Cellular Biology

Lineage Tree for the Venous Pole of the Heart
Clonal Analysis Clarifies Controversial Genealogy Based on Genetic Tracing

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Rationale: Genetic tracing experiments and cell lineage analyses are complementary approaches that give information about the progenitor cells of a tissue. Approaches based on gene expression have led to conflicting views about the origin of the venous pole of the heart. Whereas the heart forms from 2 sources of progenitor cells, the first and second heart fields, genetic tracing has suggested a distinct origin for caval vein myocardium, from a proposed third heart field.

Objective: To determine the cell lineage history of the myocardium at the venous pole of the heart.

Methods and Results: We used retrospective clonal analyses to investigate lineage segregation for myocardium at the venous pole of the mouse heart, independent of gene expression.

Conclusions: Our lineage analysis unequivocally shows that caval vein and atrial myocardium share a common origin and demonstrates a clonal relationship between the pulmonary vein and progenitors of the left venous pole. Clonal characteristics give insight into the development of the veins. Unexpectedly, we found a lineage relationship between the venous pole and part of the arterial pole, which is derived exclusively from the second heart field. Integration of results from genetic tracing into the lineage tree adds a further temporal dimension to this reconstruction of the history of venous myocardium and the arterial pole. (Circ Res. 2012;111:1313-1322.)

Key Words: cell lineage ■ caval vein ■ pulmonary vein ■ arterial pole myocardium ■ second heart field

Gene expression patterns and genetic tracing experiments provide important insight into regional differences within a tissue or organ and between the progenitor cell populations that contribute to such subregions. Definition of differences in the underlying regulatory circuits by mutant analysis further reinforces the distinction between contributing cell sources. However, approaches based on gene expression and function are not necessarily informative about lineage and can lead to confusing or conflicting conclusions in this cellular context.1

The formation of the venous pole of the heart provides a striking example of this sort of dilemma. In the mature heart, caval veins drain oxygen-poor blood from the systemic circulation into the right atrium, while pulmonary veins drain oxygen-rich blood from the lungs into the left atrium, as illustrated for the mouse model (Figure 1A). These veins, like the heart itself, are sheathed in myocardial cells, which surround the endothelial tube of these blood vessels. The origin of this myocardium continues to excite debate.2–4

The mouse heart is formed from 2 mesodermal cell populations, the first and second heart fields.5,6 The second heart field (SHF) is a major source of cells that are added into the primary heart tube which has a mainly left ventricular (LV) identity.7 Thus, the arterial pole of the heart forms from the pharyngeal splanchnic mesoderm of the SHF, from its anterior domain characterized by the expression of Fgfl.8 The posterior part of the SHF contributes to the myocardium of the atria.8 As the venous pole of the heart tube develops, the sinus venosus forms posteriorly, with symmetrically protruding sinus horns from which the caval veins derive. Myocardium is only added to the pulmonary vein from about E12.5, potentially also by recruitment of mesenchymal cells from the posterior SHF.9

Three types of myocardium are molecularly distinguishable at the venous pole of the heart, characterized by different expression of genes10–12 or transgenes.13,14 Based on overlapping expression profiles, it has been proposed that atrial myocardium grows out along the endothelial surface of the vein15,16 or that the caval vein and pulmonary vein myocardium have a common origin.12 Examples of different expression patterns, for myosin transgenes and for the Pitx2C transcription factor, which is expressed on the left side of the embryo, are illustrated in Figure 1B.

Cardiac progenitor cells in the SHF, which contribute to the atria and to the pulmonary vein, express the transcription factor Islet1, which has been regarded as a marker of the SHF,17 as well as Nkx2–5 also expressed in the myocardium.11 In contrast, caval vein myocardium is distinguished...
by the absence of Nkx2–5 and the presence of another transcrip- 
tion factor, Tbx18, also detectable in surrounding mes-
enchymal cells from about E8.25. These cells are mainly 
negative for Isl1. Mutant analysis shows that Tbx18 is es-
sential for the formation of caval vein myocardium.\cite{18,19} In
an elegant series of genetic tracing experiments, using the 
Rosa26 reporter line, Christoffels et al\cite{11,18,19} conclude that 
pulmonary vein myocardium does not derive from atrial myo-
cardium because it is not labeled with a 
Nppa-Cre (Figure 1C). They show that caval vein myocardium forms from 
cells that do not activate a Nkx2–5-IRES-Cre, which gives label-
ing in the atria and pulmonary vein (Figure 1C).\cite{11,18} Cells in the 
posterior heart field adjacent to the sinus venosus, which 
are Tbx18 positive, are also marked by a transgene expressed 
in a territory located laterally to the cardiac crescent at earlier 
stages. Dye labeling experiments at this early lateral location 
marked cells that express Tbx18 in the venous pole of the 
heart at later stages, after embryo culture. These series of 
experiments therefore led to the conclusion that caval vein 
myocardium has a distinct origin, potentially from a third 
heart field that is distinguished from the SHF by its transcrip-
tional regulation.\cite{18,19}

Lineage studies, using retrospective clonal analysis, have 
established that 2 myocardial cell lineages contribute to the 
formation of the heart. These segregate very early and their 
contributions, the first to the primitive left ventricle and the 
second to the outflow tract at the arterial pole, with overlapping 
contributions to the right ventricle and atria, are analogous to 
those of the first and second heart fields, respectively.\cite{5,20} The 
venous pole of the heart, which forms later, was not investi-
gated in detail in this analysis. The question of a third heart 
field, potentially emanating from a third lineage, has not been 
addressed.

