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Movement and commitment of primitive streak precardiac cells during cardiogenesis

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ABSTRACT Fate maps are required to address questions about the commitment and differentiation of precardiac cells. Here, we report a detailed study of the precardiac cells located at the level of the primitive streak, employing different experiments with a variety of techniques combining double transplantations, microinjections and immunocytochemistry. Most cells of the more rostral segments of the primitive streak were found to contribute cells to the endodermal layer, adjacent to precardiac mesodermal cells of the heart forming region whose provenance was in the immediately more caudal segments of the primitive streak. We established a close spatio-temporal relationship between the two cell layers and the expression of their specific cardiac markers (*cNkx-2.5, Bmp2, Cripto, Usmaar, dHand, GATA4, Pitx2, Hex, Fgf8, AMHC1* and *VMHC1*). We also analyzed the ability of precardiac cells to differentiate when they are transplanted to ectopic locations or are subjected to the influence of the organizer. We propose that the precardiac cells of the primitive streak form at least two groups with different significance. One, regulated by mediation of the organizer, is located preferentially in the more rostral region of the primitive streak. It consists of the prospective cells of the endoderm layer, with a hierarchic pattern of expression of different genes characterized by its capacity for induction and regulation of a second group of cells. This second group is located preferentially in the more caudal segments, and is fated to form the precardiac mesoderm, whose differentiation would be characterized by the expression of various specific genes.

KEY WORDS: *cardiogenesis, chick embryo, fate map, gastrulation, precardiac cell*

Introduction

One of the main goals of the study of cardiac development has been to determine the location of groups of cells which, from gastrulation, contribute to the formation of the concentric layers that characterize the primitive heart tube. In avian embryos at stage PS1-5 (Lopez-Sanchez *et al.*, 2005), or HH2-3 (Hamburger and Hamilton, 1951), cells from the epiblast and primitive streak invaginate to form mesodermal and endodermal layers and contribute to cardiac development (Rosenquist and DeHaan, 1966; Rosenquist, 1970; Garcia-Martinez and Schoenwolf, 1993; Lopez-Sanchez *et al.,* 2001; Lawson and Schoenwolf, 2003). At these stages, cells arriving from the two rostral thirds of the primitive streak, excluding Hensen's node, migrate bilaterally to constitute the heart forming region (HFR) in both anterolateral areas of the embryo at stage PS11-14, or HH5-6 (Rawles, 1936; Rawles,1943; Rudnick, 1948; DeHaan and Urspung, 1965; Rosenquist, 1966; Schultheiss *et al.,* 1995; Fishman and Chien, 1997; Ehrman and Yutzey, 1999; Redkar *et al.*, 2001). This is also called the primary cardiac field (PCF) (Harvey, 2002; Brand, 2003). This bilateral area, HFR or PCF, with limits still not well defined, close to Hensen's node in the anterior half of the embryo, is characterized structurally by the presence of an area of mesodermal precardiac cells (MesoPC) between the ectoderm and underlying area constituted by the adjacent endodermal cells (AEndoC). It has been proposed (Ehrman and Yutzey, 1999) that the HFR at stage HH5 extends practically up to the border between the area opaca and the area pellucida, on both sides of

Abbreviations used in this paper: AEndoC, adjacent endodermal cells; CFSE, carboxyfluorescein diacetate succinimidyl ester; CRSE, 5-carboxytetramethylrhodamine succinimidyl ester; HFR, heart forming region; ISH, in situ hybridization; MesoPC, mesodermal precardiac cells; PCF, primary cardiac field.

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the embryo. From stage HH4, fate maps of the bilateral HFR have been completed (Redkar *et al.,* 2001) until the formation of both primitive endocardial tubes (stage HH8). Moreover, especial attention has been paid to the rostrocaudal or anteroposterior position of the precardiac cells, with the more rostral precardiac cells of the primitive streak forming mainly the arterial pole of the primitive heart tube, and the more caudal precardiac cells of the primitive streak contributing to the venous pole (Garcia-Martinez and Schoenwolf, 1993). It is still a matter of controversy, however, whether this organization is (Fishman and Chien, 1997) or is not (Redkar *et al.,* 2001) maintained in the MesoPC from stage HH4 through stage HH8. Finally, fate maps have been constructed following stage HH8 (Colas *et al*., 2000; Kirby *et al*., 2003), analyzing the role of left and right primitive endocardial tubes in the formation of the primitive heart after fusing in the midline, involving also the so-called secondary cardiac field (Maeda *et al.,* 2006).

Although the origin of definitive endoderm and mesoderm has been mapped in the chick (Schoenwolf *et al.,* 1992; Sawada and Aoyama, 1999; Lawson and Schoenwolf, 2003; Kimura *et al*., 2006; Chapman *et al*., 2007), little attention has been focussed on the precardiac cells of the primitive streak. In the present work we analyze the precise location, movement, and commitment of the precardiac cells at early stages of gastrulation, PS2-5. Furthermore, we establish a correlation between the expression pattern of different genes that are likely to be involved in cardiogenesis (*cNkx-2.5*, *Bmp2*, *Hex*, *Fgf8*, *Cripto, Usmaar*, *dHand, GATA4, GATA4-5-6,* and *Pitx2)* and the location of precardiac cells following invagination through the primitive streak. The aim was to try to compare their molecular characteristics with their commitment and differentiation during the development of the heart, because it is still unknown precisely which genes play key roles in the induction and differentiation of cardiac cells, and their implication in the establishment of the different cardiac layers.

Using various groups of techniques with combinations of double transplantations and double microinjections with different fluorescent dyes, double immunocytochemistry, and *in situ* hybridization (Lopez-Sanchez *et al*., 2004), we carried out a precise analysis of the location, movement, and commitment of precardiac cells, and of the participation of the organizer (rostral end of the primitive streak, or Hensen's node) in the control and regulation of the process of cardiogenesis. We believe that this is the first time that such an extensive group of techniques has been used to reveal the spatio-temporal behaviour of precardiac cells in relation with a broad series of specific cardiac genes, showing the ability of precardiac primitive streak cells to self-differentiate, as well as their regulation by factors located around them.

Results

A detailed picture was obtained of the location and movement of precardiac cells using a variety of techniques to construct a precise fate map. Also, with the design of different experimental procedures, we established several mechanisms involved in precardiac cells' commitment and ability to differentiate when they are transplanted to ectopic locations, as well as shedding further light on the role of the organizer in the cardiogenic process.

Fate mapping of primitive streak cells

The location and movement of different groups of cells of the primitive streak during gastrulation was analyzed by means of double microinjections, double transplantation, and double immunocytochemistry. Simultaneously, we analyzed the expression of several genes related with precardiac areas using *in situ* hybridization (ISH). As seen in Fig. 1, four levels of the primitive streak (C0.5-C2) were subjected to double microinjection or transplantation, and subsequently identified by immunocytochemistry to show the pattern of migration through several locations in the developing chick, including precardiac and cardiac structures. Cells from the primitive streak were found to migrate bilaterally in

