This latter observation is supported by a recently reported requirement for JAK–STAT signalling in cardiomyogenesis[9]. In the case of BMP4, 5 days of treatment was optimal (Fig. 1b); both shorter and longer periods of treatment proved detrimental to the development of beating cardiac colonies (data not shown). Although its precise role in direct cardiac differentiation remains to be clarified, BMP4 is most probably driving cardiac induction from nascent precursors during a critical developmental window[10,11]. It could further be speculated that the cardioinductive effect of JI1 treatment might result from an indirect expansion of this precursor pool; by preventing cells from becoming primed for induced pluripotency, a larger number may ultimately become available for BMP4 to act on and prime for cardiogenesis instead.

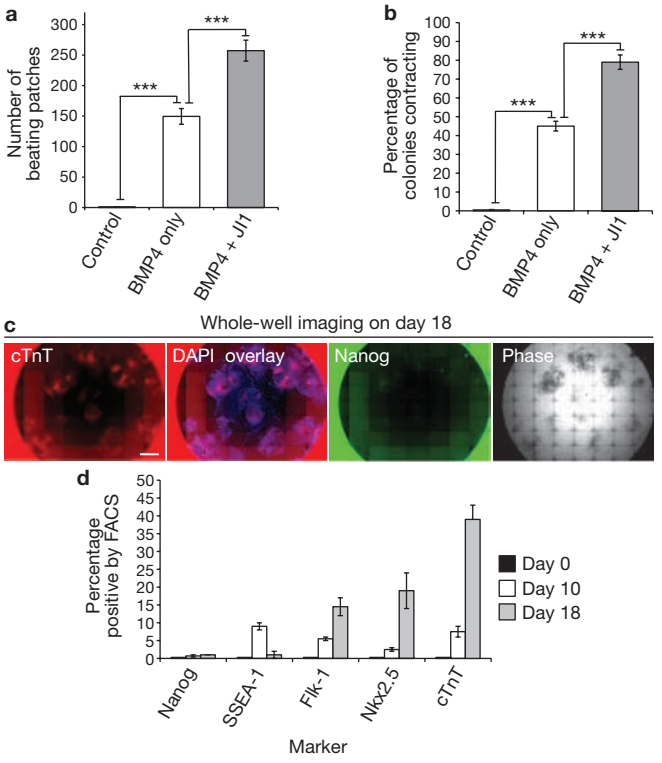

Figure 2 Gauging reprogramming efficiency and success by incidence of beating and marker expression. (**a**–**d**) MEFs were treated as indicated in Fig. 1b. (**a**, **b**) BMP4 and the JAK inhibitor JI1 were included in the media as indicated and changes in contracting colony number (**a**) and percentage (**b**) were quantified. Colony numbers in **a** and **b** correspond to incidence per 100,000 MEFs plated and were recorded on day 18. Data are means ± s.e.m. (**a**, $n = 6$; **b**, $n = 5$). Asterisks indicate $P < 0.001$. (**c**) Whole-well imaging (cells were grown in 96-well plates) demonstrating the relative expression of cTnT and Nanog on day 21. (**d**) FACS counts of cells positive for the indicated markers at 0, 10 and 18 days. Scale bar, 900 μm.

Transdifferentiation yields a substantial number of highly differentiated and functionally responsive cardiomyocytes

Although contracting colony number functions as a very convenient metric for inter-sample comparisons of reprogramming success and efficiency, it does not allow for a quantification of the individual cardiomyocyte yield. Therefore, we used fluorescence-activated cell sorting (FACS) on days 0, 10 and 18, examining both precursor (Flk-1$^+$ and Nkx2.5$^+$) and more mature (cTnT$^+$) cardiac populations. A modest increase was observed for all three markers by day 10, with significant increases by day 18. Flk-1$^+$ and Nkx2.5$^+$ cells did not increase to the same extent as cTnT$^+$ cells, presumably because the former are expressed at a higher level during the earlier stages of cardiac development[12]. Remarkably, nearly 40% (39 ± 2%, $n = 5$) of cells had become cTnT$^+$ by day 18 (Fig. 2d). Based on these data, the final yield of cTnT$^+$ cells was conservatively estimated to be 120,000 per 100,000 MEFs plated or 1.2 cardiomyocytes per fibroblast (100,000 starting MEFs reproducibly translated into a day 18 harvest of approximately 300,000–350,000 total cells).

To more closely examine the degree of differentiation among the population of contracting cells, we examined their beating frequency, calcium flux patterns and response to chronotropic agents. Contraction frequency of cardiomyocytes on day 18 varied from a minimum of 4 beats per minute (BPMs; data not shown) to a maximum of 130 BPMs (Supplementary Movie S4). Although this pattern implied a wide range of development/differentiation, the latter observation suggested that at least a subset of cardiomyocytes were acquiring highly differentiated traits[13]. Accordingly, many contracting patches exhibited characteristic calcium transients[1,14] (Fig. 3a), the frequency of which could be reversibly modulated with 1 μM isoproterenol or 10 μM carbachol, indicating proper responsiveness to β-adrenergic and muscarinic signalling, respectively[13] (Fig. 3b). Addition of isoproterenol significantly increased the frequency of spontaneous calcium transients and shortened the decay period (τ), whereas carbachol had the opposite effects (Fig. 3c). Total contracting colony number consistently levelled off beyond day 18, but most colonies continued to contract well beyond the end point of our assays (day 21), with some patches still contracting after 5 weeks (data not shown).