We now report cell lineage results, based on retrospective 
clonal analysis at a later stage, for atrial, pulmonary, and caval 
vein myocardium, which lead to the construction of a lineage 
tree for the venous pole of the heart. This is consistent with 
a common contribution from the second myocardial cell lin-
eage (SHF), which segregates into left and right components, 
contributing to the dorsal left atrium, pulmonary vein, and left 
superior caval vein or to the dorsal right atrium and right 
superior caval vein, respectively, with no indication of an inde-
pendent sublineage for caval vein myocardium. Unexpectedly,
the left lineage also contributes to the arterial pole, indicating 
that the posterior domain of the SHF also contributes to the 
most anterior part of the heart. We also demonstrate that gene 
expression and function are secondary features of these car-
diac cell lineages.

**Methods**

**Mice**

Animal care was in accordance with national and institutional guide-
lines. The \(\alpha\text{-actin}\)\textsuperscript{Cre}\textsubscript{Rosa26} line was used in conjunction with an 
\(\alpha\text{MHC}-\alpha\text{-actin}\) transgene so that cardiac actin was present in the 
heart and homozygote \(\alpha\text{-actin}\)\textsubscript{Rosa26\textsubscript{R-nlacZ}} mice were viable and could 
be mated.\cite{20} We used a mouse line in which an inducible Cre-ERT2 
sequence had been targeted to the Rosa26 locus \(\text{R26CreERT2}\) from Lars Grotewold and Austin Smith. The \(\text{Rosa26R-nlacZ}\) 
\(\text{R26r-nlacZ}\) line, in which an \(\text{nlacz}\) sequence is conditionally 
expressed on recombination by Cre, was used as a reporter line (J-F. 
Nicolás, E. Tzouanacou, and V. Wilson, unpublished). In these mice 
\(\text{R26CreERT2}\) or \(\text{R26r-nlacZ}\), both alleles of \(\text{Rosa26}\) are mutated, 
without any detectable phenotype.\cite{21} The T4 mouse line expresses an 
\(\text{nlacz}\) reporter under the regulation of \(\text{S}\) sequences of the \(\text{\alpha\text{-cardiac-}
actin}\) gene that lead to expression throughout the myocardium.\cite{22} 
These mouse lines are all on a mixed genetic background (mainly 
C57/B6/DBA2/129/SJL). Embryonic day (E) 0.5 was counted from 
the appearance of the vaginal plug.

Detailed Methods are provided in the Online Data Supplement.

**Results**

**Clonal Analyses at E14.5**

The retrospective clonal analysis used here avoids precon-
ceived ideas on lineage relationships and relies on the random 
intragenic recombination of a nonfunctional \(\text{nlacz}\) sequence into a functional \(\text{nlacz}\), which in this case is targeted to the 
\(\text{\alpha\text{-cardiac actin}}\) (\(\text{\alpha\text{-actin}}\) gene expressed throughout 
the myocardium (Online Figure I). The recombination event itself 
is totally independent of gene expression. To follow pulmo-

The expected frequency of double labeling (labeling 
between the observed frequency of double labeling (labeling 
results in a targeted condition-

We complemented this analysis with a retrospective ap-
proach, which permits more precise control of the temporal 
parameter. In this approach, an inducible Cre recombinase 
from a Rosa26\textsubscript{CreERT2} allele is used to target a condition-

A total of 631 embryos were produced by tail vein 
injections of a low dose of 4-hydroxytamoxifen at E6.5, 
which gave only 9.8% of labeling at the venous pole (atria 
and veins), thus permitting clonal analysis (with a proba-

The left lineage also contributes to the arterial pole, indicating 
that the posterior domain of the SHF also contributes to the 
most anterior part of the heart. We also demonstrate that gene 
expression and function are secondary features of these car-
diac cell lineages.
No labeled cells were detected 6 hours after injection, and the first labeled cells were observed 12 to 18 hours after injection (data not shown), suggesting that the clone was born between E6.75 and E7.25. We made use of this approach as a temporal indicator, in conjunction with the more extensive \( \alpha_c\text{-actinin}^{\text{lacZ1.1/+}} \) clonal analysis.

**Atrial and Venous Myocardium Are Clonally Related**

We first examined whether atrial and venous myocardium are clonally related. Because atrial myocardium may be regionalized, we have distinguished the dorsal and ventral parts of the atria. We obtained 9 \( \alpha_c\text{-actinin}^{\text{lacZ1.1/+}} \) hearts with double labeling in the dorsal left atrium (dLA) and in the left superior caval vein (LSCV) (Online Figure II). An example of such a clone is shown in Figure 2A. In this and other 3D reconstructions of the heart, high-resolution episcopic microscopy (HREM) permits visualization of \( \beta\)-galactosidase–positive cells in the vessel walls at the venous pole, where localization cannot be precisely described by standard whole-mount techniques. We have also observed 8 \( \alpha_c\text{-actinin}^{\text{lacZ1.1/+}} \) hearts with double labeling in both the dLA and pulmonary vein (PV) (Figure 2B and Online Figure II). Labeling in the right atrium (RA) was accompanied by labeling in the right superior caval vein (RSCV) (n=13) (Figure 2C and Online Figure II). To assess whether a clonal relationship exists between atrial and venous myocardium, we compared the observed frequency of labeling in the 2 different regions with the expected frequency of double recombination events. This frequency was estimated on the basis of the law of independent probabilities: this is equal to the product of the frequency of labeling in each region. Double labeling events between the veins (LSCV, RSCV, or PV) and a region of the atrium (dorsal-LA, ventral-LA, dorsal-RA, or ventral-RA) were analyzed one by one (Figure 2D and the Table). A high ratio (>1) between the observed and
expected frequencies of double labeling is indicative of a clonal relationship.