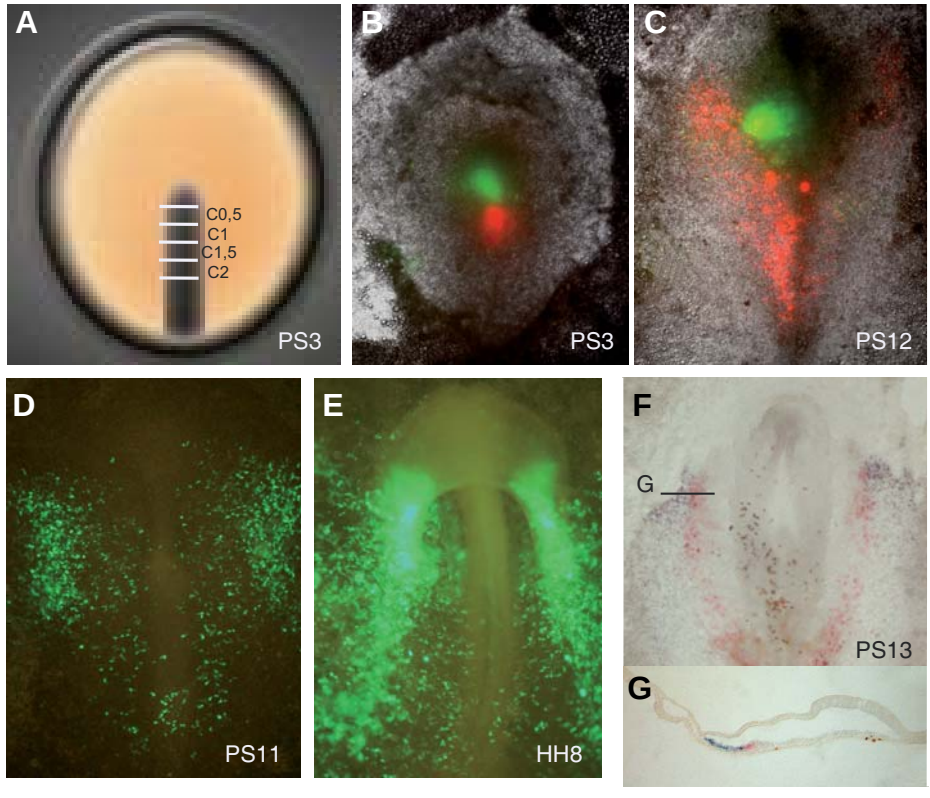

Fig. 1. Fate mapping of primitive streak cells. **(A)** *Schematic diagram showing the four analyzed sites of the primitive streak (C0.5-C2).* **(B)** *Double microinjection of site C0.5 (CFSE, green spot) and C1.5 (CRSE, red spot), to show the rostro-lateral distribution of cells* **(C)**. *Electroporated embryo at the level of segment C1.5 showing the subsequent distribution of cells at the level of the HFR* **(D)** *and then at the level of the two primitive endocardial tubes* **(E)**. *Whole mount* **(F)** *and section* **(G)** *of an embryo subjected to double transplantation of sites C1 and C2 after ISH and double immunocytochemistry, showing in the mesoderm the expression of* Usmaar *(blue) and the distribution of cells from C2 (red), and in the endoderm the cells from C1 (brown).*

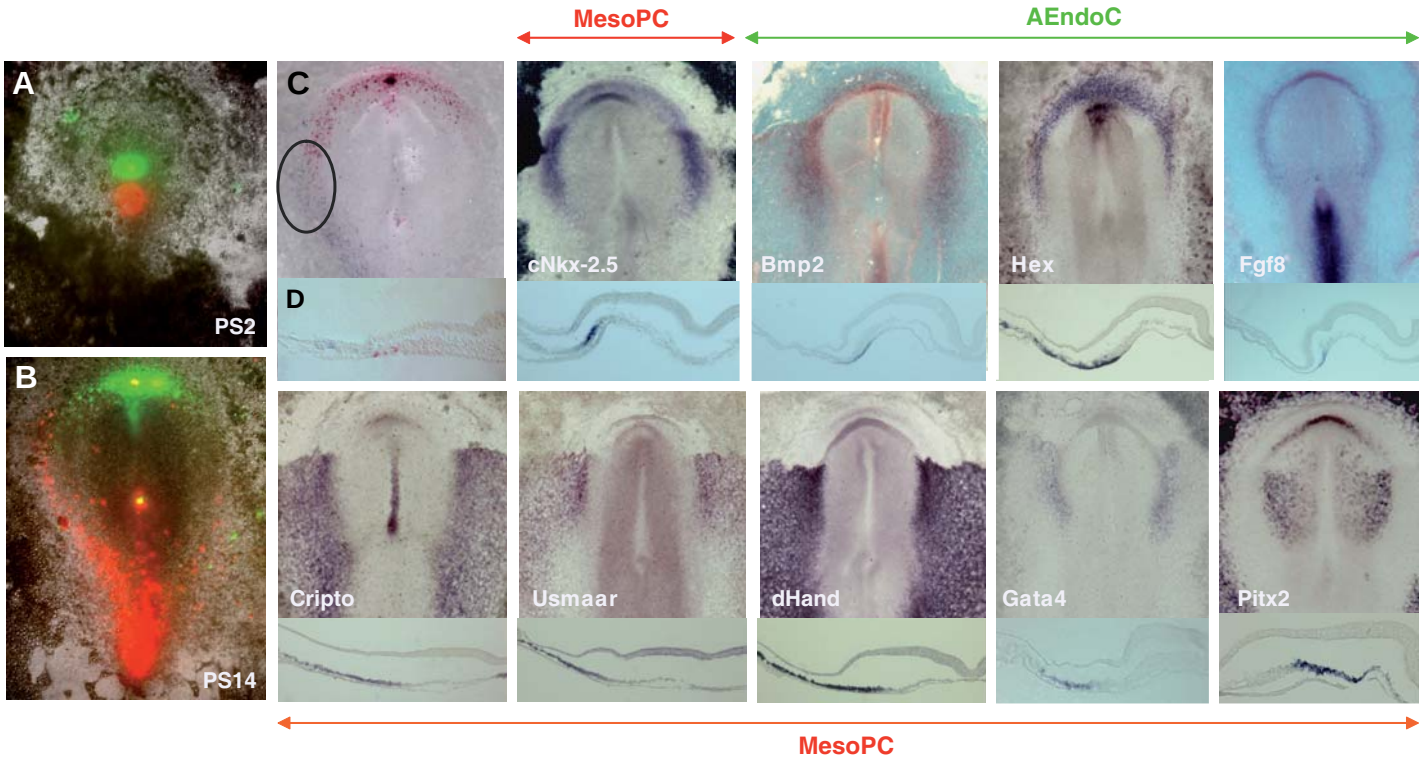

Fig. 2. Correlation between cells from sites C0.5 or C1.5, distributed in the HFR, and gene expression patterns. (A) *Double microinjection of CFSE (green spot) and CRSE (red spot) to identify 12 hours later the rostro-lateral distribution of the cells* **(B)**. *Double immunocytochemistry* **(C,D)** *shows the AEndoC (red) arriving from site C0.5, and the MesoPC (blue) from site C1.5. Note the overlap of the two groups of cells at the level of the HFR (ellipse). Whole mount and sections of embryos (PS14) subjected to ISH show at the level of the MesoPC the expression of* cNkx-2.5, Cripto, Usmaar, dHand, GATA4, *and* Pitx2, *and at the level of the AEndoC the expression of* Bmp2, Hex, *and* Fgf8. *A crescent-shaped pattern is observed in the expression of* cNkx-2.5, Bmp2, *and* Hex, *similar to the cell distribution observed after labeling the primitive streak cells.*

a rostro-lateral direction, giving rise to the endodermal layer and mesodermal structures, and following a precise pattern of migration until their final location into the heart forming region (HFR) or primary cardiac field (PCF), where their differentiation takes place.

A few hours later, stages PS11-14 (Fig. 2), most cells located at the rostral site (C0.5) contributed in the anterior half of the embryo to forming the more anterior and lateral endodermal area, in a crescent-shaped pattern; while cells from a more caudal site (C1.5) at the same time contributed to forming bilateral mesodermal areas, reaching the border with the area pellucida, and overlapping with the caudal limits of the aforementioned crescent. At these stages, PS11-14, several specific markers are closely related to this cell distribution. A group of genes, including *cNkx-2.5*, *Bmp2*, and *Hex*, showed in whole mount a crescent of expression, similar to the cell distribution observed after the primitive streak cell labeling. At the level of the HFR (or PCF), we observed that *cNkx-2.5* initially expressed at the level of the MesoPC, where there was expression of some other genes, including *Cripto, Usmaar*, *dHand*, *GATA4*, and *Pitx2.* The expression of *Bmp2* and *Hex*, however, was at the level of the AEndoC, the location also observed for the expression of *Fgf8*.