Next, we enzymatically dissociated contracting patches into individual cardiomyocytes (Fig. 3d and Supplementary Movie S5) and carried out electrophysiological measurements (Fig. 3e). The spontaneous action potentials generated in these cells closely resembled 'atrial-like' (and to a lesser extent 'pacemaker-like') action potentials reported elsewhere[15], with a mean diastolic potential (MDP) of –61.0 ± 1.2 mV and a mean overshoot of 13.9 ± 4.9 mV ($n = 3$). As such, these data corroborate immunocytochemistry results suggesting a mostly atrial phenotype for the cardiomyocytes being generated (Fig. 1d).

Of note, we repeatedly observed a small number of contracting single cells exhibiting characteristic cTnT staining even before enzymatic dissociation, indicating that nascent cardiomyocytes can differentiate, and perhaps even arise, outside of the context or 'niche' of a developing colony (data not shown). Such a phenomenon would suggest that a subset of cells might be taking a more direct transdifferentiation path (that is, perhaps not having to rely on extracellular cues from neighbouring cells in colony), a hypothesis consistent with the stochastic nature of reprogramming with the transcription factors Oct4, Sox2, Klf4 and c-Myc.

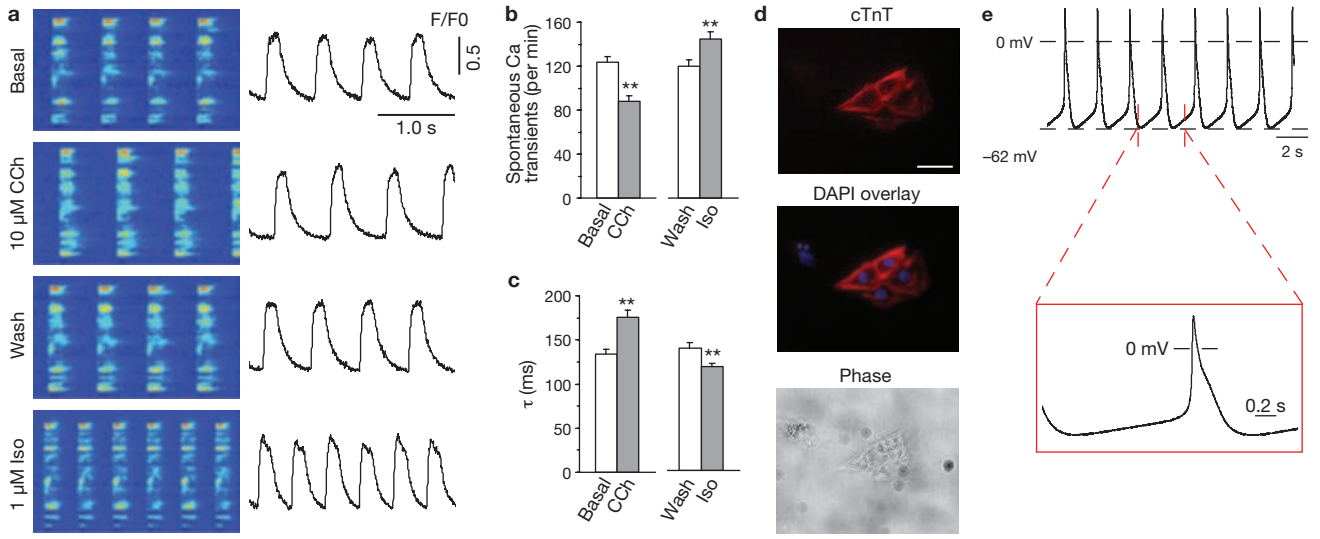

Figure 3 Calcium flux and electrophysiological characterization of contracting cardiomyocytes. (**a**) Spontaneously contracting colonies were generated, and calcium transients were recorded at basal levels, before treating the cells with carbachol (CCh), followed by a wash step and then isoproterenol (Iso) treatment. Left; linescan image of calcium transients. Right; traces of calcium transients. F/F0; transient amplitude. (**b**, **c**) Quantification of calcium transient frequency (**b**) and decay rate (**c**) from experiments performed as in **a**. Data are means ± s.e.m., $n = 22$. Asterisks indicate $P < 0.01$. (**d**) Immunostaining and microscopy of individually contracting cells isolated from a large colony from an MEF treated using our reprogramming protocol. A corresponding movie can be found in Supplementary Information (Movie S5). (**e**) Spontaneous action potentials recorded from an individual cardiomyocyte at 20–22 °C. The waveform of a single action potential is shown in the lower inset using an expanded time scale. Dashed lines indicate resting potential (–62 mV) and 0 mV, respectively.

Next, we conducted chromatin immunoprecipitation (ChIP) assays to ascertain the nature and extent of certain epigenetic changes taking place at four loci: *Oct4*, *Actn2* (encoding α–actinin), *Ryr2* (encoding the cardiac ryanodine receptor) and *Tnnt2* (encoding cardiac troponin T). Relative levels of trimethylation at histone H3 Lys 4 (H3K4me3) and Lys 27 (H3K27me3) in the promoter regions of these genes were examined. As expected, owing to marked exogenous expression of *Oct4*, *Sox2*, *Klf4* and *c-Myc* during the initial part of the protocol, we observed an initial burst of activating H3K4me3 (with a concomitant decrease in H3K27m3) at the *Oct4* promoter by day 10, followed by a return to the H3K27me3-dominated, repressed state seen in the starting MEFs by day 19 (Fig. 4a). The promoters of the three cardiac genes, all of which are expressed at the later stages of cardiomyogenesis, also exhibited enrichment in H3K4me3 by day 10. However, unlike *Oct4* this trend did not stop or reverse; by day 19, H3K4me3 enrichment was extensive when compared with the starting MEFs. Strikingly, it even exceeded levels found in cardiomyocytes derived from mouse embryonic stem cells (mESCs; Fig. 4a). Concurrently, levels of repressive H3K27me3 at the cardiac loci had fallen far below the starting fibroblasts' levels, and were approaching those of the control cardiomyocytes.

