Differentiated adult cardiomyocytes (CMs) lack significant regenerative potential, which is one reason why degenerative heart diseases are the leading cause of death in the western world. For future cardiac repair, stem cell-based therapeutic strategies may become alternatives to donor heart transplantation. The principle of reprogramming adult terminally differentiated cells (iPSC) had a major impact on stem cell biology. One can now generate autologous pluripotent cells that highly resemble embryonic stem cells (ESC) and that are ethically inoffensive as opposed to human ESC. Yet, due to genetic and epigenetic aberrations arising during the full reprogramming process, it is questionable whether iPSC will enter the clinic in the near future. Therefore, the recent achievement of directly reprogramming fibroblasts into cardiomyocytes via a milder approach, thereby avoiding an initial pluripotent state, may become of great importance. In addition, various clinical scenarios will depend on the availability of specific cardiac cellular subtypes, for which a first step was achieved via our own programming approach to achieve cardiovascular cell subtypes. In this review, we discuss recent progress in the cardiovascular stem cell field addressing the above mentioned aspects.

Cardiovascular diseases represent the leading cause of mortality in the industrialized countries. In Germany, 2010 saw a lethal rate of 41.1% due to chronic cardiomyopathy, myocardial infarction, or congestive heart failure, and the numbers are expected to further increase in the near future. Loss of working myocardium is inevitably succeeded by negative myocardial remodeling and chamber dilatation with a decrease in ejection fraction resulting in heart failure. As fewer patients die immediately from acute myocardial infarction and population is aging, congestive heart failure is taking over as a major health issue. Nowadays, one of the main reasons underlying the high mortality of cardiovascular diseases is the limitations of treatment options resulting in a 1-yr mortality exceeding 50% in the case of end-stage heart failure (59).

A major advance in the stem cell field was the derivation and cultivation of murine embryonic stem cells (ESC) early in the 1980s, the first nontumor-derived immortal, self renewing, and at the same time pluripotent cells with the potential to develop into any adult murine cell type (26, 77). It took almost 20 years until the next major breakthrough in the field: Thompson and coworkers isolated and cultured human ESC (hESC) (117), which apparently became an invaluable tool to study development and stem cell biology. However, it quickly became clear that their translation into the clinics would become problematic: after nearly 15 years of hESC research, currently merely one FDA-approved patient trial relying on pluripotent hESCs actually exists. This trial, which addresses spinal cord injury, was initiated by the company Geron (http://www.geron.com) (32a). Yet,
surprisingly during the preparation of this article, Geron announced on their website that the study will be closed to further enrolment, although no serious adverse events hitherto are existing.

The earliest approaches relying on pluripotent stem cells for heart regeneration were performed by direct injection of undifferentiated ESC into the infarcted heart (82, 107, 108). Yet, this technique is not an option for the clinic: various reports show teratoma formation in the wall of the heart caused by the transplanted undifferentiated cells (4, 93, 110). One possible strategy to eliminate this teratogenicity may be a pre-differentiation of ESC into lineage-restricted cells before transplantation (13). Furthermore, the engrafted cells need to establish a functional and electromechanically coupled tissue to avoid arrhythmic events (80, 81). Overcoming the potential oncogenic and teratogenic hazards inherent in pluripotent stem cells continues to be an intense research field—our own forward programming strategies addressing this set of problems will be discussed further below (17, 20).

However, ESC in themselves are problematic, for ethical reasons, and in addition due to the immunological concerns: nonhaplotype-matched allogeneic cells bear the risk of immune rejection. Initially, belief existed that ESC are immune privileged and therefore will not be prone to immune rejection. Yet, for haplotype-unmatched ESC, the opposite was the case (93). Furthermore, it was found that ESC upregulate histocompatibility complex type I and II antigens while progressively differentiating (109, 111). As a result, at long term, immunosuppression may become mandatory.