Analyzing the fate several hours later (HH7), we observed that most cells located at the rostral site (C0.5), which were to form the endodermal layer, contributed to forming the endoderm of the foregut, as well as the endoderm underlying the splanchnopleure (Fig. 3). Interestingly, we observed that the second group of cells of the primitive streak (C1.5) contributed cells to this bilateral precardiac splanchnic mesoderm, to form from HH7 the primitive endocardial tubes. Specific markers closely related to this step of cardiogenesis showed restricted expression at the level of the areas related with primitive endocardial tubes. Thus, from this stage, while *cNkx-2.5* and *Bmp2* were expressed in both the precardiac splanchnic mesoderm and the underlying endoderm, *Cripto, Usmaar*, *dHand*, *GATA4*, and *Pitx2* expressed only in the former, and *Hex* and *Fgf8* only in the latter.

The study of another two groups of primitive streak cells, C1 and C2 (Fig. 4), located caudally to the foregoing sites, showed the first group of cells (coming from C1) to be distributed mainly at the level of the endoderm, underlying the second group of cells (coming from C2) located at the level of the splanchnic mesoderm of the heart rudiment, close to the amniocardiac vesicle portion of the coelom. From stage HH8, we followed the specific markers closely related to the process of cardiogenesis. At the level of the primitive endocardial tubes when they are beginning to fuse at the level of the midline, we observed expression of *cNkx-2.5* and *Bmp2* together with *Cripto*, *Usmaar*, *dHand*, *GATA4*, and *GATA4-5-6* in the splanchnic mesoderm of heart rudiment, with the characteristic asymmetric expression of *Pitx2* in the left lateral plate mesoderm. Moreover, *Hex* and *Fgf8* expressed in the underlying endoderm together with *cNkx-2.5* and *Bmp2*.

We observed that, when the two endocardial tubes finally move

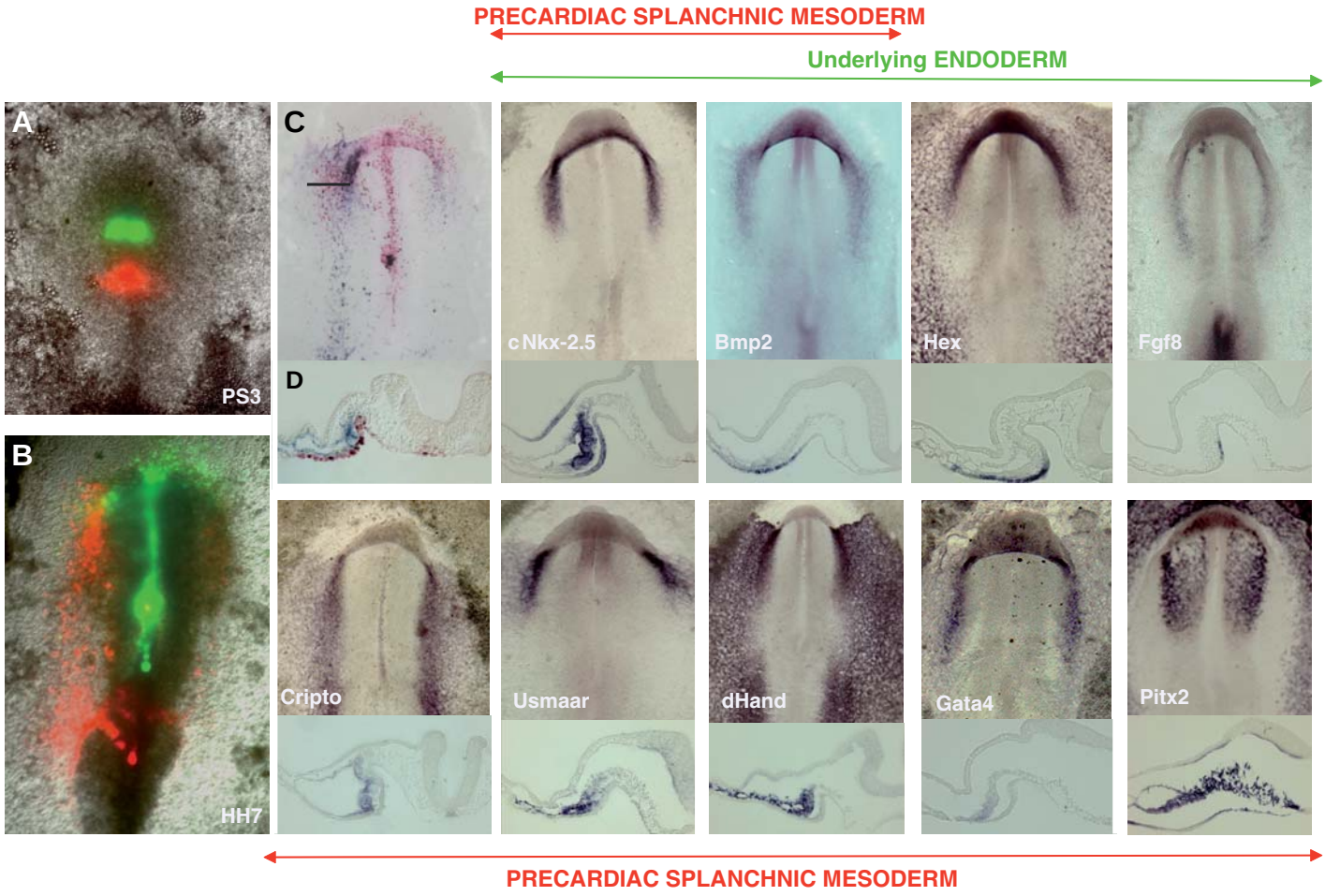

Fig. 3. Correlation between cells from sites C0.5 or C1.5, distributed in the primitive endocardial tubes, and gene expression patterns. (A) *Double microinjection of CFSE (green spot) and CRSE (red spot) to identify 14 hours later the rostro-lateral distribution of the cells* **(B)**. *Double immunocytochemistry* **(C,D)** *shows the cells from site C1.5 forming the precardiac splanchnic mesoderm (blue), and cells from site C0.5 forming the underlying endoderm (red). Whole mount and sections of embryos (HH7-8) subjected to ISH show the expression of* Cripto, Usmaar, dHand, GATA4, *and* Pitx2 *at the level of the precardiac splanchnic mesoderm, and the expression of* Hex *and* Fgf8 *at the level of the underlying endoderm. Note that* cNkx-2.5 *and* Bmp2 *show expression in both layers.*

to the midline and fuse to form the primitive heart tube, cells from the rostral-most site of the primitive streak mainly contribute to foregut endoderm and dorsal mesocardium, and the other more caudal sites analyzed form the myocardial and endocardial layers of the primitive heart tube (Fig. 5).

As in our previous fate maps, some cells from the head mesenchyme, blood vessels, ventral and dorsal aortae, and endoderm or mesoderm not involved in heart structures, were also observed in our experimental embryos.

Commitment and regulation mechanisms of precardiac cells

The state of commitment of precardiac cells located at the level of different segments of the primitive streak was studied by their transplantation into host embryos at different positions. We also studied the possible role of the rostral tip of the primitive streak as a potential regulatory mechanism in cardiogenesis.