If we consider first the LSCV, the observed frequency of double labeling in this vein and in the dorsal part of the left atrium (dLA) is 10-fold higher than the expected frequency (Figure 2D). Statistical analysis with the Fisher exact test confirms that such labeling has a very low probability of arising from independent recombination events \( (P = 2 \times 10^{-6}) \) (Table). We also conclude that double labeling in the PV and LA is the product of a unique recombination event, because the observed frequencies were significantly higher than the expected frequencies (Figure 2D) (dLA and PV: \( P = 4 \times 10^{-10} \); vLA and PV: \( P = 2 \times 10^{-3} \)) (Table).

If we now consider the RSCV, the observed frequency of double labeling with the RA is significantly higher than the expected frequency, suggesting that this reflects a unique recombination event. The statistical analysis supports the conclusion that \( \beta \)-galactosidase–positive cells in the RSCV and the RA arise from a common progenitor, with a more pronounced clonal relationship with the dorsal part of the RA (dRA: \( P = 7 \times 10^{-15} \)) than with the ventral part (vRA: \( P = 7 \times 10^{-6} \)).

Induction of clones at E6.5 also produced double labeling in the LSCV and LA, LSCV and PV, and RSCV and RA (Figure 2E and 2F), suggesting that the segregation between venous and atrial myocardial lineages arises after E6.75–E7.25. We also observed nonmyocardial \( \beta \)-galactosidase labeling (TnI negative; Figure 2E and 2F), indicating that progenitors giving rise to LSCV, LA and LSCV or RSCV and RA also contribute to other cardiac cell fates. In addition, with the exception of 1 heart, which may reflect independent recombination events, the induced labeling was found in either the left or right side of the venous pole suggesting that the segregation between the left and right lineages arises before E6.75–E7.25.

**PV and LSCV Myocardium Are Clonally Related**

We then examined whether caval and PV myocardium are clonally related. Whereas no hearts had double labeling in the RSCV and PV myocardium, we have observed 9 \( \alpha \)-actin\(^{Rosa26\ lacZ_1.1/+} \) hearts with double labeling in LSCV and PV myocardium (Figure 3A and 3B and Online Figure II). The comparison between the observed and expected frequencies of double labeling shows that the PV and LSCV arise from common progenitors, and this was confirmed by statistical analysis. The probability that double labeling arises from independent recombination events is very low \( (P = 3 \times 10^{-11}) \) (Table). However, the clonal relationship between the PV and LSCV is
more pronounced with the distal part of the LSCV ($P = 3 \times 10^{-11}$) than with the proximal part of the vein ($P = 8 \times 10^{-6}$) (Figure 3C).

A clone with $\beta$-galactosidase–positive cells in the LA, LSCV, and PV myocardium produced by injection of 4-hydroxytamoxifen at E6.5 is shown in Figure 3D, suggesting that the segregation between these different lineages arises after E6.75–E7.25.

### Table. Statistical Analysis of the Probability of an Independent Recombination Event

<table>
<thead>
<tr>
<th></th>
<th>PV</th>
<th>RSCV</th>
<th>LSCV</th>
<th>vRA</th>
<th>dRA</th>
<th>vLA</th>
<th>dLA</th>
<th>LV</th>
<th>RV</th>
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<td>0.39</td>
<td>$8 \times 10^{-5}$</td>
<td>0.42</td>
<td>1</td>
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<td>0.22</td>
<td>$1 \times 10^{-2}$</td>
<td>$6 \times 10^{-2}$</td>
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<tr>
<td>RV</td>
<td>1</td>
<td>0.54</td>
<td>0.45</td>
<td>$2 \times 10^{-2}$</td>
<td>0.86</td>
<td>1</td>
<td>1</td>
<td>0.07</td>
<td>...</td>
</tr>
<tr>
<td>LV</td>
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<td>0.16</td>
<td>0.65</td>
<td>0.88</td>
<td>0.22</td>
<td>0.36</td>
<td>0.44</td>
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<td>0.07</td>
</tr>
<tr>
<td>dLA</td>
<td>$4 \times 10^{-5}$</td>
<td>1</td>
<td>$2 \times 10^{-4}$</td>
<td>8 $\times 10^{-2}$</td>
<td>1</td>
<td>5 $\times 10^{-4}$</td>
<td>...</td>
<td>0.44</td>
<td>1</td>
</tr>
<tr>
<td>vLA</td>
<td>$2 \times 10^{-3}$</td>
<td>0.53</td>
<td>$9 \times 10^{-2}$</td>
<td>0.19</td>
<td>0.10</td>
<td>...</td>
<td>5 $\times 10^{-4}$</td>
<td>0.36</td>
<td>1</td>
</tr>
<tr>
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<td>0.10</td>
<td>0.22</td>
<td>0.86</td>
<td></td>
</tr>
<tr>
<td>vRA</td>
<td>1</td>
<td>$7 \times 10^{-4}$</td>
<td>1</td>
<td>...</td>
<td>$1 \times 10^{-5}$</td>
<td>0.19</td>
<td>8 $\times 10^{-3}$</td>
<td>0.88</td>
<td>$2 \times 10^{-2}$</td>
</tr>
<tr>
<td>LSCV</td>
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<td>1</td>
<td>...</td>
<td>0.34</td>
<td>1</td>
<td>$9 \times 10^{-2}$</td>
<td>2 $\times 10^{-2}$</td>
<td>0.65</td>
<td>0.45</td>
</tr>
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<td>1</td>
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<td>1</td>
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<td>$7 \times 10^{-5}$</td>
<td>0.53</td>
<td>1</td>
<td>0.16</td>
<td>0.54</td>
</tr>
</tbody>
</table>

AP indicates arterial pole; RV, right ventricle; LV, left ventricle; dLA, dorsal left atrium; vLA, ventral left atrium; dRA, dorsal right atrium; vRA, ventral right atrium; LSCV, left superior caval vein; RSCV, right superior caval vein; PV, pulmonary vein.