Transdifferentiated cardiomyocytes most probably do not arise from contaminating cardiac precursors or iPSC intermediates

To eliminate the possibility that beating patches might be arising from rare multipotent cardiac precursor cells in our MEF cultures, we tested our method on tail-tip fibroblasts (TTFs), which are a much more homogenous source of fibroblasts and do not contain any cardiac cells. As postnatal and adult cells are more refractory to reprogramming[16], it was necessary to use all four reprogramming factors, including c-Myc, to induce cardiac colony formation. Surprisingly, on day 12—only a day later than when MEFs were used—we observed a small number of colonies beginning to contract. Despite this striking similarity in timing, the number of beating patches generated per 100,000 cells plated (115 ± 7, $n = 6$) was lower than the yield from MEFs reprogrammed under the same conditions (145 ± 6, $n = 6$; $P < 0.01$).

The conditions used in our protocol are not conducive to the establishment or maintenance of iPSCs[17]. We do not use feeder cells or LIF, and switch to chemically defined media containing cardiogenic molecules early in the protocol. Most importantly, even with three-factor transduction, the first contracting patches emerge within 12 days, a much shorter period than the approximately three-week minimum required to generate iPSCs with three factors[18,19]. Strikingly, cardiac reprogramming of TTFs also proceeds at the same pace, even though such postnatally derived cells would normally be expected to reprogramme more slowly[20]. Despite these considerations, we sought to more rigorously rule out the possibility that our method might depend on the generation of a transient pluripotent intermediate, the re-differentiation of which could conceivably give rise to cardiomyocytes.

First, we reasoned that if generation of iPSCs before the switch to CDM was critical, using standard reprogramming media with LIF for the first 9 days should yield a greater number of contracting colonies. However, this substitution actually resulted in a 4-day delay in the onset of beating and a ~25-fold decline in beating colony number (Fig. 4b). A critical implication of this result is that early reprogramming events—brought about primarily by the overexpression of Oct4, Sox2, Klf4 and c-Myc—seem to be generating a heterogeneous population of cells with diverse developmental potential, as even standard reprogramming media allows for the generation of a very small number of cardiac progenitors. Furthermore, conditions that favour the development of iPSCs must do so at the expense of other potential outcomes of the reprogramming process. Therefore,