Grafts of prospective cardiac cells into the germ cell crescent

Groups of cells corresponding to sites C0.5 and C1 at stage PS3 when transplanted to the germ cell crescent at stage PS8 were able to form an endodermal-like layer, accompanied by a closely related tissue, which expressed the earliest specific cardiac marker *cNkx-2.5* (Fig. 6A-D). Furthermore, groups of cells corresponding to sites C1.5 and C2 (Fig. 6E) were able to differentiate, expressing not only *cNkx-2.5* but also specific markers of atrial, *AMHC1* (Fig. 6F), and ventricular, *VMHC1* (Fig. 6G,H), cardiac segments.

Grafts of prospective cardiac cells into prospective somitic sites, and vice versa

Transplantation of cells (Fig. 7) from segment C1.5 of donor embryos at stage PS3 (precardiac cells) to site C1.5 at stage PS8 (presomitic site; Schoenwolf *et al.*, 1992) showed the precardiac cells to change their fate, to form the somites. Furthermore, they expressed the specific somitic marker *paraxis* (Fig. 7B,C). Transplantation of cells from segment C1.5 of donor embryos at stage PS8 (presomitic cells) to site C1.5 at stage PS3 (precardiac site) showed the presomitic cells to also change their fate, to form

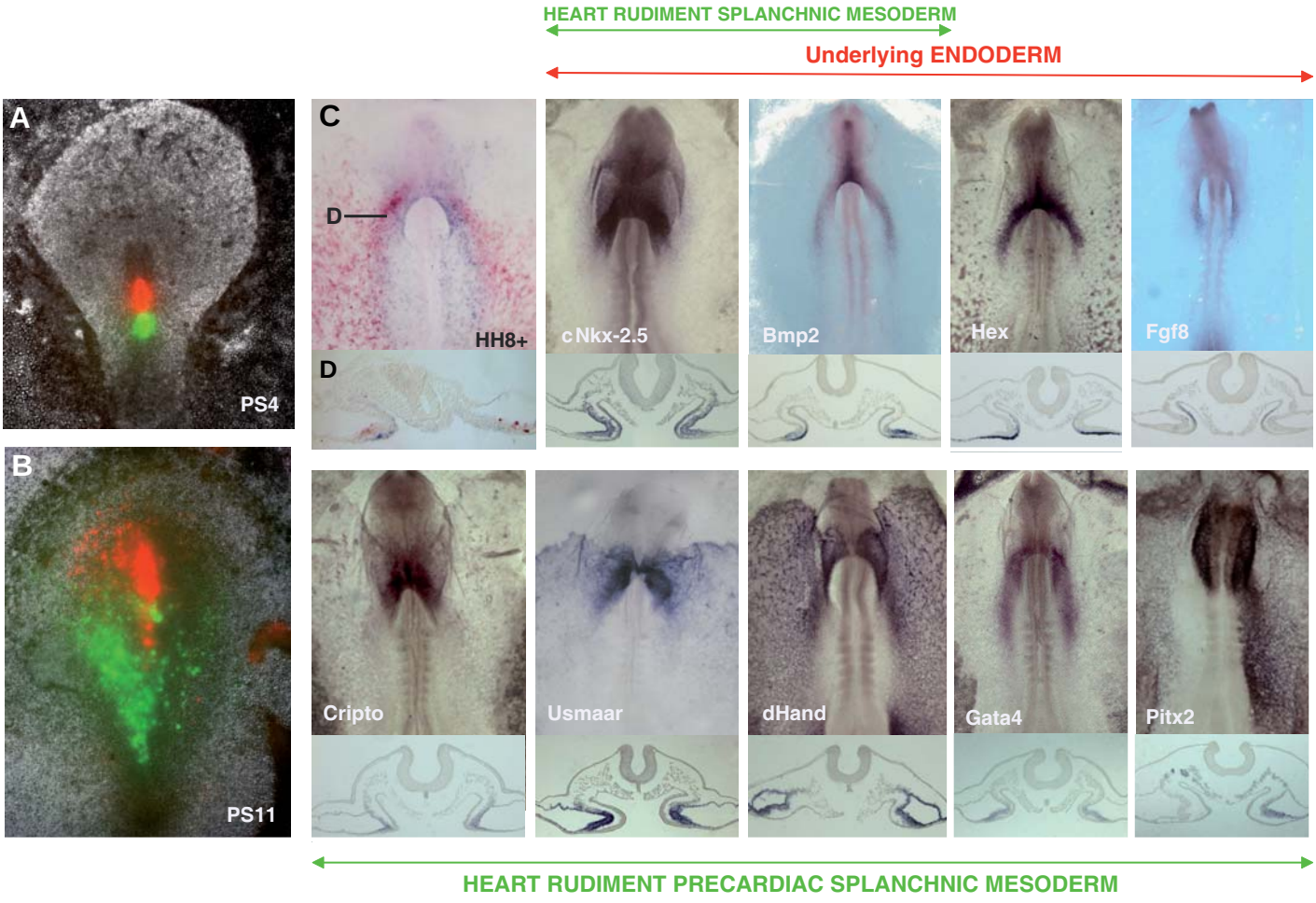

Fig. 4 (Top). Correlation between cells from sites C1 or C2, distributed in the primitive endocardial tubes, and gene expression patterns. (A) *Double microinjection of CRSE (red spot) and CFSE (green spot) to identify 16 hours later the rostro-lateral distribution of the cells* **(B)**. *Double immunocytochemistry* **(C,D)** *shows the cells from site C2 forming the splanchnic mesoderm of the heart rudiment (red), and cells from site C1 forming the underlying endoderm (blue). Whole mount and sections of embryos (HH8-9) subjected to ISH show the expression of* Cripto, Usmaar, dHand, GATA4, *and* Pitx2 *at the level of the heart rudiment splanchnic mesoderm, and the expression of* Hex *and* Fgf8 *at the level of the underlying endoderm. Note that* cNkx-2.5 *and* Bmp2 *show expression in both layers.*

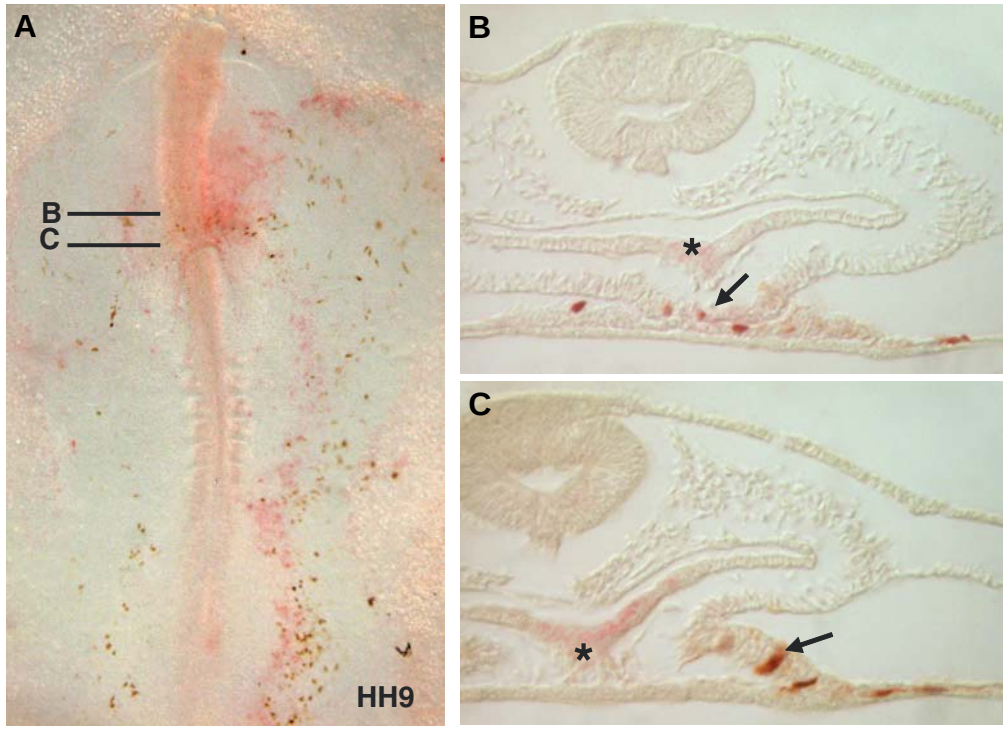

Fig. 5 (Right). Distribution of cells from sites C1 and C2 in the primitive heart tube. *Whole mount* **(A)** *and sections* **(B,C)** *after double transplantation and immunocytochemistry showing the distribution of cells from site C1 (red) at the level of the dorsal mesocardium and foregut endoderm (asterisks), and cells from site C2 (brown) at the level of the endocardial and myocardial layers (arrows).*