We used the nonparametric Fisher exact test to assess whether double labeling results from 2 independent events. Each region was tested for independence with another region of the heart. P values are indicated in the table.

*P value was lower than $5 \times 10^{-2}$, leading to the conclusion that the labeling is likely to derive from a single recombination event and that the 2 regions are clonally related. The large range of significant P values reflects different cellular components with distinct clonal relationships within a compartment, as shown for the pulmonary trunk at the arterial pole.

### Arterial and Venous Myocardium Are Clonally Related

Arterial pole myocardium is exclusively derived from the second myocardial cell lineage, whereas both first and second lineages contribute to atrial myocardium. To distinguish first versus second lineage contributions, we therefore examined whether the veins share common progenitors with the arterial pole.
myocardium surrounding the great arteries (pulmonary trunk and aorta). We found 9 $\beta$-actinnlaacZ1.1+ hearts with colabeling in the myocardium surrounding the veins and at the base of the great arteries (Figure 4A and Online Figure II). The comparison between the observed and expected frequencies of double labeling demonstrates that arterial pole myocardium is clonally related to the dLA ($P=1\times10^{-2}$) and both the LSCV ($P=8\times10^{-5}$) and PV ($5\times10^{-4}$) myocardium (Figure 4B and the Table). This unexpected finding indicates that arterial pole myocardium shares common progenitors with the left components of the venous pole. This clonal relationship is more pronounced with the pulmonary trunk myocardium. Eight $\alpha$-actinnlaacZ1.1+ hearts had labeling in the left venous pole and in the myocardium surrounding the aorta, whereas no clones showed colabeling in the venous pole and in the myocardium surrounding only the aorta.

Right ventricular myocardium is also largely derived from the anterior SHF. Comparison between the observed and expected frequencies of double labeling did not show any significant relationship between the LSCV, RSCV, or PV with the RV (Figure 4B and the Table). However, a significant relationship was observed between the vRA and the RV ($P=2\times10^{-2}$; Figure 4B and the Table). As expected, a significant relationship was observed between the RV and arterial pole ($P=2\times10^{-3}$; Table). In contrast, no clonal relationships were observed with left ventricular myocardium that derives from the first lineage30 (Table).

Aspects of Cell Behavior in the Myocardium Surrounding the Veins
This retrospective clonal analysis also allowed us to investigate aspects of cell behavior in the myocardium surrounding the veins. We first noticed that $\alpha$-actinnlaacZ1.1+ clones were organized in oriented lines of $\beta$-galactosidase–positive cells in the LSCV, as illustrated in Figure 5A, notably in parallel to the vein in the proximal part (Figure 5B). In contrast, in the distal part of the LSCV, the clones were organized around the circumference of the vein (Figure 5C). These results therefore show that the LSCV myocardium follows 2 modes of oriented growth, whereas the RSCV and PV myocardium only shows circumferential growth (examples are shown in Online Movies I and II).

In the PV myocardium, we obtained many large clones. This is consistent with a high number of proliferative cells in this myocardium31 (Online Figure III). Notably, we observed that the frequency of clones in the PV increased with their size (Figure 5D). This is typical of a stem cell mode of growth (Online Figure IV).24 We then investigated the localization of the clones along the proximo-distal axis of the veins. Although in the left superior caval vein, clones are located randomly along the vein with no preferential regionalization (Figure 5F), almost all the clones in the pulmonary vein extend to the more proximal end, corresponding to the base of the vein (Figure 5E). This is consistent with a stem cell–like mode of growth with a self-renewing pool of cells located at the base of the vein. In the context of this different growth mode, small clones are generated over a longer time period. We checked all the PV clones in our statistical analysis. This gave similar results on clonal relationships to those shown in the Table.

Discussion
The retrospective clonal analysis described in the present study for the venous pole of the heart results in the lineage tree shown in Figure 6A. Despite molecular differences,
myocardium of the pulmonary or caval veins shares common progenitors with atrial myocardium, notably with the dorsal part of the atria. Furthermore, left superior caval vein and PV myocardium are clonally related. This is in contrast to the conclusions suggested by genetic tracing experiments (Figure 6B).

Early segregation takes place between left and right components of the venous pole, separating myocardial progenitors of the left atrium, the left superior caval vein, and the pulmonary vein from those of the right atrium and right superior caval vein. This segregation probably occurs at the time of gastrulation, when mesoderm separates on either side of the primitive streak.25,26 As expected, Pitx2C expression, which is a read-out of later left/right signaling, marks these structures at the left side of the venous pole (Figure 1B).14,27 Pitx2C is also expressed on the left side of the posterior second heart field; explant experiments monitoring the left/right myocardial potential of this domain, together with dye tracking of cells, showed that left atrial myocardium derives from the left side of the field.8

Unexpectedly, myocardium at the venous pole shows clonality with the myocardium of the pulmonary trunk at the arterial pole of the heart. This clonal relationship is seen with the left side of the venous pole. Previous clonal analysis had shown the contribution of the left pharyngeal mesoderm to a subset of skeletal muscles on the left side of the head and to pulmonary trunk myocardium,26 consistent with left segregation of progenitors.