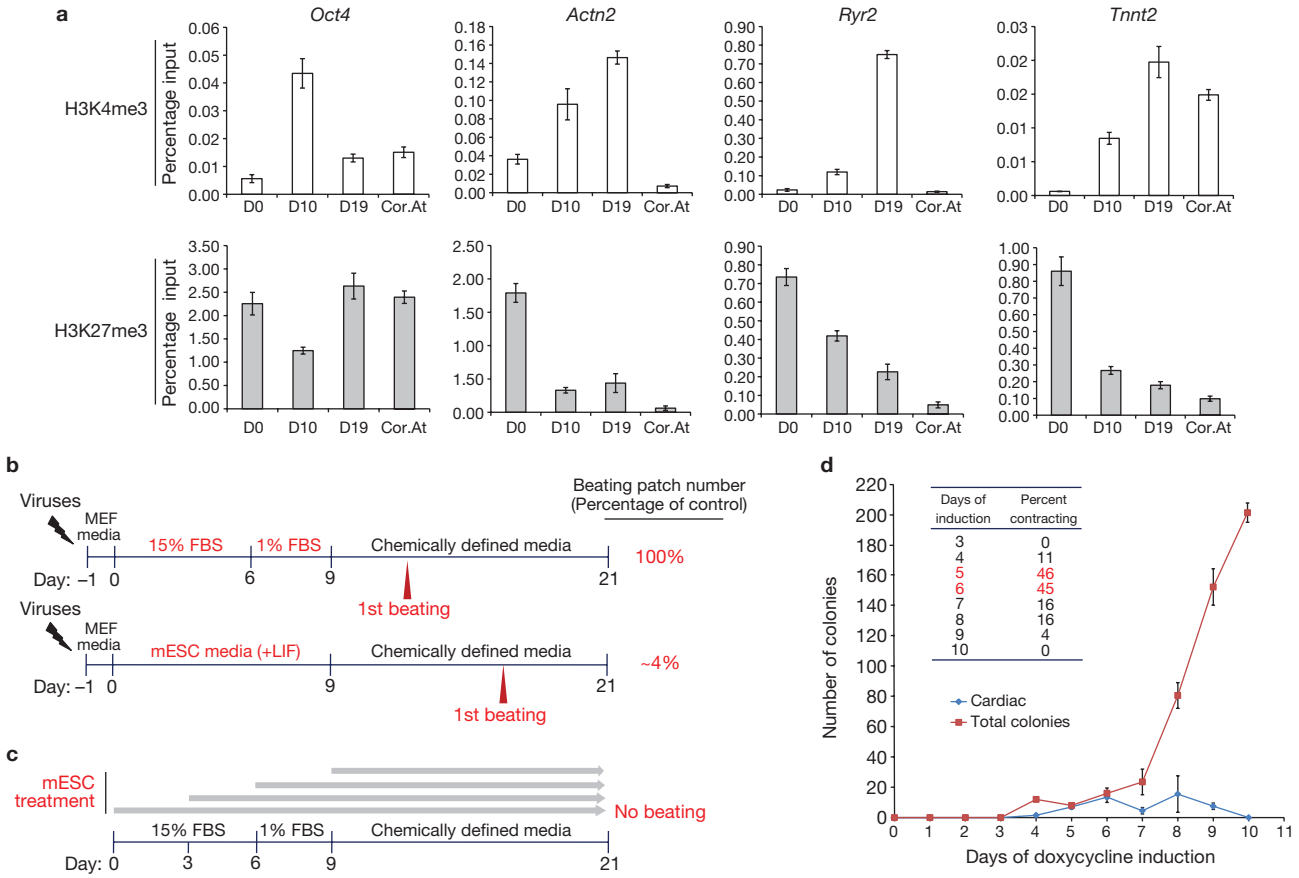

Figure 4 Development of pluripotency is detrimental to cardiogenesis. (**a**) ChIP assays examining epigenetic modifications at four relevant loci during reprogramming at day 0 (D0), day 10 (D10) and day 19 (D19). Quantitative PCR was used to assess changes in histone H3 K4 (top) and K27 (bottom) trimethylation levels. Cor.At denotes commercially available pure cardiomyocytes (Axiogenesis). Data are means ± s.d. $n = 3$. (**b**) Effect of conditions promoting iPSC generation on cardiac reprogramming. Schematic representation of maximised protocol for production of cardiac cells (top left) and modifications to these conditions (bottom left) with corresponding relative efficiencies (right). (**c**) Schematic representation of protocol used to test whether cardiogenesis could be induced in pluripotent cells. mESCs were cultured using the protocol as indicated by the grey arrows. (**d**) Differential effects of prolonged reprogramming factor expression on beating versus total colony number. MEFs were generated that harbour doxycycline-inducible transgenes encoding the reprogramming factors. Cells were treated with doxycycline over the indicated days and total number of colonies, number of cardiac cells (graph) and percentage of contracting cells (inset table) were quantified. Data are means ± s.d., $n = 5$. Red numbers in table indicate optimal duration of drug treatment.

although marginally permissive, standard reprogramming media is a very poor choice for the initiation of direct cardiac reprogramming.

Prolonged induction of the pluripotency programme inhibits transdifferentiation

The rapid induction of beating suggests that lineage-commitment decisions are made quite early and further supports the suggestion that the cardiomyocytes are not generated from a pluripotent intermediate. We speculate that the reprogramming factors (especially Oct4) mostly function to 'erase' cell identity by epigenetic mechanisms, and do not directly activate lineage-specific genes. Conversely, exposure to external, cardiac-specific signals (in this case BMP4 treatment) probably results in early lineage specification and/or the induction of terminal differentiation. In any case, overexpression of reprogramming factors should be only transiently required to allow for activation of lineage-specific gene networks, and prolonged expression is probably detrimental. To test this hypothesis, we generated secondary MEFs harbouring doxycycline-inducible transgenes encoding reprogramming factors[21], and a Nanog–GFP reporter[22] to monitor establishment of pluripotency[23]. Strikingly, a mere 4 days of doxycycline treatment was sufficient to induce beating on day 11. The optimal duration of drug treatment was between 5 and 6 days, with treatment longer than 9 days not producing any contracting cardiac cells at all (Fig. 4d). Conversely, generation of iPSC colonies using this particular system requires a minimum 12 days of transgene induction[21,24]. Even the shortest transgene expression requirement reported to date is 7–8 days[25]. Therefore, optimal cardiac reprogramming required the transgenes to be inactivated well before pluripotency could be endogenously established.

We also tested our protocol on mESCs to establish whether it had a cardiogenic effect on pluripotent cells. mESCs were cultured from day 0, 3, 6 or 9 of our protocol (Fig. 4c). However, none of the cultures produced any contracting patches by day 21 or beyond, indicating that our protocol could not bring about the development of cardiac colonies from iPSCs, even if they were arising during the direct reprogramming process (Fig. 4c). These results demonstrate the establishment of *bona fide* pluripotency during our protocol would in fact constitute a strong barrier to successful cardiac reprogramming.