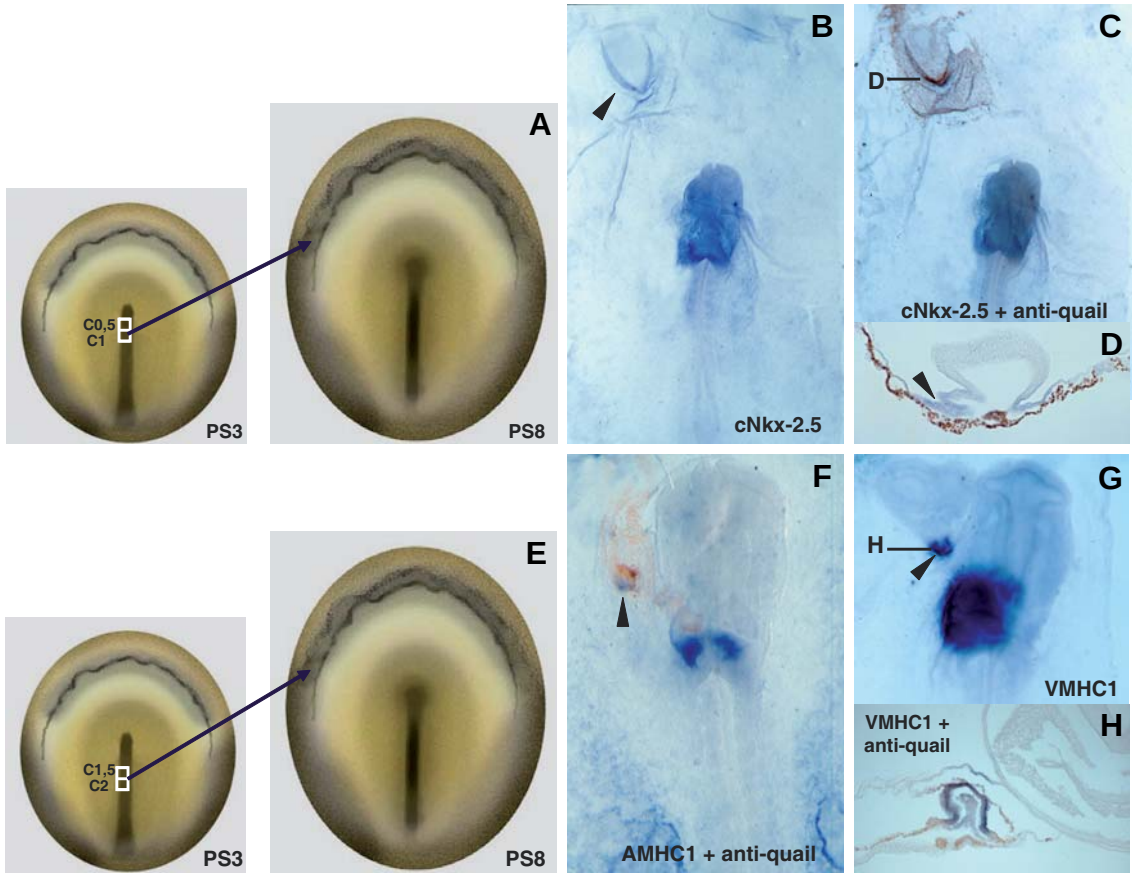

Fig. 6. Grafts of prospective primitive streak cardiac cells into the germ cell crescent. (A) *Schematic diagram showing the experimental procedure of transplantation of more rostral sites (C0.5 and C1) into the germ cell crescent.* **(B)** *Whole mount ISH showing the expression of* cNkx-2.5 *at the level of the primitive heart of the host embryo and at the level of the transplanted cells (arrowhead).* **(C,D)** *Whole mount and section after ISH and antiquail immunocytochemistry showing the quail cells (brown) forming at the level of the transplant an endodermal-like layer underlying a tissue expressing* cNkx-2.5 *(arrowhead).* **(E)** *Schematic diagram showing the experimental procedure of the transplantation of more caudal sites (C1.5 and C2) to the germ cell crescent.* **(F)** *Whole mount ISH showing the expression of the atrial cardiac marker* AMHC1 *at the level of the host embryo's heart and at the level of the transplanted cells (arrowhead). Whole mount* **(G)** *after ISH showing the expression of the ventricular cardiac marker* VMHC1 *at the level of the host embryo's heart and at the level of the transplanted cells (arrowhead). Note in the section after immunocytochemistry* **(H)** *the quail cells (brown) expressing* VMHC1 *(blue).*

heart, expressing *cNkx-2.5* (Fig. 7D-G).

Grafts of the rostral tip of the primitive streak into HFR

As can be seen in Fig. 8, the rostral tip of donor embryos at stage PS3 transplanted to HFR of a host embryo at stage PS11 gave rise a few hours later to an area of loss of expression of the early cardiac marker *cNkx-2.5* at the level of the HFR in the host embryo (Fig. 8B). On the contrary, the Hensen's node of donor embryos at stage PS8 transplanted to HFR (PS11) induced an increased area of expression of this marker (Fig. 8D).

Discussion

One of the main questions in the process of cardiac development is what is the origin and behaviour of precardiac cells before this organ has formed. It is known (Lopez-Sanchez *et al.*, 2001) that they are detectable in the initial stages of gastrulation (PS1) at the level of the epiblast and later during their invagination through the primitive streak. Detailed fate maps have been constructed for both prospective mesodermal and endodermal cells (Schoenwolf *et al.,* 1992; Sawada and Aoyama, 1999; Lawson and Schoenwolf, 2003; Kimura *et al.*, 2006; Chapman *et al.*, 2007), however, they have been not focussed on the origin and displacement of the prospective cardiac cells during heart development. Although various workers (Fishman and Chien, 1997; Ehrman and Yutzey, 1999; Redkar *et al.*, 2001) have contributed to constructing a precardiac cell fate map, there still remain several aspects to explain, such as the correlation between the position of the precardiac cells at the level of the primitive streak with their position at the level of the HFR (or PCF), and their final position at the level of the primitive heart tube. In the present study, we have made a detailed analysis of the origin, location, and movement of precardiac cells until their fate is determined in the formation of the structure of the HFR, the endocardial tubes, and finally the primitive heart tube. Our results show that the more rostral region of the primitive streak, with the exception of Hensen's node, migrates bilaterally in a rostro-lateral direction to form part principally of the endoderm layer, including the AEndoC. The primitive streak cells positioned immediately caudal to that region follow a parallel but more lateral direction,

and constitute the MesoPC which will later become the precardiac splanchnic mesoderm. This could be a very important aspect to take into account in cardiogenesis, since it has been suggested (Schultheiss *et al.*, 1995, 1997; Ehrman and Yutzey, 1999) that the AEndoC induces the initiation of cardiogenesis, acting on the MesoPC. It could be hypothesized that the cells which invaginate at the level of the more rostral segments of the primitive streak play a role in later stages in the induction and regulation of the differentiation of the cells that have invaginated through the more caudal segments and which form the layers characterizing the structure of the primitive heart tube (Fig. 9). We have previously reported (Schoenwolf *et al.*, 1992; Garcia-Martinez and Schoenwolf, 1993) that the more rostral cells of the primitive streak mainly form the arterial pole of the heart tube, and the more caudal mainly the venous pole, and even that in stage PS8 some Hensen's node cells are capable of forming part of the arterial pole endocardium (Schoenwolf *et al.*, 1992; Kirby *et al.*, 2003). With the results of the broad group of techniques used in the present work, the hypothesis suggests itself that the cells of the primitive streak consist of two groups with different significance. One group of cells, located preferentially in the more rostral region of the primitive streak ($C_{0.5}$-C_1), would be characterized by their prospective capacity for induction and regulation (AEndoC) of a second group of cells. This second group, located preferentially in more caudal segments ($C_{1.5}$-$C_{2.5}$), is fated to form and differentiate the MesoPC, to constitute the precardiac splanchnic mesoderm, and subsequently the layers of the primitive heart tube (Fig. 9).