Outflow tract myocardium, which subsequently locates to the base of the aorta and pulmonary trunk, derives exclusively from the second myocardial cell lineage.20 In this previous analysis, the atria were shown to have contributions from both first and second lineages; the early end point (E8.5) precluded any conclusions about the pulmonary or caval veins. Since we now show that pulmonary trunk myocardium is clonally related to venous pole myocardium and that there is no clonal relationship with left ventricular myocardium that derives exclusively from the first lineage,20 we conclude that pulmonary and caval vein myocardium derive from the second lineage. This would also suggest that the dorsal part of the atria may be preferentially of this origin. Since the second lineage contribution correlates with that of the second heart field,5 this is consistent with the late recruitment of mesodermal cells to both caval and pulmonary vein myocardium,28 from the posterior second heart field.

Outflow tract myocardium is formed by progenitor cells, which are recruited from the anterior part of the second heart field, including the mesodermal core of the pharyngeal arches (Figure 6C, left), as evidenced by dye labeling and genetic tracing.5 Many genes, such as Fgf8/10, are only expressed in this part of the heart field, and their mutant phenotypes are confined to the arterial pole of the heart.29 In this context, it is very surprising that pulmonary trunk myocardium shares common progenitors with venous myocardium. This might...
imply that the myocardial lineages of the pulmonary trunk and caval and pulmonary veins had segregated before establishment of the heart fields. However, this is unlikely, given the lineage tree (Figure 6A) and the fact that there is no clonal relationship with left ventricular myocardium derived from the first heart field. Furthermore, right ventricular myocardium, which is also mainly derived from the anterior SHF, shows no clonal relationship with venous pole myocardium, with the exception of the ventral right atrium. However, it is the dorsal part of the atrium that has strong clonal relationships with the caval and pulmonary veins. Alternatively, some outflow tract progenitors may move from the posterior to the anterior region of the heart field, where they then express typical anterior markers. Dye injections in the posterior SHF also showed a contribution to the proximal and distal outflow tract.³⁰ Moreover, a recent report on HoxB1-expressing cells is in favor of this alternative.³¹

This would suggest that posteriorly located HoxB1-positive cells have moved anteriorly to contribute to arterial pole myocardium and specifically to pulmonary trunk myocardium, as also observed in the clonal analysis. As a word of caution, it is possible that in both cases, a contribution to the base of the aorta may have been missed because this myocardial sleeve is much less prominent than that of the pulmonary trunk. It is also possible, since the SHF gives rise to smooth muscle and the aorta has a major smooth muscle sleeve, that part of this aortic smooth muscle has a clonal relationship with the venous pole, not revealed in the α-actinlacZ+/− analysis.

Clonal analysis also gives information about cell behavior. Thus, we had previously observed oriented clonal cell growth, which we had proposed underlies cardiac morphogenesis.¹² In the case of the arterial pole of the heart, clones in the embryonic outflow tract were oriented in parallel with this tubular structure, except in the region proximal to the ventricle where they assumed a circumferential orientation. Subsequently, after septation and formation of the pulmonary trunk and aorta, myocardial clones were oriented around the
base of these arteries. In the case of the venous pole, we observed that clones are oriented parallel to the wall of the left superior caval vein, except in the distal region, furthest from the atrial chamber, where they are circumferential. Pulmonary vein and right superior caval vein myocardium showed circumferential growth. This difference is consistent with the major elongation of the LSCV, which extends across the mature heart. Strikingly, the clonal analysis reveals that PV myocardium has undergone a stem cell–like mode of proliferation, unlike other myocardial domains at the venous or at the arterial pole. This may reflect an early role of the pulmonary pit, which is the point of connection between the pulmonary vein and the heart, as a proliferative center for PV outgrowth, in which is the point of connection between the pulmonary vein and the heart,34,35 as a proliferative center for PV outgrowth, in additional to recruitment of proliferating cells from the second heart field.

In conclusion, we have established a lineage tree for the venous pole of the heart. The clonal relationships revealed by our analysis do not always correlate with differences between progenitor populations shown by genetic tracing. This underlines the distinction between gene expression and gene regulatory networks compared with lineage per se, in these complementary aspects of the history of a cell. Thbx18-positive, Nkx2–5-negative cells contribute to caval vein myocardium, but this domain of gene expression, initially positioned laterally to the cardiac crescent, does not reflect an earlier lineage segregation from atrial or pulmonary vein progenitors. However, the onset of gene expression in subpopulations of progenitor cells provides important insight into the segregation of sublineages. Thbx18 expression and the absence of Nkx2–5 mark the right and left caval vein branches of the lineage tree. Because this expression is observed in progenitor cells from E8.25, it indicates that segregation takes place before E8.25 (Figure 6C). For clonal analysis, the tamoxifen-inducible approach also gives temporal information. In the experiments described here, the left/right segregation of lineages must take place before tamoxifen activation of the Cre recombinase in the time window of E6.75–E7.25, in keeping with the establishment of left/right territories at the time of gastrulation. The unexpected lineage relationship, revealed by our clonal analysis, between the venous myocardium and the pulmonary trunk at the arterial pole of the heart, which is supported by Hoxb1 genetic tracing, leads us to propose a lineage tree that underlines their common origin (Figure 6C, right). Hoxb1 expression is initiated at E7.25, indicating that the segregation of anterior and posterior sublineages precedes this time point.

We had previously shown that arterial pole myocardium and skeletal muscles of the head share common progenitors, such that a first sublineage contributes to the right ventricle and to masticatory muscles and a second sublineage contributes to myocardium at the base of the aorta and pulmonary trunk and to facial expression muscles. The question therefore arises of clonality between head muscles and venous pole myocardium. We did not find any evidence of this and therefore conclude that pulmonary trunk myocardium derives from 2 distinct sublineages. These sublineages may correspond to the anterior and posterior second heart field that have been described previously (Figure 6C).

