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BRIEF COMMUNICATION

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Fsp1 cardiac embryonic expression delineates atrioventricular endocardial cushion, coronary venous and lymphatic valve development

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Abstract

Fsp1 (a.k.a S100A4 or Metastatin) is an intracellular and secreted protein widely regarded as a fibroblast marker. Recent studies have nonetheless shown that Fsp1 is also expressed by other cell types, including small subsets of endothelial cells. Since no detailed and systematic description of Fsp1 spatio-temporal expression pattern in cardiac vascular cells is available in the literature, we have used a transgenic murine line (Fsp1-GFP) to study Fsp1 expression in the developing and postnatal cardiac vasculature and endocardium. Our work shows that Fsp1 is expressed in the endocardium and mesenchyme of atrioventricular valve primordia, as well as in some coronary venous and lymphatic endothelial cells. Fsp1 expression in cardiac venous and lymphatic endothelium is progressively restricted to the leaflets of cardiac venous and lymphatic valves. Our results suggest that Fsp1 could play a role in the development of atrioventricular valves and participate in the patterning and morphogenesis of cardiac venous and lymphatic vessel valves.

KEYWORDS

cardiac atrioventricular valves, coronary endothelium, mouse embryo, venous and lymphatic valves

1 | INTRODUCTION

The transformation of the embryonic cardiac tubular primordium into a functional four-chambered heart requires the contribution of multiple cardiac progenitor cell lineages, and the coordinated activity of different developmental programmes (Kelly and Evans, 2010). Among such programmes, those controlling the formation of the cardiac valves (Eisenberg and Markwald, 1995; Timmerman *et al.*, 2004), and the development of heart vascular (coronary) and lymphatic vessels (Ishii *et al.*, 2009; Red-Horse *et al.*, 2010; Wu *et al.*, 2012; Klotz *et al.*, 2015; Cano *et al.*, 2016) are of extreme importance, as all these cardiac structures are crucial to heart homoeostasis. Understanding the molecular and cellular mechanisms that control the morphogenesis of these structures is a relevant goal, since the disruption of any of the transcriptional and signalling programmes controlling embryonic heart formation is likely to result in cardiac congenital disease (CHD), a heterogeneous group of heart conditions that roughly affect 1% of newborns (Williams *et al.*, 2019). Alteration of developmental mechanisms has been shown to have an impact on acquired adult valvular disease also (Nus *et al.*, 2011), therefore expanding the relevance of embryonic morphogenetic mechanisms to adult life. Moreover, recent studies have convincingly shown that cardiac lymphatic vessels regulate tissue fluid balance

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and immune reactions in the adult damaged heart (Henri *et al*., 2016), and that VEGF-C-induced lymphangiogenesis improves cardiac function and reduces ventricular remodelling after injury (Klotz *et al*., 2015). In accordance with all these findings, the deciphering of the roles played by specific molecules during coronary vasculature, cardiac lymphatics and cardiac valve development could be key for the identification of new CHD and adult cardiac disease diagnostic markers, and the development of new therapies to treat the diseased heart.

Fsp1 (a.k.a. S100A4 or Metastasin) is a cytoplasmic protein that can both accumulate in the cell nucleus (Flatmark *et al*., 2003) and be secreted to the extracellular space (Cabezón *et al*., 2007). Fsp1 has for a long time been regarded as a specific marker for cardiac fibroblasts (Strutz *et al*., 1995). However, different reports indicate that only a subset of cardiac fibroblasts expresses Fsp1 (Ruiz-Villalba *et al*., 2015), and that Fsp1 expression is also characteristic of various blood circulating (Osterreicher *et al*., 2011), and some endothelial cells (Ochiya *et al*., 2014). Fsp1 functions in all these different cell types are not well understood. Fsp1-myosin IIA interactions are known to influence cell migration during metastasis (Zhang *et al*., 2005), and Fsp1-dependent cell motility has also been shown for macrophages (Dulyaninova *et al*., 2018), endothelial cells (Ochiya *et al*., 2014), and fibroblasts (Cunningham *et al*., 2010). In addition to cell migration, the expression of Fsp1 in endothelial cells relates to angiogenic phenomena (Ambartsumian *et al*., 2001), and Epithelial-to-Mesenchymal Transition (EMT; Schäfer and Heizmann, 1996). Pleiotropic Fsp1 functions are supposed to depend on its ability to interact with multiple proteins (Ning *et al*., 2018).