Various workers (Fishman and Chien, 1997; Ehrman and Yutzey, 1999; Redkar *et al.*, 2001) have tried to establish the configuration and arrangement of the cells constituting the HFR, and to delimit this area using techniques of DiI crystal implantation and DiI microinjections. There appeared evident discrepancies between these studies with respect to the boundary and organization of precardiac cells in the HFR, since there are no clear anatomical limits to define the region. In an attempt to contribute to understanding the process of the constitution, delimitation, and migration of the cells in the HFR, we have here described a fate map study of precardiac cells from the primitive streak, together with a detailed analysis of the expression of different specific genes of the heart or intimately related with its development. Our results show that precardiac cells, from their localization in the primitive streak to their position in the primitive heart tube, and the cells intimately related with them (AEndoC), are characterized by presenting a hierarchic pattern of expression of different genes. The expression of *Bmp2*, which has been proposed to be the inductor of cardiogenesis (Lough *et al.*, 1996; Schultheiss *et al.*, 1997; Andrée *et al.*, 1998; Schlange *et al.*, 2000; Alsan and Schultheiss, 2002; Lopez-Sanchez *et al.*, 2002) begins in the AEndoC, which is the first group of cells coming from the rostral sites of the primitive streak, followed a few hours later in both the precardiac splanchnic mesoderm and the underlying endoderm. However, the earliest cardiac marker *cNkx-2.5* (Schultheiss *et al.*, 1995) was first expressed at the level of the MesoPC, whose cells come from more caudal segments of the primitive streak, followed later by expression at the level of the MesoPC and AEndoC simultaneously. In view of these data, together with the gene expression analysis at the level of the primitive streak at these stages (PS1—14, Lopez-Sanchez *et al.*, 2005), we could hypothesize that the second group of cells of the primitive streak (prospective MesoPC) is regulated by the first group (prospective AEndoC), which in turn would be regulated by mediation of the rostral tip or Hensen's node (Fig. 9). Moreover, this process of initiation of regulation of cardiogenesis could be controlling genes that require expression at the level of the AEndoC, such as *Fgf8* (Alsan and Schultheiss, 2002) and *Hex* (Zhang *et al.*, 2004), while other genes, such as *Cripto* (Colas and Schoenwolf, 2000), *Usmaar* (Colas *et al.*, 2000), *dHand* (Srivastava *et al.*, 1995), *GATA4, GATA4-5-6*

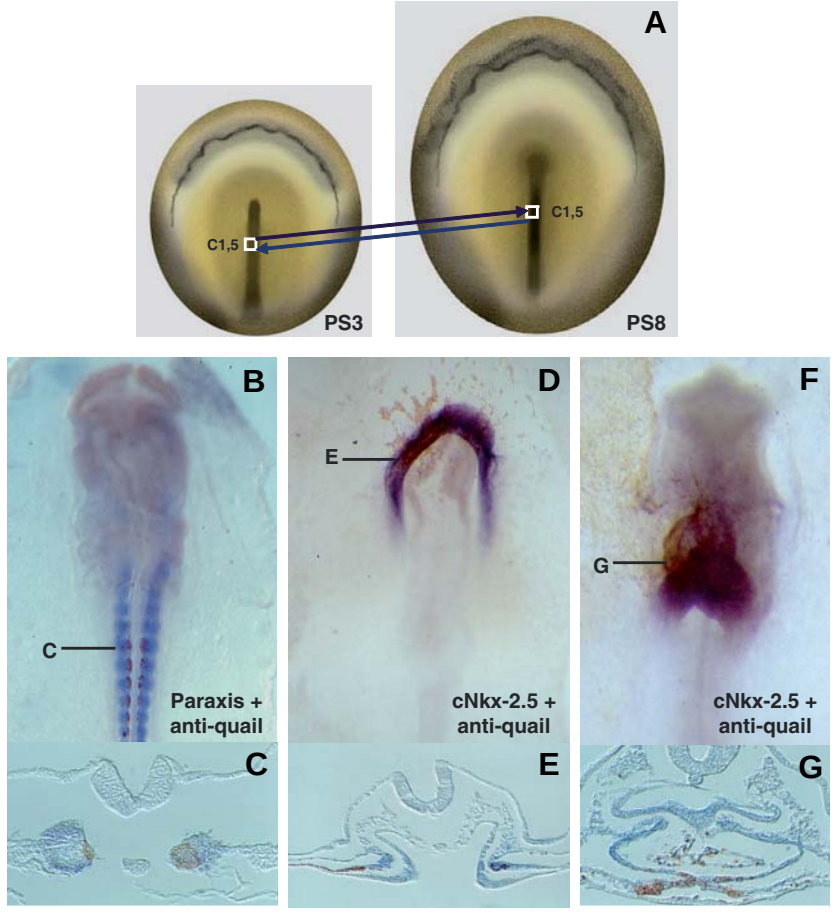

Fig. 7. Grafts of prospective primitive streak cardiac cells into the prospective somitic cells, and vice versa. (A) *Schematic diagram showing the experimental procedure of the transplantation of site C1.5, between embryos at stages PS3 (precardiac cells) and PS8 (presomitic cells). Whole mount and section* **(B,C)** *after ISH and immunocytochemistry showing the quail precardiac cells (brown) transplanted into presomitic site expressing* paraxis *(specific somitic marker).* **(D-G)** *Whole mount and section after ISH and immunocytochemistry showing the expression of* cNkx-2.5 *at the level of the precardiac splanchnic mesoderm* **(D,E)** *and endocardium and myocardium of the primitive heart tube* **(F,G)**. *Note the quail presomitic cells (brown) transplanted into the precardiac site expressing* cNkx-2.5 *at the new locations in cardiac layers.*