Congenital heart defects at the arterial pole are particularly frequent (about 30% of the affected 1% of neonates) in the human population and in some cases are linked to a venous pole defect that was frequently not detected and therefore not corrected in the past. This was ascribed to a contribution of the second myocardial cell lineage also to the venous pole. Our current study clarifies this connection and underlines the importance of characterizing the arterial pole defects that arise from a failure in the pulmonary trunk/venous pole sublineage, which should also be diagnostic of potential venous pole defects.

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Disclosures

None.

References


**Novelty and Significance**

- **What Is Known?**

  - The mouse heart is formed from 2 myocardial cell lineages, where the second lineage, which correlates with the second heart field, contributes all the myocardium of the outflow region at the arterial pole and also contributes to the right ventricle and both atria.

- **What New Information Does This Article Contribute?**

  - The relationship between the myocardium that surrounds the caval and pulmonary veins has remained controversial and it has been proposed that caval vein myocardium has a distinct origin from the rest of the venous pole.

  - The left and right components of the venous pole and that the dorsal part of the right or left atrial myocardium is clonally related to the right or left superior caval vein myocardium, respectively. Pulmonary vein myocardium belongs to the left sublineage because it is clonally related to left atrial and left superior caval vein myocardium. We also found that arterial pole myocardium, at the base of the pulmonary trunk, belongs to this lineage, demonstrating the relation of venous pole myocardium to the second myocardial lineage. The current study clarifies the origin of the venous myocardium and illustrates the difference between genetic tracing experiments and lineage analysis. These findings are relevant to congenital heart defects that affect not only the arterial pole but also the venous pole of the heart.

  - Arterial and venous myocardium also share common progenitors, demonstrating the relation with the second myocardial cell lineage, which is the only source of arterial pole myocardium.

**Calcium and Myocardial Function** It is known that myocardial function is maintained through a balance of calcium influx and efflux. In this study, they investigated the role of a particular calcium channel in the regulation of myocardial function in the developing mouse heart. Their results showed a significant decrease in calcium influx when the channel was not functioning properly, leading to a reduction in overall contractility. This finding has implications for understanding the role of calcium channels in cardiac development and could potentially inform future treatments for congenital heart defects.
Lineage Tree for the Venous Pole of the Heart: Clonal Analysis Clarifies Controversial Genealogy Based on Genetic Tracing

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Online detailed Methods
Online Table I: related to Table 1
Online Figure I: related to Figure 2
Online Figure II: related to Table 1
Online Figure III: related to Figure 5
Online Figure IV: related to Figure 5
Online Movie I: related to Figure 5
Online Movie II: related to Figure 5
Online detailed Methods

Mice

Animal care was in accordance with national and institutional guidelines. The αc-actin\textsuperscript{nlaacZ1.1/nlaacZ1.1} line was used in conjunction with an αMHC-αc-actin transgene so that cardiac actin was present in the heart and homozygote αc-actin\textsuperscript{nlaacZ/nlaacZ} mice were viable and could be mated \(^1\). We used a mouse line in which an inducible Cre-ERT2 sequence had been targeted to the Rosa26 locus (R26CreERT2 from Lars Grotewold and Austin Smith). The ROSA26R-nlacZ (R26R-nlacZ) line in which an nlacZ sequence is conditionally expressed upon recombination by Cre, was used as a reporter line (J-F. Nicolas, E. Tzouanacou and V. Wilson, unpublished). In these mice (R26CreERT2 or R26R-nlacZ) both alleles of Rosa26 are mutated, without any detectable phenotype \(^2\). The T4 mouse line expresses an nlacZ reporter under the regulation of 5’ sequences of the α-cardiac-actin gene that lead to expression throughout the myocardium \(^3\). These mouse lines are all on a mixed genetic background, mainly (C57B6/DBA2/129/SJL). Embryonic day (E) 0.5 was counted from the appearance of the vaginal plug.

X-gal staining

Embryos were dissected from the uterus, fixed in 4% paraformaldehyde and X-Gal staining was performed as previously described \(^4,5\).

Retrospective clonal analysis

2018 αc-actin\textsuperscript{nlaacZ1.1/+} embryos, at E14.5, were obtained by crossing homozygous αc-actin\textsuperscript{nlaacZ1.1/nlaacZ1.1} ; αMHC-αc-actin males with superovulated wildtype females (B6SJL/F1) \(^1\). Labelled cells were produced spontaneously after recombination of the nlaacZ sequence expressed in the myocardium into a functional nlacZ reporter. We excluded from the statistical analysis, hearts with extensive labelling in all components of the venous pole (see Online Figure II), since this is indicative of a very early recombination event and is therefore not informative for distinguishing relationships between the different regions. We have scored in our lineage analysis only clones with clusters of more than 8 labelled cells, since smaller clusters probably derive from a late recombination event, and are therefore less informative about progenitor cell populations. We estimated the expected frequency of double recombination events in two different regions, which, according to the law of independent probabilities, is equal to the product of the frequency of labelling in each region. For example, the expected frequency of double recombination events between the dorsal left atrium (dLA) and the left superior caval vein (LSCV) is based on the frequency of labelling in each region in the collection of embryos (number of embryos with labelling divided by the total number of embryos) (f(dLA)=0.021 and f(LSCV)=0.015 – see Table1) so that the expected frequency of a double recombination event is, f(dLA+LSCV)=0.021x0.015=3x10\textsuperscript{-4}.The ratio between the observed and expected frequency was used to identify clonal relationships. In order to decide whether the observed frequency of common labelling in two distinct regions was consistent with the expected frequency, we used the non-parametric Fisher’s exact test that allows us to work with small numbers of labelled embryos as previously described \(^6\). The null hypothesis is that the labelling in both regions results from two independent events. When the p-value is lower than 5x10\textsuperscript{-2}, the null hypothesis may be confidently rejected, leading to the conclusion that the labelling is likely to derive from a single recombination event.