In this work we report the previously undescribed expression of Fsp1 in cardiac valve primordia, coronary venous and lymphatic valves, and suggest unexpected roles for this protein in the formation of all these important cardiac structures.

2 | METHODS

2.1 | Mouse breeding, crossing and genotyping

All animals used in this study were handled in compliance with institutional and European Union guidelines for animal care and welfare under a specific experimental procedure approved by the Ethics Committee of the University of Málaga. In this work, we used a previously described Fsp1-GFP mouse strain (Iwano *et al*., 2002).

2.2 | Embryo extraction and histological processing

Mouse embryos were staged considering the presence of the vaginal plug as embryonic day (ED) 0. Pregnant females were sacrificed by cervical dislocation, and embryos isolated from the uterus and washed in PBS. Embryonic hearts for whole mount analysis or cryostat/vibratome sectioning were excised, washed in PBS and fixed in 4% formalin overnight (4°C). In order to obtain frozen sections, tissues were cryoprotected in 15% and 30% sucrose:PBS solutions, embedded in OCT, snap-frozen in liquid N_2-cooled isopentane and sectioned in a cryostat. For microtome sections, hearts were dehydrated in an ethanolic series, embedded in paraffin and sectioned in a microtome. At least three hearts were analysed for each embryonic stage.

2.3 | Immunohistochemistry

Immunofluorescence analyses were performed by blocking non-specific binding sites with 16% sheep serum, 1% bovine serum albumin and 0.5% Triton X-100 in PBS (SBT) and incubating either tissue slides or whole hearts with primary antibodies (see Table 1) overnight at 4°C. Then, samples were washed in PBS (3 × 5 min for

TABLE 1 Primary antibodies used in this work

Epitope	Host	Clonality	Working dilution	Reference
Lyve-1	Rat	Monoclonal	1/100	Santa Cruz Biotechnology sc-65647
Troponin I	Rabbit	Polyclonal	1/100	Santa Cruz Biotechnology sc-15368
Fsp1	Rabbit	Polyclonal	1/100	DAKO A5114
CD31	Rat	Monoclonal	1/100	BD Pharmingen 550274
GFP	Chicken	Polyclonal	1/500	Abcam ab13970
Endomucin	Rat	Polyclonal	1/300	Santa Cruz 65495

TABLE 2 Secondary antibodies used in this work

Epitope	Host	Conjugated molecule	Working dilution	Reference
Chicken IgG	Donkey	FITC	1/200	Jackson IR 703-095-155
Rat IgG	Donkey	Alexa Fluor® 647	1/200	Jackson IR 712-545-153
Rabbit IgG	Goat	Alexa Fluor® 647	1/200	Jackson IR 711-605-152

tissue sections; 3 × 1 hr for whole hearts) and incubated with the secondary antibody (see Table 2) for 1 hr at room temperature (tissue sections) or overnight at 4°C (whole hearts). Samples were finally washed in PBS, mounted in a 1:1 PBS/glycerol solution and analysed under a SP5 laser confocal microscope (LEICA). Additional endothelial counterstain was performed using a specific TRITC-coupled lectin (*Griffonia simplicifolia*, IB4).

2.4 | Quantifications

Distances between adult venous valves were measured using the ImageJ software. These values were measured from the central point of each GFP$^+$ structure to the next one, by analysing whole mount images of seven different adult hearts (n = 7). Each replicate would correspond to the mean value of all measured distances over one adult heart surface.