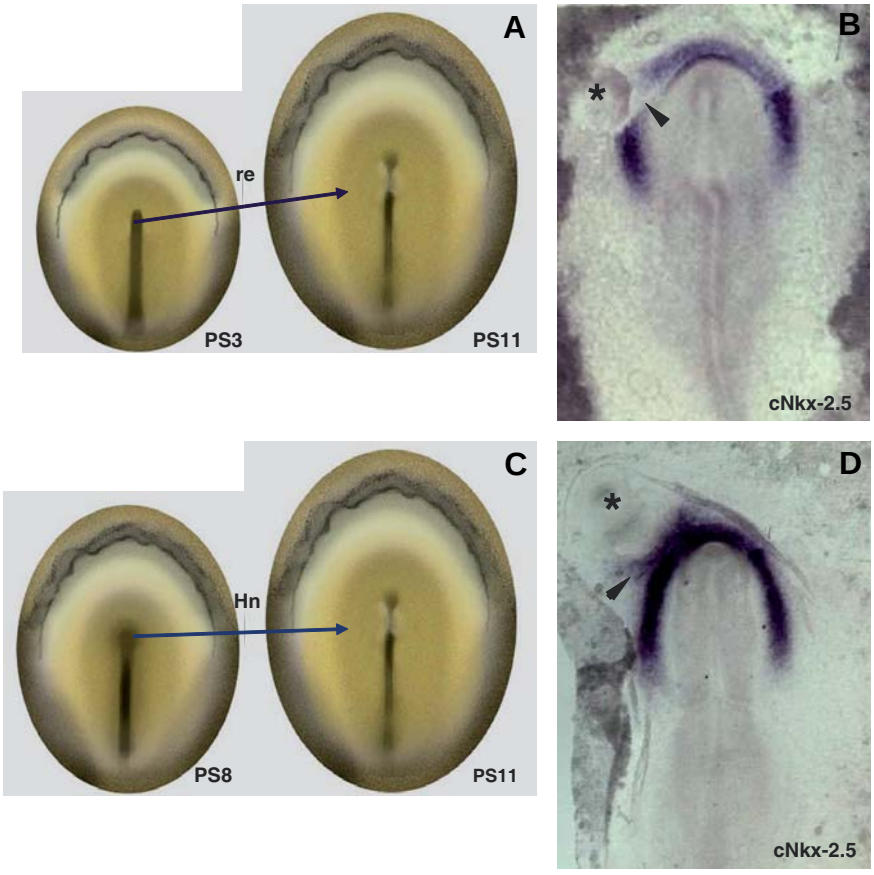

Fig. 8. Grafts of the tip of the primitive streak into the HFR. (A) *Schematic diagram showing the experimental procedure of the transplantation of the rostral end (re) from a donor embryo at stage PS3 into the HFR of a host embryo at stage PS11.* **(B)** *ISH showing the area of lost expression of* cNkx-2.5 *(arrowhead) in the HFR in the host embryo due to the transplanted rostral end (asterisk).* **(C)** *Schematic diagram showing the experimental procedure of transplantation of Hensen's node (Hn) from a donor embryo at stage PS8 into the HFR of a host embryo at stage PS11.* **(D)** *ISH showing the increased area of expression of* cNkx-2.5 *(arrowhead) in the host embryo due to the transplanted Hensen's node (asterisk).*

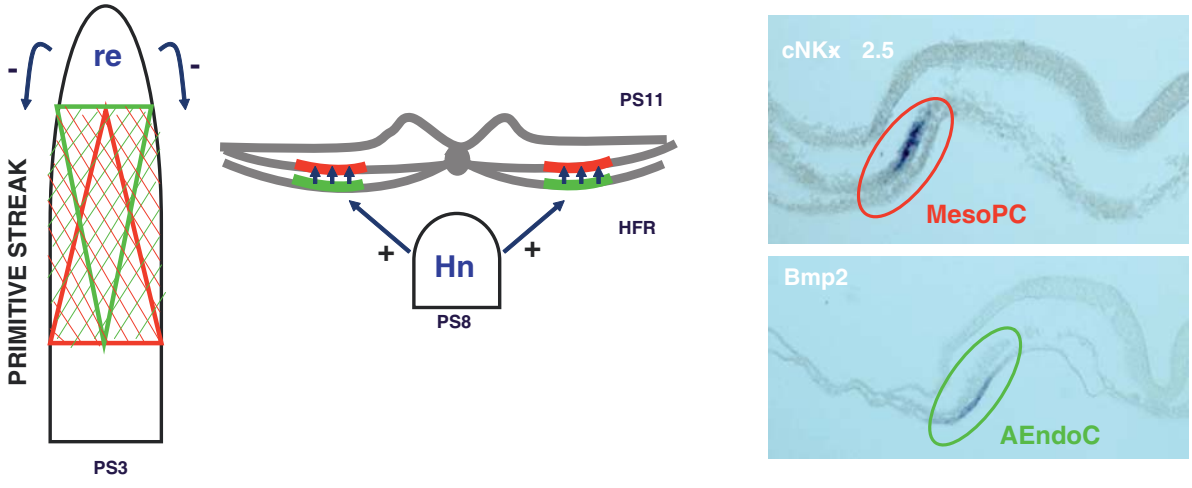

Fig. 9. A model of the significance of primitive streak precardiac cells. *The precardiac cells of the primitive streak are distributed into two groups with different significance. The group of cells located in the more rostral sites of the primitive streak, with a decreasing gradient towards more caudal sites (green triangle), is regulated by the rostral end (re), and is responsible for the formation of the endoderm layer (green line) characterized by the expression of specific genes, such as the initial expression of* Bmp2 *(green ellipse). The second group is located in more caudal sites of the primitive streak, with a decreasing gradient towards the more rostral sites (red triangle). This group will be responsible for the formation of the MesoPC (red line), differentiating subsequently to form the precardiac splanchnic mesoderm and primitive heart tube. It is regulated by the cells of the previous group, and expresses specific genes, such as the initial expression of* cNkx-2.5 *(red ellipse). At later stages, the Hensen's node (Hn) regulates the cell differentiation of the HFR.*

(Laverriere *et al.*, 1994; Jiang *et al.*, 1998), and *Pitx2* (Piedra *et al.*, 1998), would be regulated to be expressed in the MesoPC and to maintain their expression in the course of the differentiation of these cells towards precardiac splanchnic mesoderm and primitive endocardial tubes. Some other specific genes are required in later phases for cardiac morphogenesis, including *AMHC1* (Yutzey *et al.*, 1994; Somi *et al.,* 2006), *VMHC1* (Bisaha and Bader, 1991; Somi *et al.,* 2006), *Tbx1*, (Huynh *et al.*, 2007), and *Tbx5* (Bruneau *et al.*, 1999; Plageman and Yutzey, 2004).

Altogether, these data indicate that the potentiality of primitive streak cells to differentiate towards heart will depend not only on their own spatio-temporal characteristics, but also on the inductive and/or regulatory factors with which they maintain an intimate relationship. For this reason, in the present work we also analyzed the following two aspects: the commitment of the precardiac cells of the primitive streak, and their regulatory mechanisms. Our results showed that the more rostral precardiac cells of the primitive streak, transplanted to the germ cell crescent, are able to constitute an endodermal-like layer, accompanied by a closely related tissue, which expresses the earliest specific cardiac marker *cNkx-2.5*, whereas the more caudal cells have the capacity for differentiation and *cNkx-2.5* expression as well as cardiac sector markers, *AMHC1* and *VMHC1*. The data suggest that the precardiac cells of the primitive streak already carry information for their fate towards cardiogenesis, and lend support to the hypothesis put forward above (Fig. 9). Nonetheless, if the precardiac cells of the primitive streak are homotopically and heterochronically transplanted to presomitic sites of the primitive streak, they lose their capacity to migrate towards the heart and their expression of specific cardiac genes (and vice versa). These data support the hypothesis that, at the level of the primitive streak, there have to exist factors that determine the mechanisms establishing the regulation of the movement and differentiation of its cells, with the capacity to modify their initial information. In our attempt to contribute new data on the possible factors involved in this process of induction and regulation, we have been able to demonstrate the participation of the rostral tip of the primitive streak, showing that this end of a stage PS2—5 primitive streak negatively regulates the expression of genes involved in the initiation of cardiogenesis. The properties of a later stage Hensen's node, with different morphological and molecular characteristics (Lopez-Sanchez *et al.*, 2005), are needed for this process to begin. This finding supports our earlier results (Lopez-Sanchez *et al.*, 2002) of the capacity of Hensen's node to mediate the induction of the process of cardiogenesis.