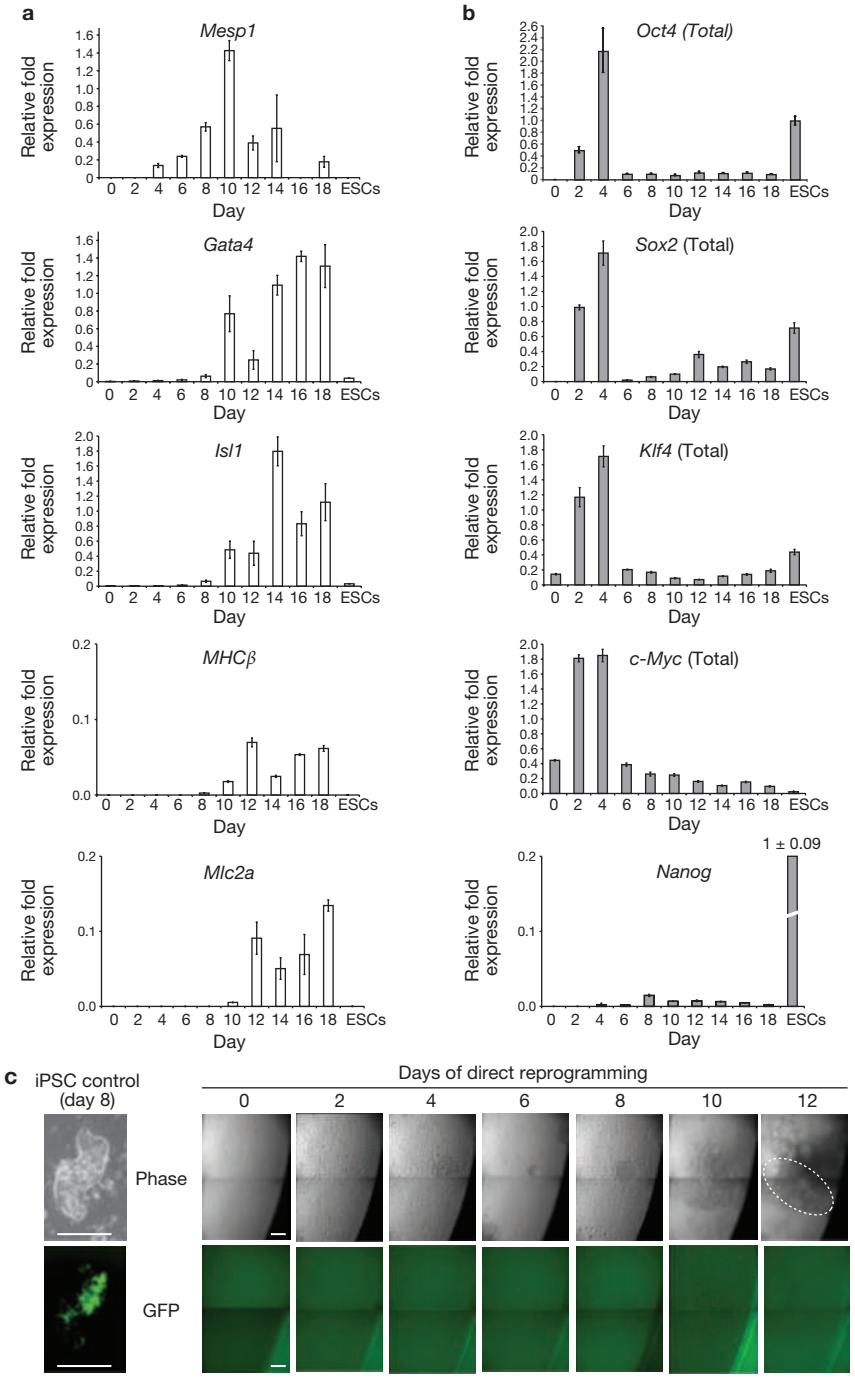

Figure 5 Direct cardiac reprogramming represents a parallel process that occurs in the absence of iPSC generation. (**a**, **b**) Time course analysis of cardiac lineage (**a**) and pluripotency (**b**) marker expression by quantitative RT–PCR. Secondary MEFs harbouring Nanog–GFP and doxycycline-inducible transgenes encoding reprogramming factors were cultured using our protocol. Data are means ± s.d. ($n = 3$). (**c**) Images from phase-contrast microscopy (top) and fluorescence microscopy using the FITC channel (bottom) of cardiac colony formation and maturation in Nanog–GFP reporter MEFs at the indicated days of the reprogramming protocol. Spontaneous contraction was first detected on day 12 in the encircled portion of the colony (see Supplementary Movie S6 for video). Robust early activation of the Nanog–GFP marker using standard reprogramming methods is provided as a positive control (left). Scale bars, 100 μm.

Cardiac transdifferentiation and iPSC generation represent parallel, non-overlapping processes

Finally, to rule out the possibility that our method might depend on the generation of a transient pluripotent intermediate, we quantified the expression of cardiac genes in secondary MEFs that had undergone the reprogramming protocol. Results from quantitative PCR with reverse transcription (RT–PCR) show that the cardiogenic programme is already underway by days 4–6, as indicated by the marked expression of *Mesp1* and *Gata4* transcripts (Fig. 5a). We did note that the randomness and asynchrony inherent in reprogramming perhaps resulted in a heterogeneous population of cardiac mesoderm cells and, in turn, higher inter-sample variation. Conversely, levels of reprogramming factor transcripts

decrease precipitously following doxycycline withdrawal and generally remain very low (Fig. 5b). Endogenous *Nanog* transcripts are not detectable until days 8–9, and at very low levels. Consequently, most colonies do not express any Nanog–GFP at all; only a small subset show levels of fluorescence scarcely above background, starting on days 9–12 (data not shown). Thus, any low-level activation of Nanog coincides with early expression of mature cardiac markers such as the β isoform of myosin heavy chain (MHCβ) and the initiation of spontaneous contraction on day 11 (Fig. 5a). Although this concurrent activation of late-stage iPSC and cardiomyocyte markers effectively rules out the possibility that the former could somehow give rise to the latter, we sought to definitively show that the development of spontaneous contraction does not require activation of the endogenous pluripotency network. We acquired daily images of large numbers of cells undergoing direct reprogramming and retrospectively evaluated Nanog–GFP expression in colonies that eventually began contracting. Unsurprisingly, these colonies had not expressed even minute amounts of Nanog–GFP at any time during their formation or differentiation (Fig. 5c and Supplementary Movie S6).