Therefore, research has long aimed to overcome immunology problems as well as ethical concerns accompanying ESC by creating autologous pluripotent stem cells via reprogramming terminally differentiated somatic cells. So-called somatic cell nuclear transfer (SCNT), initially performed in amphibians, finally led to the first SCNT-derived cloned mammalian specimen, the now famous sheep Dolly (reviewed in Ref. 36).

After the cloning of Dolly, there was great hope that the SCNT technology would be the future way for generating autologous pluripotent cells from patients via “therapeutic cloning” (43). Yet, obstacles are its low efficiency and incomplete reprogramming, leading to early manifestation of various degenerative old-age diseases as well as ethical concerns due to the need of human blastocysts. Therefore, the perspectives to take advantage of this approach for the generation of human pluripotent stem cells nowadays appear speculative, although success has been reported for SCNT in many other species (12). However, the ultimate generation of iPSC (induced pluripotent stem cells; “reprogrammed cells”) has greatly benefitted from the lessons learned in this pilot work.

The first creation of murine and human iPSC 5 years ago was seminal: Yamanaka and coworkers described a bona fide pluripotent cell type, highly resembling ESCs, yet unaffected with ethical cloaks, potentially autologous, and in addition easily produced and propagated (113, 114). Subsequently, reprogramming of human somatic cells was reproduced by various groups leading to iPSC that are very similar to human ESC (73, 96, 130, 132) (FIGURE 1).

The rapid progress in the field has caused a dramatic increase in reprogramming reports describing improvements such as reprogramming based on piggyBAC models (128), integration-independent viruses (42), and transfection of recombinant proteins (135), a synthetic mRNA (124), and combinations of small molecules with reprogramming factors (134). All of these novel approaches are increasing the confidence to ultimately create iPSC devoid of any genetic alteration or integration of extrinsic DNA (reviews in Refs. 23, 73). However, severe remaining caveats persist (see below), and a more realistic perspective for the near future are ex vivo autologous models in reverse translational medicine to bring patient-specific diseases into the tissue culture dish (85). Thus iPSC definitely are an outstanding tool for human disease research.
For use in the clinic, iPSC would have to meet the most severe safety and efficiency criteria; yet, for a long time, concerns over the oncogeneity of pluripotent cells have existed—these are particularly increasing for iPSC now (73). First-generation iPSC were based on lentiviral overexpression of the four factors including the oncogene c-Myc. In addition, the integrating lentiviral system per se can be oncogenic due to its potential for insertional mutations (94, 113). As a consequence, mouse chimeras partially derived from lentivirally reprogrammed iPSC had a higher tendency to form tumors (7). As described, a number of approaches were successful in generating iPSC devoid of genomic manipulations or DNA integration (42, 135); the most promising may rely on future administration of small molecules only to reprogram somatic cells. In this regard, many laboratories have successively reduced the number of reprogramming factors, yet Oct3/4 along with chemical compounds is still required (134). On the other hand, reprogramming based on miRNA transfection was successful without Oct3/4 (1, 84).

A further issue to be clarified is consequences of reprogramming with respect to functionality and, in particular, to what extent iPSC differ from native pluripotent cells such as ESC. Evidence is increasing that severe differences between ESC and iPSC do exist and that reprogramming so far does not fully reset the epigenetic memory. Therefore, human iPSC bear important epigenetic discrepancies compared with human ESC leading to inconsistent differentiation potential characteristics (3, 34, 45, 52, 56, 70). By now there is no direct hint that the tumorigenic potential of iPSC-derived refractory cells is based on epigenetic memory, yet the crucial role of epigenetics in cancer implies a possible important role (83). However, gene expression patterns also are divergent, and iPSC may actually stand for a new class of pluripotent stem cells (15, 22).

Apart from epigenetic alterations of human iPSC, reprogramming was accompanied by genetic deletions of tumor-suppressor genes, and in addition further culture was associated with duplications of oncogenes and, even during differentiation, duplications arose (65). Overall, careful epigenetic screening based on standardized analyses to understand the results of reprogramming would have to be developed before use in large-scale clinical trials, making iPSC-based therapies unjustifiable, at least in the near future (73, 96).