Fate map studies and analyses of the capacity for differentiation and/or induction of the cells of the primitive streak are not enough to provide precise knowledge of the factors that determine the process of cardiogenesis and cardiac morphogenesis. Also required is experimental analysis of the molecular factors that regulate the MesoPC and AEndoC that arise at the level of the primitive streak. This work is currently under development in our laboratory.

Materials and Methods

Chick and quail embryos were used in this study, following different procedures in accordance with our protocols described previously (Darnell *et al.,* 2000; Lopez-Sanchez *et al.*, 2004).

For whole embryo culture, fertile eggs were obtained from reliable suppliers and incubated at 38°C in forced-draft, humidified incubators, until the embryos reached the desired stage. The embryos were then removed from the egg and cultured on an agar-albumen bed in modified New (New, 1955) or EC (early chick, Chapman *et al.*, 2001) cultures.

Location and movements of precardiac cells

Chick blastoderms at stages PS2-5 (Lopez-Sanchez *et al.*, 2005) were subjected to microinjection of fluorescent dyes, transplantation of labeled groups of quail cells, and electroporation (Fig. 1). Four sites (125 μm square fragments) were selected within the primitive streak (Fig. 1A), designated as sites C0.5 through C2 as previously described (Garcia-Martinez and Schoenwolf, 1993).

Double microinjection of fluorescent dyes

To determine the initial location and the movement of different groups of cells, several pre-selected primitive-streak sites were injected simultaneously with different fluorescent dyes (Lopez-Sanchez *et al.,* 2004). Into each chick blastoderm (n=92; n refers to the number of embryos successfully labeled and studied), two solutions were pressure-injected using a Picospritzer II (General Valve Corp., Fairfield, NJ) and a micromanipulator (Fig. 1B,C). A first single small bolus of a solution of DiI/CRSE (Molecular Probes, Inc., Eugene, OR) was microinjected into a selected area of the primitive streak (red fluorescent spot), and a second small bolus of a solution of DiO/CFSE (Molecular Probes, Inc., Eugene, OR) was microinjected immediately after the first injection (green fluorescent spot). Each injection was made across the width of the primitive streak. Embryos were immediately examined with a fluorescence microscope to confirm the size and site of each injection, and they were then reincubated for periods up to 24 hours, during which they were examined at regular intervals.

Double transplantation of labeled groups of cells

Grafts of two pre-selected primitive-streak segments from donor quail embryos were obtained using a cactus needle, and transplanted homotopically and isochronically to host chick embryos to construct chimeras (n=44, n refers to the number of host embryos successfully labeled and studied). Two groups of donor embryos were labeled with fluorescent markers (Lopez-Sanchez *et al.*, 2004). One group was labeled with a solution of CRSE. The other was labeled with a solution of CFSE. Grafts were transferred to a host embryo in which a similar fragment had previously been removed. Two grafts were transplanted, one labeled with CRSE (red fluorescent spot), and the other with CFSE (green fluorescent spot).

Electroporation of selected primitive-streak sites

For electroporation (Colas and Schoenwolf, 2003), each embryo (n=18, n refers to the number of embryos successfully labeled and studied) was transferred ventral-side-up to an agarose culture plate that contained the tungsten cathode electrode (tip 2 mm long and 100 μm diameter) embedded in the agarose. The primitive streak of the embryo was aligned with the cathode located underneath. Next, a plasmid DNA solution (pCAGGS-EGFP vector) was microinjected using an Inject + Matic microinjector system (INJECT + MATIC, Geneva) into the desired site of the primitive streak. Immediately after DNA injection, the anode (platinum, tip 2mm long and 300 μm diameter) was placed just over the injected precardiogenic primitive streak area, and electric pulses were applied. A train of five square wave pulses (40-ms duration at 5V with 999-ms intervals) was applied using a CUY21 EDIT square electroporator (NEPA GENE, Tokyo). This technique allows the cells of the different segments of the primitive streak to be precisely marked, and then followed in whole mount under fluorescence microscopy until their localization in the heart tube (Fig. 1D,E).

State of commitment of precardiac cells and mechanisms of regulation

To determine the state of commitment of precardiac cells, as well as possible mechanisms involved in early cardiogenesis, quail primitive

streak segments were transplanted heterochronically, and hetero- or homo-topically, to chick host blastoderms.

Grafts of prospective cardiac cells into the germ cell crescent

Grafts of site C0-5-C2 segments of quail embryos at stage PS3 (Fig. 6) were transplanted into the germ cell crescent of chick embryos at stage PS8 (n=28, n refers to the number of host embryos successfully labeled and studied).

Grafts of prospective cardiac cells into prospective somitic sites and vice versa

Grafts of the site C1.5 segment of quail embryos at stage PS3, which contributes to forming the heart tube (Garcia-Martinez and Schoenwolf, 1993), were transplanted to site C1.5 of chick embryos at stage PS8 (n=8), which contributes cells to form the somites (Schoenwolf *et al.*, 1992). And vice versa, grafts of the site C1.5 segment of quail embryos at stage PS8 were transplanted to site C1.5 of chick embryos at stage PS3 (n=11) (Fig. 7).

Grafts of the tip of the primitive streak into HFR

The rostral end (125 μm in length) of quail embryos at stage PS3 was transplanted into the HFR of chick embryos at stage PS11 (n=18). This experiment was also performed (n=12) with the quail Hensen's node (125 μm in length) from stage PS8 (Fig. 8).

Identification of precardiac cells and gene expression pattern

After 4-16h of incubation, experimental embryos were fixed overnight in 4% PFA, and subjected to one or more of the following techniques.

Immunocytochemistry

Two antibodies were used for immunocytochemistry to determine the location of the transplanted quail cells: QH1 (which identifies quail endothelial and endocardial cells) and antiquail antibody (Darnell *et al.*, 2000).

In order to identify the fluorescence (i.e., CRSE and CFSE) labeled cells at the end of the incubation period, embryos subjected to microinjection or transplantation were processed for double whole mount immunocytochemistry. To detect cells labeled with CRSE and CFSE, we used an alkaline phosphatase conjugated goat anti-rhodamine, and a horseradish peroxidase conjugated sheep anti-fluorescein, respectively, according as described previously by us (Lopez-Sanchez *et al.*, 2004).

In situ hybridization (ISH)

To determine the expression pattern of several genes known to involved in cardiogenesis, a group of control embryos from stage PS14 through stage HH9 were fixed and processed for ISH (Nieto *et al.*, 1996) using a broad series of chick riboprobes for *cNkx-2.5*, *Bmp2*, *Cripto, Usmaar, dHand, GATA4, GATA4-5-6, Pitx2, Hex,* and *Fgf8*. A group of experimental embryos, previously subjected to transplantation, was also analyzed using the riboprobes *VMHC1, AMHC1,* and *paraxis* (Burgess *et al.*, 1995), as well as by immunocytochemistry which provides double and triple labeled areas that can be detected in whole mounts and histological sections (Fig. 1F,G).

Finally, embryos for paraffin histology were dehydrated with an ethanol series and then cleared in isopropanol and processed for paraplast embedding. Serial transverse or longitudinal sections were cut at 15 μm.

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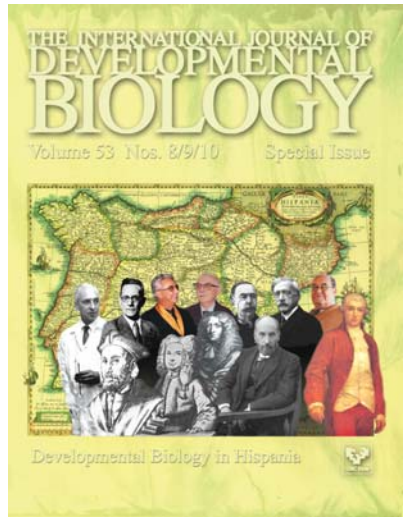

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