FACS analyses of SSEA-1$^+$ and Nanog$^+$ cells on days 0, 10 and 18 corroborated the above results: even on day 18, Nanog$^+$ cells only comprised 1% of the population. SSEA-1$^+$ cells made up a similarly small fraction of the cells on day 18. On day 10, these latter cells represented close to 10% of the population (Fig. 2d). However, as SSEA-1 is one of the earliest markers induced on overexpression of Oct4, Sox2, Klf4 and c-Myc[4], and is in fact expressed in a range of differentiated precursor cells[26,27], it is not a marker of *bona fide* pluripotency. This notion is especially valid given the lack of concomitant Nanog expression[23] and the precipitous decline of the SSEA-1$^+$ population by day 18. We surmise that the higher levels on day 10 may result from a delayed downregulation of exogenously induced SSEA-1 following doxycycline withdrawal.

Taken together, these results indicate that any iPSC-like cells generated in our protocol are a minor by-product and most probably do not contribute to cardiomyocyte formation. The observation that JAK–STAT pathway inhibition by JI1 can shift the balance considerably in favour of cardiomyogenesis suggests that the relationship between iPSC and cardiomyocyte formation is zero-sum in nature, that is, that they represent mutually exclusive outcomes.

DISCUSSION

We have shown that brief reactivation of reprogramming factors in embryonic and adult fibroblasts can be used to rapidly generate contracting cardiomyocytes—almost certainly without going through a pluripotent intermediate. Compared with transdifferentiation by overexpression of lineage-specific factor, our protocol is nearly three times as fast. The first spontaneous contractions begin after 11 days versus 4–5 weeks[1]. Moreover, the efficiency in terms of cTnT$^+$ cardiomyocyte yield is several fold higher; we obtained approximately 1.2 cTnT$^+$ cells for each fibroblast plated (versus an estimated maximum of 0.2; ref. 1). This latter feat is most likely made possible by the generation of mitotically active precursor cells akin to multipotent *Isl1*$^+$cardiovascular progenitors (MICPs[12]), as suggested by gene expression data. It is tempting to speculate that these intermediate cells, if successfully isolated and stabilized in culture, could eventually become an expandable and renewable source for not just cardiomyocytes, but many other terminally differentiated cardiovascular cells as well.

The process we have discovered bears a striking resemblance to regeneration blastema formation in zebrafish and frogs, during which the stage is set for transdifferentiation by transient low-level expression of pluripotency factors (especially Oct4 and Sox2) rather than a re-establishment of pluripotency[28]. Although this suggests that the two processes could share a common mechanism, confirmation of this hypothesis will require a more detailed understanding of the molecular underpinnings of reprogramming and its various intermediate stages[4]. The key role here for reprogramming factors is probably the induction of a developmentally more naïve, open-chromatin state marked by high epigenetic instability[29–31]. Highly unstable intermediate populations may give rise to a multitude of cell types as they rapidly 'relax' back into epigenetically more stable states, among which pluripotency is one of many possible outcomes (Fig. 6). This model underscores the unique versatility and potential of our transdifferentiation scheme when compared with lineage-specific transcription factor ovexpression, namely the likelihood that precursors and/or fully differentiated cell types from many different lineages could be derived simply by using different inductive signals.

For the generation of autologous tissue, transdifferentiation offers a potentially very attractive alternative to the rather circuitous iPSC methodology. To fulfil this role, our protocol will need to be improved on in the following ways: first, the most suitable method for transgene expression without permanent genetic modification[32,33] needs to be determined. A distinct advantage of our protocol in this regard is that the requirement for factor overexpression is only transient, which should make finding functional substitute(s) easier. Next, transplant studies need to be conducted to ensure that the cardiomyocytes have the required functional longevity and that no cells capable of teratoma formation are being generated. Finally, the overexpression of lineage-specific transcription factors[34] may greatly improve yield and efficiency, especially if properly regulated temporally . Increasing protocol effectiveness would be critical for adaptation to the human system and eventual therapeutic use. □

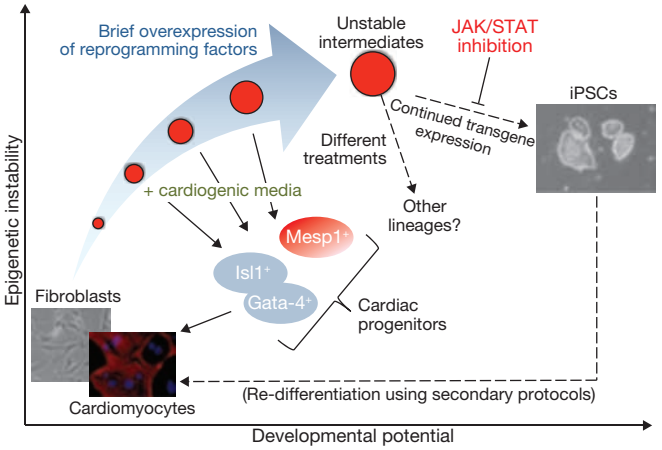

Figure 6 A model for direct reprogramming to alternative fates. A brief burst of reprogramming factor overexpression leads to the formation of various highly transient and epigenetically unstable (that is, less restricted, more naïve) intermediates. These cells (shown as red circles) then spontaneously 'relax' back into more stable state(s). Owing to the very complex nature of complete dedifferentiation, the generation of iPSCs is not a likely outcome of this process, and requires continued overexpression of exogenous pluripotency factors. Conversely, a direct switch to multipotent lineage progenitors—or even terminally differentiated cells—represents the path of least resistance, especially under culture conditions that allow and/or promote their genesis and proliferation, and simultaneously inhibit establishment of pluripotency (JAK/STAT inhibition).