**iPSC in the Cardiovascular Field**

In the cardiovascular field, the description of iPSC has been very exciting. Various groups have shown that mouse iPSC use the same developmental pathways as ESC and have the potential to yield all three cardiovascular cell types typically found in the heart, i.e., cardiomyocytes, endothelial cells, smooth muscle cells (79, 89, 103). Subsequently, this was also transferred to human iPSC (30, 133, 137). Cardiomyocytes were even derived from iPSC generated without the reprogramming factor c-Myc (78). Not only was the oncogene c-Myc dispensable for reprogramming of fibroblasts but Myc-independent iPSC lines were even more prone to efficient cardiogenesis and had improved ability to integrate into host myocardium compared with c-Myc-reprogrammed iPSC (78). Likewise, injection of conventionally iPSC-derived cardiomyocytes ameliorated left ventricular performance post-MI in animal models (90). Here, iPSC restored contractile properties, wall thickness, as well as electric parameters via in situ reformation of cardiomyocytic, smooth muscle, and endothelial tissue. These animal studies are definitely promising, yet transference to patients would depend either on clonally derived cardiovascular lineage committed cells or highly efficient purification strategies to avoid any hazard of terATOMA formation. Furthermore, greatly variant cardiovascular differentiation potentials of diverse iPSC lines have been reported even exceeding a factor of one hundred-fold (133).

For murine ESC, a number of strategies to improve cardiac differentiation have long been described: these include the addition of retinoic acid or ascorbic acid to the cultures (115, 127) as well as exposure to a low-frequency magnetic field (121). Likewise, under appropriate culture conditions, hES cells and hiPS cells have been shown to yield high numbers of cardiomyocytes. Here, an important step forward was the use of chemically defined medium to overcome the inconsistencies existing between different serum lots. This approach was combined with specific inducers to direct differentiation toward desired cell types. Growth factors and small molecules described are TGF-β family members, BMP and activin, Wnt and FGF family members, all of which are known to be crucial for cardiac development. In addition, VEGF, a p38 MAPK inhibitor, SCF, the Wnt-inhibitor Dkk1, and ascorbic acid have been shown to be useful (27, 32, 35, 60, 88, 131). In addition, sulfonyl-hydrazone, a GSK3 inhibitor, and dorsomorphin have also been reported to enhance cardiac differentiation (39, 98, 99). Further improvements include coculturing with murine visceral endoderm-like cells (END-2) (87). In addition, combining the techniques of directed differentiation with selection for endogenous surface markers or transgenic markers such as EGFP under control of specific promoters has allowed yields of stem cell-derived cardiomyocytes with up to 95% purity (Ref. 86; review in Ref. 54).
Future challenges now need to focus on the maturation and derivation of specific cardiomyocytic subtypes, which will be crucial for their clinical use in the cardiovascular field.

In addition, development of many organs depends on mechanical inputs during embryogenesis (review in Ref. 76). This also includes the heart (28, 44, 123). It notably affects heart looping independently of the initial distribution of the transcription factor Pitx2 (66, 67, 71). Conversely, loss of myosin-based tension early during development impairs heart morphogenesis (125). Molecules involved in the underlying mechanisms also include the cytoskeletal protein titin (62, 97) and lamins (64). Correspondingly, mutations in various lamin genes as well as other nuclear structural factors are underlying diverse human congenital cardiovascular diseases, including dilated cardiomyopathy and Hutchinson-Gilford progeria syndrome (63).