3D-imaging of hearts
Hearts with clones of labelled cells at the venous pole, from αc-actin\(^{\text{nlacZ1.1/+}}\) embryos at E14.5, were dehydrated, embedded in JB4 resin that contains eosin and sectioned every 2 µm. HREM (High-Resolution Episcopic Microscopy) was performed as previously described. Morphology of the hearts was observed in the green channel (GFP filter set), with X-gal staining visualized through the red channel (TX2 filter set). Data sets were normalized and sub-sampled prior to 3D reconstruction using OsiriX. This approach was used for clones produced by the αc-actin\(^{\text{nlacZ1.1/+}}\) line. In the case of induced clones, it was necessary to identify myocardium by immunohistochemistry on sections, precluding use of the HREM approach.

**Inducible clonal analysis**

Homozygous R26CreERT2 females were crossed with homozygous male R26R-nlacZ mice to produce embryos (R26CreERT2/R26R-nlacZ). Tail vein injections were performed on gravid females, 6.5 days after coitus, with a clonal dose of 4-hydroxytamoxifen (4-OHT) (Sigma) (1 to 2.25µg per gram body weight, the precise dose was adjusted for each male). 4-OHT was prepared in cremophor solution (Sigma) as described. 631 embryos were produced, of which 131 (20.8%) had a cluster of β-galactosidase positive cells in the heart. 9.8% of embryos had a cluster at the venous pole. Statistical analysis shows that, at this frequency, the probability of a double recombination event in the venous pole is equal to 0.098 x 0.098=1x10\(^{-3}\), thus permitting clonal analysis. Moreover, even if we consider that the half-life of the 4-OHT is long, the probability of a second event in more recent cells is also unlikely as it corresponds to the probability of two events of recombination (p=1x10\(^{-3}\)). Embryos collected without 4-OHT injection, had no β-galactosidase positive cells, showing that this Cre allele is not leaky (n=84 embryos).

**Proliferation assay**

To assess the proliferation rate in the different components of the venous pole, pregnant mice (14.5 days after coitus) were injected intraperitoneally with BrdU (100µg per g), followed by EdU (50µg per g) after an interval, Ti, of 90 min. Embryos were collected 30 min later. The length of the cell cycle (Tc) was estimated based on the formula: Tc=Ti/(N\(_{\text{BrdU+Edu}}\)/N\(_{\text{prolif}}\)) with N\(_{\text{BrdU+Edu}}\) the number of cells stained positively only by the BrdU antibody and N\(_{\text{prolif}}\) the total number of proliferative cells as estimated by immunohistochemistry with a Ki67 antibody that marks all cycling cells.

**Histology and Immunohistochemistry**

Fixed hearts from E14.5 embryos, after Brdu/Edu injection or Cre induction of R26CreERT2; R26R-nlacZ crosses, were dehydrated in ethanol, incubated 2x15 min in histoclear, further incubated in 50:50 parafin:histoclear and then embedded in standard parafin. Frozen T4 αc-actin-nlacZ hearts were sectioned using a cryostat. Immunohistochemistry was performed with the following antibodies: mouse anti-BrdU (1/50 – BD Biosciences 347580), rabbit anti-cTn I (1/100 - abcam ab47003), mouse anti-α–actinin (sarcomeric) (1/400 – SIGMA A7732), mouse anti-actin (α-Sarcomeric) (1/400 – SIGMA A2172), rabbit anti-smooth muscle MHC (1/300; Biomedical Technologies BT-562), chicken anti-β-galactosidase (1/300 – abcam ab9361) and mouse anti-Ki67 (1/400 - BD Biosciences 556003). Edu staining was revealed with the Invitrogen Edu Click-It assay. Immunohistochemistry on labelled induced clones was performed with the rabbit Vectastain ABC kit. Peroxidase activity was revealed with SIGMAFAST DAB tablets.
Online Table I: Frequency of labelling (retrospective clonal analysis)

<table>
<thead>
<tr>
<th></th>
<th>AP</th>
<th>RV</th>
<th>LV</th>
<th>dLA</th>
<th>vLA</th>
<th>dRA</th>
<th>vRA</th>
<th>LSCV</th>
<th>RSCV</th>
<th>PV</th>
</tr>
</thead>
<tbody>
<tr>
<td>number of</td>
<td>46</td>
<td>304</td>
<td>395</td>
<td>42</td>
<td>71</td>
<td>71</td>
<td>78</td>
<td>31</td>
<td>21</td>
<td>17</td>
</tr>
<tr>
<td>embryos with β-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gal+ cells</td>
<td>(2.3)</td>
<td>(15.1)</td>
<td>(19.6)</td>
<td>(2.1)</td>
<td>(3.5)</td>
<td>(3.5)</td>
<td>(3.9)</td>
<td>(1.5)</td>
<td>(1.0)</td>
<td>(0.8)</td>
</tr>
</tbody>
</table>