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturecellbiology/

Note: Supplementary Information is available on the Nature Cell Biology website

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AUTHOR CONTRIBUTIONS

J.A.E. and S.D. conceived the project and wrote the manuscript. J.A.E. designed and carried out experiments. S.H., J.K., H.Z., K.O., G.W. and J.C. provided materials and assisted with experiments.

COMPETING FINANCIAL INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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METHODS

Cell culture and media. All MEFs (129S2/SvPasCrlf) were derived according to WiCell protocols (https://www.wicell.org/index.php?option=com_docman&task=doc_download&gid=687). TTFs were a gift from H. Schöler (Max Planck Institute for Biomolecular Medicine, Germany). Secondary MEFs were derived as previously described[21]. All results were initially obtained and confirmed with the Nebulette–LacZ MEFs. To ensure reproducibility and minimize inter-experimental variation, reported results almost exclusively derive from experiments using secondary doxycycline-inducible MEFs for the optimum induction period. MEFs were initially passaged on gelatin-coated (0.1%) tissue-culture dishes for 2 h at 37 °C in DMEM supplemented with 10% FBS, 2 mM Glutamax and 0.1 mM NEAA (non-essential amino acids; all components from Invitrogen). Before viral transduction, cells were seeded onto Matrigel (BD Biosciences) or Geltrex (Invitrogen)-coated plates (1:40, overnight at 4 °C) at 3.5×10^4 cells per well of a six-well plate (7×10^4 cells for TTFs) in the same media. After the addition of virus (12–24 h), cells were washed with PBS and switched to reprogramming media: knockout DMEM with 1–15% knockout serum replacement, 1–15% ES cell-qualified FBS, 1% Glutamax, 1% nonessential amino acids, 0.1 mM β-mercaptoethanol and 1% ESC-qualified nucleosides (all components from Invitrogen except nucleosides, which were from Millipore). When required for a control experiment, LIF (Millipore) was also added at 10^3 units ml^{-1}. At the indicated times, cells were again washed with PBS and switched to CDM: RPMI-1640 supplemented with 0.5 × N2, 1 × B27 (without vitamin A), 0.05% BSA (bovine serum albumin) fraction V, 0.5% Glutamax and 0.1 mM β-mercaptoethanol (all components from Invitrogen). BMP4 (Stemgent) and/or JI1 (EMD) were added at multiple timepoints for different durations. Fresh media was added at least once every 48 h throughout all experiments. For the inducible reprogramming of secondary MEFs, 2 μg ml^{-1} doxycycline was used, with the exception of 4-day induction experiments, where the first 2 days of treatment were done at 4 μg ml^{-1}.

Retroviral packaging and transduction. Retroviruses encoding *Oct4*, *Sox2*, *Klf4* and *c-Myc* were individually packaged in PLAT-E cells using pMXs-based vectors (Addgene) and infections were carried out as previously described[35].

LacZ assays. Nebulette–LacZ expression was detected *in situ* using a β-galactosidase staining kit (Stratagene).

Immunocytochemistry and fluorescence microscopy. Cells were fixed in 4% paraformaldehyde (Sigma-Aldrich) for 15 min, washed three times with PBS, and incubated in PBS containing 0.3% Triton X-100 (Sigma-Aldrich) and 5% donkey serum (Jackson ImmunoResearch) for 1 h at room temperature. All primary antibody incubations were done overnight at 4 °C: cardiac troponin T (CT3, Developmental Studies Hybridoma Bank; 1:1,000); Flk1 (AF-644, R&D Systems; 1:100); Gata-4 (sc-25310, Santa Cruz Biotechnology; 1:200); Myosin heavy chain (MF20; 1:200); Nkx2.5 (sc-8697, Santa Cruz Biotechnology; 1:200); Mlc2v (aka Myl7; sc-34488, Santa Cruz Biotechnology; 1:50); Connexin-43 (610061, BD Biosciences; 1:100) and α-actinin (NBP1-40428, Novus; 1:100). Following three PBS washes, cells were incubated with the appropriate Alexa Fluor-conjugated secondary antibodies (Invitrogen) for 1 h at room temperature and nuclei were stained with DAPI (4,6-diamidino-2-phenylindole; Sigma-Aldrich). Images were acquired using a Zeiss AX10 microscope equipped with an Axiocam HRm camera and processed using Axiovision 4.7.1 software. Colony-tracking experiments and whole-well microscopic imaging were done on a Pathway 435 system (BD Biosciences). Cells expressing GFP were imaged using the FITC (fluorescein isothiocyanate) channel.