Indeed, it has recently been shown that differentiation of hESC and human iPSC exposed to uniaxial mechanical stress yield twofold increased cardiomyocytes with improved matrix fiber alignment and enhanced myofibrillogenesis as well as sarcomeric banding. In addition, cyclic stress administration increased cardiomyocyte hypertrophy and proliferation. Addition of endothelial cells further enhanced cardiomyocyte proliferation. This indicates that mechanical load as well as vascular cell coculture also influence stem cell derived in vitro generated cardiomyocytes with respect to their proliferation and phenotypes (119). Yet, likewise here, the authors did not assay for cardiomyocytic subtype distribution. Therefore, although the differentiation methods have definitely been greatly improved, the cardiomyocytes derived are electrophysiologically and phenotypically very heterogeneous and often more similar to those of a fetus (137). Our own work on subtype-specific forward programming strategies, which we will describe below, was developed against this background (17, 20).

**Direct Cardiovascular Programming**

Clearly, the original work by Yamanaka’s group still represents a groundbreaking finding since it initially defined the quartet of Oct4, Sox2, Klf4, and Myc as the minimal required classical reprogramming factors. However, scientists recently aimed at finding combinations of factors to drive lineage-specific reprogramming for a number of reasons: first, as discussed above, full reprogramming to the pluripotent state is accompanied by severe genetic and epigenetic aberrations. There are first indications in the cardiac field that a bona fide milder direct programming approach may have less dramatic unwanted side-effects at least on the epigenetic level: H3K4 and H3K27 trimethylation signatures at promoters of cardiac structural genes resembled those of native cardiomyocytes (25, 48). Second, direct reprogramming may increase the speed and simplicity of differentiation protocols since the time necessary for generation, expansion, characterization, and differentiation of pluripotent cells iPSC could possibly be avoided.

In this regard, seminal work, which was long neglected in the field of regenerative medicine, dates back already to the late 80s and early 90s: Weintraub et al. showed that MyoD as a sole specific factor transforms fibroblast cells as well as other cell types into skeletal muscle cells. The underlying process is independent of normal developmental pathways (21, 116, 126). Another intriguing paradigm is the fact that Pax6 as a master regulator is sufficient to induce ectopic formation of eyes in *Drosophila* legs (38). More recently, we have shown that the cardiovascular key regulator MesP1 (58, 100–102) of human origin is sufficient to induce ectopic cardiac tissue in frog tadpoles in vivo (17). Briefly thereafter, Zhou et al. demonstrated conversion of exocrine cells into endocrine β-cells in the pancreas of mice (136).

The year 2010 saw two studies showing direct in vitro lineage programming achieving neurons (122) and blood precursor cells (112). In addition, and most crucial for this review, a third paper demonstrated the conversion of murine fibroblasts toward cardiomyocytes (48) (FIGURE 2A). This group started off with a cocktail of 14 lentiviruses encoding transcriptional regulators crucial for cardiogenesis. Initially, the fibroblasts for factor screening were derived from mice bearing marker genes under control of the cardiac-specific alpha myosin heavy chain (αMHC) promoter. From the initial 14 factors, Mef2c, Gata4, and Tbx5 were minimally required to induce the transgenic reporter as well as endogenous cardiac troponin T. The effect was further increased by addition of MesP1. The resulting cardiac cells [induced cardiomyocytes (iCM)] could be cultivated for over 1 wk in vitro, even after shutting off the three programming factors. Lineage-mapping using inducible reporters (MesP-Cre/R26R-YFP; Isl1-Cre/R26R-YFP) implied direct reprogramming of the fibroblasts to iCM, circumventing an intermediate, early mesodermal stage. When injected into the myocardium of live mice at an early stage of reprogramming, the fibroblasts were able to differentiate in situ into cardiomyocyte-like cells positive for the myofilament marker α-actinin. Caveats of the study are the very low numbers of iCMs found in vivo; in addition, these donor cells were unlikely coupled electrically to cardiomyocytes in the recipient heart. Furthermore, spontaneous contractions and electrical activity in vitro appeared only signifi-
Significantly when the iCM were derived from cardiac fibroblasts; therefore, a contamination with cardiomyocytes from the donor mice cannot be fully excluded. Electrophysiologically, the authors found the cells to resemble adult ventricular cardiomyocytes based on intracellular electrical recordings. However, it remains unclear whether their protocol actually promotes ventricular differentiation or whether cardiomyocytic subtypes appear stochastically because Ieda et al. analyzed only four cells (48). It is known, e.g., from ES cell work, that the ventricular subtype is by far the most frequently represented via spontaneous differentiation (17, 41, 74, 75).