Total number of embryos: n=2018
Percentage are indicated in brackets. AP, arterial pole; RV, right ventricle; LV, left ventricle; dLA, dorsal left atrium; vLA, ventral left atrium; dRA, dorsal right atrium; vRA, ventral right atrium; LSCV, left superior caval vein; RSCV, right superior caval vein; PV, pulmonary vein; β-gal+, β-galactosidase positive.
Large clones with all components of the venous pole labelled were not scored in our analysis as they are not informative for distinguishing relationships between the different regions.
Online Figure I: \( \alpha_c \)-actin expression in the myocardium at the venous and arterial poles of the heart. A-C'. Immunostaining on heart sections of a T4 \( \alpha_c \)-actin-nlacZ embryo \(^3\) at E14.5, in which \( \alpha_c \)-actin expression is recapitulated by the expression of \( \beta \)-galactosidase, showing that \( \alpha_c \)-actin is expressed in the myocardium of the pulmonary vein (A), caval vein (B) and arterial pole (C). We used an anti-\( \beta \)-galactosidase antibody (\( \beta \)-gal), together with antibodies against myocardial specific proteins (anti-\( \alpha \)-sarcemic actin (\( \alpha \)-sarc. act.) (A) or anti-\( \alpha \)-actinin (sarcomeric) (\( \alpha \)-actinin) (B, C) or against smooth muscle myosin heavy chain (sm-MHC) (A', B', C'). While all \( \beta \)-galactosidase positive cells co-express myocardial markers in the veins, very few smooth muscle heavy chain (sm-MHC) positive cells are found and those that are positive are negative for \( \beta \)-galactosidase (arrows) thus demonstrating that \( \alpha_c \)-actin is expressed in the myocardium but not in smooth muscle at the venous (A, B) and arterial poles (C) of the heart. * Labelling of a vessel wall with sm-MHC antibody provides a positive control. PV, pulmonary vein; RA, right atrium; RSCV, right superior caval vein; pt, pulmonary trunk.
Online Figure II: Summary of α-actin<sup>nlaecZ/+</sup> hearts with β-galactosidase positive cells in the venous pole of the heart. All hearts with clones of β-galactosidase-positive cells are summarized (only clones with more than 8 cells were analyzed as small clones probably derive from a recent recombination event within the myocardium (born after E12.5, assuming a cell cycle time of 15h<sup>4</sup> and a proliferative mode of growth). The presence of labelled cells within a region of the heart is indicated by a coloured square (the intensity of the colour reflects the number of labelled cells). In the left column, hearts that have been analyzed with High Resolution Episcopic Microscopy (HREM) are indicated (3D). The identification number of the embryo is indicated in the second column. Nine categories of clones are observed: large clones where almost all regions of the venous pole are positively stained; clones where the left atrium (LA), left superior caval vein (LSCV) and pulmonary vein (PV) are labelled (LA+LSCV+PV); clones where the LA and LSCV are labelled (LA+LSCV); clones where the LSCV and PV are labelled (LSCV+PV); clones with labelling only in the LSCV (LSCV); clones where the LA and PV are labelled (LA+PV); clones with labelling only in the PV (PV); clones where the right atrium (RA) and right superior caval vein (RSCV) are labelled (RA+RSCV); clones with labelling only in the RSCV (RSCV). AP, aterial pole myocardium; RV, right ventricle; LV, left ventricle; dLA, dorsal LA; vLA, ventral LA; dRA, dorsal RA; vRA, ventral RA. Large clones (in red) were not scored in our analysis, as they correspond to very early recombination events and are not informative for distinguishing relationships between the different regions.
Online Figure III: Cell Proliferation in the myocardium of the venous pole of the heart

We went on to investigate the proliferative properties of venous and atrial myocardium. We investigated the cell cycle length in the different myocardium with co-injection of BrdU and EdU \(^9, 10\). As previously shown, the pulmonary vein myocardium has a high proliferation rate, which is twice that of atrial or caval vein myocardium \(^11\). We also estimated that the cell cycle length is relatively similar in atrial (around 15 hours), caval vein (around 14 hours) and pulmonary vein (around 13 hours) myocardium.

Percentage of BrdU (2 hours of incorporation) and EdU (30 minutes of incorporation) positive cells in atrial (A), caval vein (CV) and pulmonary vein (PV) myocardium (n=3 hearts).* (p=3x10\(^{-3}\)), ** (p=1x10\(^{-3}\)), *** (p=4x10\(^{-4}\)), **** (p=8x10\(^{-4}\)). The length of the cell cycle (Tc) was estimated and is indicated below.
Online Figure IV: Proliferative versus Stem cell modes of growth.
A theoretical model of growth and predicted distributions of clone size. In a proliferative mode of growth the pool of progenitors (in red) is smaller than in a stem cell mode of growth. The frequency of large clones is thus frequent in a proliferative mode of growth (A) than in a stem cell mode of growth (B).
Supplemental references


Online Movie I: Cell behaviour in the Right Superior Caval vein
3D reconstruction using High Resolution Episcopic Microscopy of the Right Superior Caval vein (RSCV) of an $\alpha_c$-actin$^{nlacZ+y}$ heart (number 1638) showing $\beta$-galactosidase positive cells (red) oriented circumferentially. The lumen of the vein is depicted in yellow. Three $\alpha_c$-actin$^{nlacZ+y}$ hearts were analysed and showed a similar circumferential mode of growth.

Online Movie II: Cell behaviour in the Pulmonary Vein
3D reconstruction using High Resolution Episcopic Microscopy of the Pulmonary Vein (PV) of an $\alpha_c$-actin$^{nlacZ+y}$ heart (number 1374) showing $\beta$-galactosidase positive cells (red) oriented circumferentially. The lumen of the vein is depicted in yellow. Five $\alpha_c$-actin$^{nlacZ+y}$ hearts were analysed and showed a similar circumferential mode of growth. This particular heart showed dispersed clusters of $\beta$-galactosidase positive cells suggesting that the circumferential mode of growth was preceded by a dispersive growth of progenitors.