Quantitative PCR. For each sample or timepoint, total RNA from two wells of a six-well plate was extracted using the RNeasy Plus Mini Kit with Qiashredder columns (Qiagen), after which 1 μg of RNA was reverse transcribed with the iScript cDNA Synthesis Kit (Bio-Rad). All quantitative PCR reactions were done in duplicate on a CFX96 system with iQ SYBR Green Supermix (Bio-Rad), using one twentieth of a cDNA reaction per replicate. Expression data were normalized relative to ribosomal protein L7 transcript levels. Each set of reactions was repeated using cDNA from at least three independent experiments. Primer sequences are in Supplementary Table S1.

Chromatin immunoprecipitation quantitative PCR (ChIP–qPCR). ChIP was performed using a commercially available Magna ChIP G kit (Millipore). Briefly, histones and DNA were cross-linked by incubation in 1% formaldehyde. The chromatin was then sonicated to an average DNA fragment length of 200–500 bp. Equal amounts of soluble chromatin were incubated with and without anti-normal rabbit IgG, anti-trimethyl-histone H3 lysine 4 (#17-614, Millipore; 3 μl per reaction) or anti-trimethyl-histone H3 Lys 27 (#17-622, Millipore; 4 μg per reaction) that had been pre-incubated with secondary antibodies conjugated to magnetic beads. After overnight antibody incubations, DNA–histone cross-linking was reversed and DNA fragments were purified. DNA fragments obtained without antibody were used as the input controls, whereas DNA fragments obtained with normal rabbit IgG were applied as negative controls. Of the resulting DNA solution, 1 μl from each condition was subject to real-time PCR reaction. Primer sequences used are in Supplementary Table S2.

Flow cytometry. Adherent cells on plates were washed with PBS and dissociated by treatment with trypsin (Invitrogen), Accutase (Innovative Cell Technologies) or collagenase II (Invitrogen) for 10–45 min at 37 °C. Cells were washed twice with ice-cold staining buffer (HBSS, Invitrogen; with 10mM HEPES, Sigma; 2% FBS, Invitrogen and 0.1% azide, Sigma) and put through single-cell strainers (BD Biosciences) both times. Incubation with antibodies was done at 4 °C for 30 min at the volume and concentrations suggested by the manufacturer. Cells were washed three times with staining buffer and fixed in high-grade 4% paraformaldehyde (Electron Microscopy Sciences) for 15 min at 4 °C. Cells were washed again with staining buffer and counted using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences). Final analysis was later done with FlowJo software (Tree Star). The following antibodies were used: APC (allophycocyanin)-conjugated anti-Flk1 (560070, BD Biosciences), PE (phycoerythrin)-conjugated anti-SSEA1 (560142, BD Biosciences), Alexa647-conjugated anti-Nanog (560279, BD Biosciences), anti-cTnT (ab10214, Abcam) and anti-Nkx2.5 (sc-8697X, Santa Cruz Biotechnology). Unlabelled antibodies were conjugated to PE or APC using the appropriate LYNX rapid conjugation kit (AbD Serotec). All experiments included the appropriate isotype controls, conjugated using the same kits as above when necessary.

Calcium imaging and testing of chronotropic agents. Cells were loaded with Fluo-4-AM (5 μM, 30min, 37 °C; F14201, Invitrogen), and imaged with an Olympus Fluoview 1000 inverted confocal microscope with a ×40 oil immersion lens (numerical aperture 1.3). Line-scan imaging mode was used to measure spontaneous Ca^{2+} transients at 37 °C. Image processing and data analysis were performed as previously described[36]. For drug testing, all measurements were repeated 10 min after the addition of isoproterenol (1 μM; Sigma-Aldrich) or Carbachol (10 μM; Sigma-Aldrich).

Electrophysiology. Contracting cell clusters were dissociated by treating the cells at 37 °C for 30–45 min using 1 mg ml^{-1} Collagenase type II in RPMI-1640 media supplemented with 10 mM HEPES and 5 mM sodium pyruvate (all reagents from Invitrogen). Following repeated pipetting to ensure complete dissociation, cells were allowed to adhere to fibronectin-coated Biocoat coverslips (BD Biosciences) overnight in Cor.At media (Axiogenesis, distributed by Lonza). Single-cell patch clamp recordings were performed using an Axopatch 200B amplifier and pClamp 10.0 software (HEKA Elektronik), as described previously[36]. Spontaneous action potentials were recorded under whole-cell current-clamp conditions with a patch pipette resistance of 4–6 MΩ. Standard external solution contained: 150 mM NaCl, 5.0 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10 mM HEPES and 10 mM glucose (pH 7.4, adjusted with NaOH). Intracellular pipette solution contained (in mM): 150 mM KCl, 5.0 mM NaCl, 1.0 mM MgCl2, 2.0 mM EGTA, 1.0 mM MgATP, 10 mM HEPES (pH 7.2 adjusted with KOH). All experiments were performed at room temperature (20–22 °C).

Videos. All videos were recorded using a Canon A650IS digital camera custom-mounted to a Zeiss Axiovert 40C microscope.

Statistics. *P* values for the purpose of group comparisons were calculated using Student's *t* test. Differences where $P < 0.05$ were regarded as significant.

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Supplementary Movies:

Movie 1 An example of a large, forcefully contracting colony .
Movie 2 An approximately one-minute clip showing the camera moving around a representative well, documenting the high percentage of beating colonies.
Movie 3 Video showing contraction of cTnT^{+} colonies depicted in Fig. 2c.
Movie 4 An example of very rapid contraction, 130 BPM.
Movie 5 Video accompanying Fig. 1e.
Movie 6 Contraction of the encircled region in Fig. 4c (filmed on day 12).