In addition to the work by Ieda et al., a related paper appeared likewise describing a rapid way to obtain cardiomyocytes directly from fibroblasts (25). These authors transduced murine embryonic fibroblasts with viruses bearing only three of the classical reprogramming factors: Oct4, Sox2 and Klf4 (FIGURE 2B). Subsequently, various culture conditions were assayed to come up with a medium, in which the transduced cells revealed spontaneously beating foci after ≈2 wk. After further optimizing this protocol, at day 18, nearly 40% of the cells were expressing cardiac troponinT, a sarcomeric marker for mature cardiomyocytes. The resulting cardiomyocytes revealed normal physiological characteristics, such as calcium transients and response to isoproterenol and carbachol. The protocol was also transferable to postnatal fibroblasts from tail tips, yet c-Myc had to be included in this case and the programming efficiency was lower (FIGURE 2B). The authors claim that the cardiomyocytes did not arise from transiently pluripotent stem cells but rather that direct conversion of fibroblasts appeared based on a number of classical ESC assays. However, maybe the most important indication was the group’s finding that activity of reprogramming factors for only 4 days sufficed to induce cardiomyogenesis. Normally, the generation of fully reprogrammed iPS lines takes at least 12 days, but overexpression of the factors for more than just 9 days did not yield any contracting cells at all, even under their cardio- genic conditions. Correspondingly, the authors did not find expression of the pluripotency marker Nanog at any time point during their direct cardiogenic reprogramming procedure (25). According to this group, their method exclusively generates cardiomyocytes of the atrial subtype. Yet, similar to Ieda et al. this group subjected only three cells to patch-clamp analyses, and it has to be seen whether the finding is actually specific for their protocol. Overall, these two publications are important first steps, and the direct programming approaches need to be transferred to the human system now.

In this regard, it is noteworthy that first attempts have been reported at a Keystone Symposium in February 2011: the group of Bob Schwartz transduced human dermal fibroblasts in a sequential manner with the transcription factor ETS-2 and the cardiovascular key inducer MesP1 (17, 58, 100–102), thereby for the first time converting human cells into cardiomyocytes (50) (FIGURE 2C).

**Specific Programming Toward Cardiovascular Subtypes**

It remains to be seen whether full reprogramming or direct programming will be the strategy of choice; in any case, future applications require a

![FIGURE 2. Current status of direct in vitro lineage programming toward cardiomyocytes](http://physiologyonline.physiology.org/)

A: from 14 initially tested molecules, the early cardiac transcription factors Mef2c, Gata4, and Tbx5 were minimally required to directly reprogram murine cardiac as well as tail-tip fibroblasts into cardiomyocytes. Additional administration of MesP1 enhanced the efficiency of the protocol (48). B: three of the classical reprogramming factors (Oct4, Sox2, and Klf4) are sufficient to partially reprogram murine embryonic fibroblasts so that optimized culture conditions allow transdifferentiation into cardiomyocytes bearing normal physiological characteristics. Yet, for transference to postnatal tail-tip fibroblasts, c-Myc had to be included (25). C: transduction of human dermal fibroblasts initially with the transcription factor ETS-2 and subsequently with the cardiovascular key inducer MesP1 was reported to convert human cells into cardiomyocytes at a recent Keystone meeting (50).
better understanding of cardiovascular subtype-specific differentiation of the cells taking into account that stem cell-based cardiac repair will depend on high yields of various subtypes for transplantation or tissue engineering as well as for very innovative goals such as reseeding entire de-cellularized hearts (95). The latter will likely depend on the earliest cardiovascular precursors to reconstitute the whole myocardium plus the vasculature, whereas the former may rather require specific ventricular cells, e.g., to repair a typical infarction. In addition, nodal cells are eagerly sought after to ultimately obtain biological pacemakers for curative treatment of the “sick sinus syndrome.” Furthermore, the specific generation of these cardiovascular cellular subtypes is a major goal not only for various clinical applications but also to transfer specific diseases to in vitro models. In general, the cardiac action potential consists of five distinct phases: a rapid upstroke (phase 0), a subsequent transient repolarization (phase 1), a slowly decaying plateau (phase 2), a rapid repolarization (phase 3), and a resting state (phase 4). One has to take into account that the various cardiomyocytic subtypes express differential sets of genes (transcription factors, structural proteins, ion channels, etc.). A number of genes encoding ion channels underlying the different action potential characteristics, i.e., between atrial and ventricular myocytes, have been identified; these comprise sodium channels (i.e., Nav1.5), potassium channels (i.e., Kv1.4, Kv1.5, Kv1.7, Kv3.4, Kv4.2, and Kv4.3), and L-type calcium channels. The so-called funny current (I_f), mediated by hyperpolarization-activated, cyclic nucleotide-gated (HCN) channels, is almost absent in atrial as well as ventricular cardiomyocytes and highly characteristic for nodal and to a lesser extent for early/intermediate cells (review in Ref. 92).

In a first attempt of “forward programming” pluripotent stem cells toward cardiovascular fates, we have recently characterized the early cardiovascular transcriptional regulator MesP1, which had been described as the earliest molecular sign and crucial for cardiogenesis earlier (58, 100–102). Our work revealed that MesP1 is even the first factor sufficient to induce ectopic cardiovascularaglobuslation when overexpressed in vivo (17). Transference to ES cells resulted in highly increased beating foci within the cultures and simultaneously enhanced the formation of endothelial cells (17). This finding was also independently obtained by others (8, 9, 68). Our patch-clamping data obtained from isolated beating cardiomyocytes revealed all subtypes of cardiac ES cell differentiation, but with a high proportion of multipotent early/intermediate cell types (74, 75) compared with controls (17). Correct timing and dosage of wnt signaling during early cardiovascular induction had long been postulated (review in Refs. 33, 120). Indeed, we found an important function of MesP1 within a gene regulatory pathway inducing Dkk-1-driven suppression of canonical Wnt-signaling. Thereby, we identified the Dkk-1 promoter as a direct target, which is activated by MesP1 (17). We have also shown that MesP1-driven cardiomyogenesis depends on initial mesoderm induction, as evident from Flk-1 (VEGFR-2, KDR) expression analyses, the earliest surface marker for the lateral mesoderm (51). Briefly thereafter, it was shown that MesP1 in addition directly induces the early cardiovascular key transcriptional machinery with factors such as Hand2, Myocardin, Nkx2-5, and Gata4. Simultaneously MesP1 represses genes inducing pluripotency, early mesoderm, and endoderm (9). Others have reported that MesP1 expression promotes changes associated with epithelial-mesenchymal transition (EMT) and induction of Snail consistent with a role in gastrulation. Thereby, MesP1 expression restricted potential cell fates derived from ESCs to mesoderm progenitors with cardiovascular, but not hematopoietic, potential (68). Therefore, on the mechanistic level, MesP1 is acting in parallel in non-cell-autonomous as well as in cell-autonomous fashions. For a long time, upstream regulators of MesP1 were unknown. However, recently, the early mesodermal T-Box transcription factors Brachyury(T) and Eomesodermin have been shown to be direct inducers of MesP1 (16, 19). Subsequent repression of Brachyury(T) by MesP1 itself (see above), therefore, represents a negative feedback loop, most likely enabling further progression of the cardiovascular differentiation program.

Encouraged by our findings, we then chose Nkx2-5/Csx as a candidate for directed “forward programming” of ES cells toward ventricular cardiomyocytes. In the embryo, this transcriptional regulator plays an important role in specification and maturation of ventricular cardiomyocytes (40, 57, 72). Its high evolutionary conservation is evident from the fact that homologs of Nkx2-5/Csx exist in various vertebrate species from frogs to humans (14, 69, 104, 106, 118) as well as in invertebrates: Drosophila tinman, the fly homolog of Nkx2-5/Csx is crucial for cell fate specification of the dorsal vessel, which is regarded as the equivalent of the vertebrate heart (2, 6). In vertebrates, Nkx2-5/Csx plays a dominant role in lineage specification and maturation of ventricular cardiomyocytes as evident from murine Nkx2-5/Csx knockout models (72).

The mechanism underlying Nkx2.5 action has been ascribed to transcriptional regulation of target genes dependent on specific binding partners,
FIGURE 3. Programming of ES cells to enrich electrophysiologically functional cardiomyocyte subtypes

Single-cell patch-clamp and Funny Channel current ($I_f$) density analyses at −110-mV activation showed all cell types described for cardiomyocytic ES cell development in MesP1 and Nkx2.5 programmed cells as well as in control cells, yet at highly distinct proportions. More than 79% (19 of 24) of the analyzed Nkx2.5 programmed cells were identified as near-mature differentiated ventricular-like cardiomyocytes, whereas in the MesP1 programmed cells only 25% (7 of 28) and in the control-cell population 45% (13 of 29) of ventricular cells were present. In the MesP1 cell population, the dominant cell type is represented by the early/intermediate type (“inter”; 57%; 16 of 28) compared with only 4% (1 of 24) among the Nkx2.5 cells and 24% among the controls (7 of 29). Notably, both Nkx2.5 as well as MesP1-based programming led to a strong decrease of nodal cells (“SA/AV”), whereas the content of atrial cells remained greatly unaffected. All analyses were performed at day 18 of differentiation. MDP, maximum diastolic potential; DDR, diastolic depolarization rate; AP, action potential.

<table>
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<th>Controls</th>
<th>Ventricular (n=13)</th>
<th>Atrial (n=3)</th>
<th>SA/AV (n=6)</th>
<th>Inter (n=7)</th>
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with which Nkx2.5 forms heterodimers. In particular, atrial natriuretic factor (ANF), which is critical for embryonic chamber formation, is transcriptionally induced by a Nkx2-5/GATA4 dimer (24) and inhibited by an Nkx2-5/Tbx-2 dimer (37). The NK-2 domain (also called the NK-2-SD domain) mediates dimer formation with other proteins (49, 53) and may have a role when NK-2 family members repress rather than activate gene expression (105). In addition, expression of the ventricular-specific homeobox gene Irx4 requires correct Nkx2-5/Csx expression (129). Strikingly, loss of Nkx2-5/Csx leads to formation of only a single atrial chamber during embryogenesis with complete absence of the ventricles (129). Therefore, we compared the effect of Nkx2-5/Csx-based stem cell programming with that of the early cardiovascular fate inducer MesP1 (20). Our hypothesis, based on the embryological background described above, was that Nkx2-5/Csx may specifically induce ventricular subtypes, whereas MesP1 may rather promote the formation of the earliest yet multipotent cardiovascular precursors.

Compared with control ES cells, an approximately fivefold increased appearance of beating foci was observed in MesP1 as well as Nkx2.5 programmed cells. However, in contrast to MesP1, no increase of the endothelial lineage within the cardiovascular mesoderm was induced via Nkx2.5. Electrophysiological parameters revealed highly different distributions of the various cell types in Nkx2.5 vs. MesP1 programmed cells: only ~4% of the former corresponded to early/intermediate cardiomyocytes, whereas in the MesP1-cell population almost 60% represented this type (FIGURE 3). Although various types of “early/intermediate type cells” have been reported (74), all of them have rapid and robust diastolic depolarisation rates (DDRs) and brief but distinct plateaus.

In addition, a small \( I_d \) in these intermediate type cells sets them apart from nodal cells, which have an at minimum three times higher \( I_d \) density, as well as from atrial- or ventricular-like cells, which basically do not show any \( I_d \). Contrary to these multipotent intermediate type cells, we found near-mature differentiated ventricular cell types in almost 80% of the Nkx2.5 programmed cells, whereas only 25% of the MesP1 programmed population corresponded to this type. In each case, the controls were ranking in the middle, with 24% of early/intermediate cells and 45% of ventricular-like cells (20) (FIGURE 3). The ventricular subtype is clearly distinguishable from the other differentiated cell types by various action potential parameters, such as a distinct plateau phase with at least one-third of total action potential (AP) duration, which is shorter in atrial as well as in intermediate cells and entirely absent in nodal cells. The ventricular-like cells also responded to isoproterenol, as expected, with a further prolongation of their plateau phase and a lowering of the AP rate (20).

Overall, our data confirm the molecular hierarchy for cardiovascular specification initiated via MesP1 with factors such as Nkx2.5 further downstream. Although they also show proof of principle for specific programming of stem cells toward cardiovascular subtypes, it becomes evident that a single factor unlikely will be sufficient to achieve pure cell populations for therapy or in vitro applications. Therefore, future work will require the characterization of additional factors to be used in combination to drive uncommitted cells toward one particular cardiac subtype. Eventually, this will have to be supplemented by genetic or non-genetic cell purification strategies as well as exposure to mechanically applied load (10, 18, 119) to set the basis for the availability of these urgently needed, highly specified cellular subtypes at large scale.

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References


106. Shiojima I, Komuro I, Mizuno T, Aikawa R, Aka-


108. Single OK, Lyons GE, Kamp TJ. Transplanted em-

109. Swijnenburg RJ, Tanaka M, Vogel H, Baker J,

110. Takahashi K, Yamanaka S. Induction of pluripo-

111. Thayer MJ, Tapscott SJ, Davis RL, Wright WE,

112. Tuohlo NL, Muskheili V, Razumova MV, Korte FS,

113. Tzahor E. Wnt-beta-catenin signaling and cardio-

114. Ueda M, Maioi M, Asara Y, Santoni D, Mesaric

115. Udrioli G, Scurati S, Gullans SR, Lee RT. Ascorbic acid en-


117. Thomson JA, Itskovitz-Eldor J, Shapiro SS, 

118. Tonissen KK, Drysdale TA, Lints TJ, Harvey RP,

119. Tsujimoto T, Negishi M, Shimizu Y, Nakamura

120. Tzahor E. Wnt-beta-catenin signaling and cardio-

121. Ventura C, Masioli M, Asara Y, Santoni D, Mesaric

122. Vierbuchen T, Ostermeier A, Pang ZP, Kokubu Y,

123. Voronov DA, Alford PW, Xu G, Taber LA. The

124. Warren L, Manos PD, Ahfeldt T, Li H, Lau

125. Weintraub H, Tapscott SJ, Davis RL, Thayer MJ,

126. Weintraub H, Benos P, Slawson S, Pigula FA,

127. Wobus AM, Kaomei G, Shan J, Wellner MC, Ro-

128. Woltjen K, Michael IP, Mohseni P, Desai R, Mil-

129. Yamagishi H, Yamagishi C, Nakagawa O, Harvey

130. Yamanaka S. Strategies and new developments

131. Yang L, Soo Dana MH, Adler ED, Roepeke TP, Katt-

132. Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-


135. Zhi L, Caspi O, Arbel G, Huber I, Gepstein A,


137. Zwi L, Caspi O, Arbel G, Huber I, Gepstein A,

138. Zuber M, Tidmore J, Gono A, Heilman M, 

139. Zacharias KL, Caspi O, Arbel G, Huber I, Gepstein A,

140. Zacharias KL, Caspi O, Arbel G, Huber I, Gepstein A,

141. Zacharias KL, Caspi O, Arbel G, Huber I, Gepstein A,

142. Zacharias KL, Caspi O, Arbel G, Huber I, Gepstein A,

143. Zacharias KL, Caspi O, Arbel G, Huber I, Gepstein A,