3 | RESULTS AND DISCUSSION

3.1 | Embryonic and postnatal AV cushions express Fsp1

To investigate Fsp1 expression pattern in the endocardium and other cardiac endothelia we have used a well-established Fsp1-GFP murine transgenic line (Iwano *et al*., 2002). These transgenic mice express GFP under the control of the murine *Fsp1/S100A4* gene promoter. Earliest Fsp1 cardiac expression was identified in the atrioventricular (AV; Figure 1a) and proximal cardiac outflow tract (OFT) cardiac valve primordia (a.k.a. endocardial cushions; Figure 1b) of ED11.5 mouse embryos. At this stage, only a few OFT endocardial were found to be GFP$^+$ (Figure 1c). GFP expression, however, was frequent in the AV endocardium (CD31$^+$, Figure 1d). The majority of ED11.5 AV or OFT GFP$^+$ cells were found within the mesenchymal core of the cushions (Figure 1d). Colocalization of GFP and the Fsp1 protein confirms that the Fsp1-GFP transgene faithfully recapitulates native Fsp1 spatio-temporal expression pattern in the cardiac OFT (Figure 1e). Fsp1 expression in the ED11.5 AV and OFT endocardial cushions is compatible with the described role of Fsp1 protein in EMT (Schäfer and Heizmann, 1996), as well as with fibroblast activation (Ruiz-Villalba *et al*., 2015). At ED13.5, GFP expression was conspicuous in AV and OFT cushion mesenchymal cells (Figure 1f,g and Figure 1h, respectively). Expression of GFP in AV (Figure 1g,g') and OFT (Figure 1i,i') CD31$^+$ endocardial cells is also evident at these stages. GFP expression was maintained at perinatal and postnatal stages (ED18.5, Figure 1j,k). Endomucin (End) counterstaining of GFP$^+$ AV valve mesenchyme revealed Fsp1 protein accumulation in both endocardial (End$^+$) and (End$^-$) mesenchymal cells (Figure 1j). GFP/Fsp1 colocalization was evident in both tissues (Figure 1k). This same expression pattern was observed in 4-day-old postnatal hearts (P4, Figure 1l–o). The consistent Fsp1 expression in atrioventricular and OFT valve primordia suggests an involvement for this molecule in the endocardial EMT that gives rise to a significant part of the cushion mesenchymal tissue. We cannot, however, exclude the participation of this molecule in other parallel phenomena like the mobilization of the cushion mesenchyme in the developing valves. Taken together, these results suggest that Fsp1 is not a marker for a single cell type (e.g. cardiac fibroblasts), but rather a developmental or physiological effector with specific embryonic and postnatal functions displaying a unique spatio-temporal expression pattern.

3.2 | Cardiac venous endothelial cell Fsp1 expression delineates venous valves

Detailed analysis of GFP expression in the heart revealed the presence of significant numbers of GFP$^+$ cells associated to the prenatal and postnatal coronary vasculature at ED15.5. No GFP$^+$ coronary cells could be recorded in the coronary endothelium before this time point. These GFP$^+$ coronary cells were evenly distributed in the coronary tree. At these stages, the majority of GFP$^+$ cells in the developing cardiac walls were nonvascular ones (CD31$^-$), most probably representing blood-borne infiltrated cells (Osterreicher *et al*., 2011; Figure 2a). Endothelial GFP expression was first found in discrete populations of vascular endothelial cells (Figure 2b–f'), both on the subepicardium (heart surface, Figure 2b,d) and intramyocardially (Figure 2c,e); the GFP reporter and the Fsp1 protein extensively colocalized in these blood vessels (Figure 2f,f'). By E18.5, GFP expression was observed at specific locations of the CD31$^+$ coronary vasculature (Figure 2g), while adult GFP vascular endothelial expression was restricted to cardiac venous valves and some discrete endothelial domains close to these structures (Figure 2h–j). GFP$^+$ valves in the adult heart vascular network appeared regularly along the vessel lumen, so that the average distance between these valves was 200 μm (Figure 2k). Additional research is needed to clarify whether Fsp1 is involved in the spatial patterning and/or the morphogenesis of cardiac venous valves.

3.3 | Cardiac lymphatic endothelial cells express Fsp1

Since cardiac lymphatic vessels are also known to develop valves to prevent lymph backflow, we inspected Fsp1 expression in the forming heart lymphatics. Cardiac lymphatic vessels are known to form from a heterogeneous pool of progenitors including venous endothelium and follow coronary morphogenesis in time (Klotz *et al*., 2015). The first consistent GFP expression in cardiac lymphatic vessels (Lyve1$^+$) was found at E18.5, and shows a clear trend to concentrate on specific regions of the developing lymphatic vessels (Figure 2l,m). From postnatal stages to adulthood, GFP expression was progressively confined to lymphatic valves (Figure 2n). Double counterstain with IB4 lectin (for vascular endothelium) and Lyve1 (for lymphatic

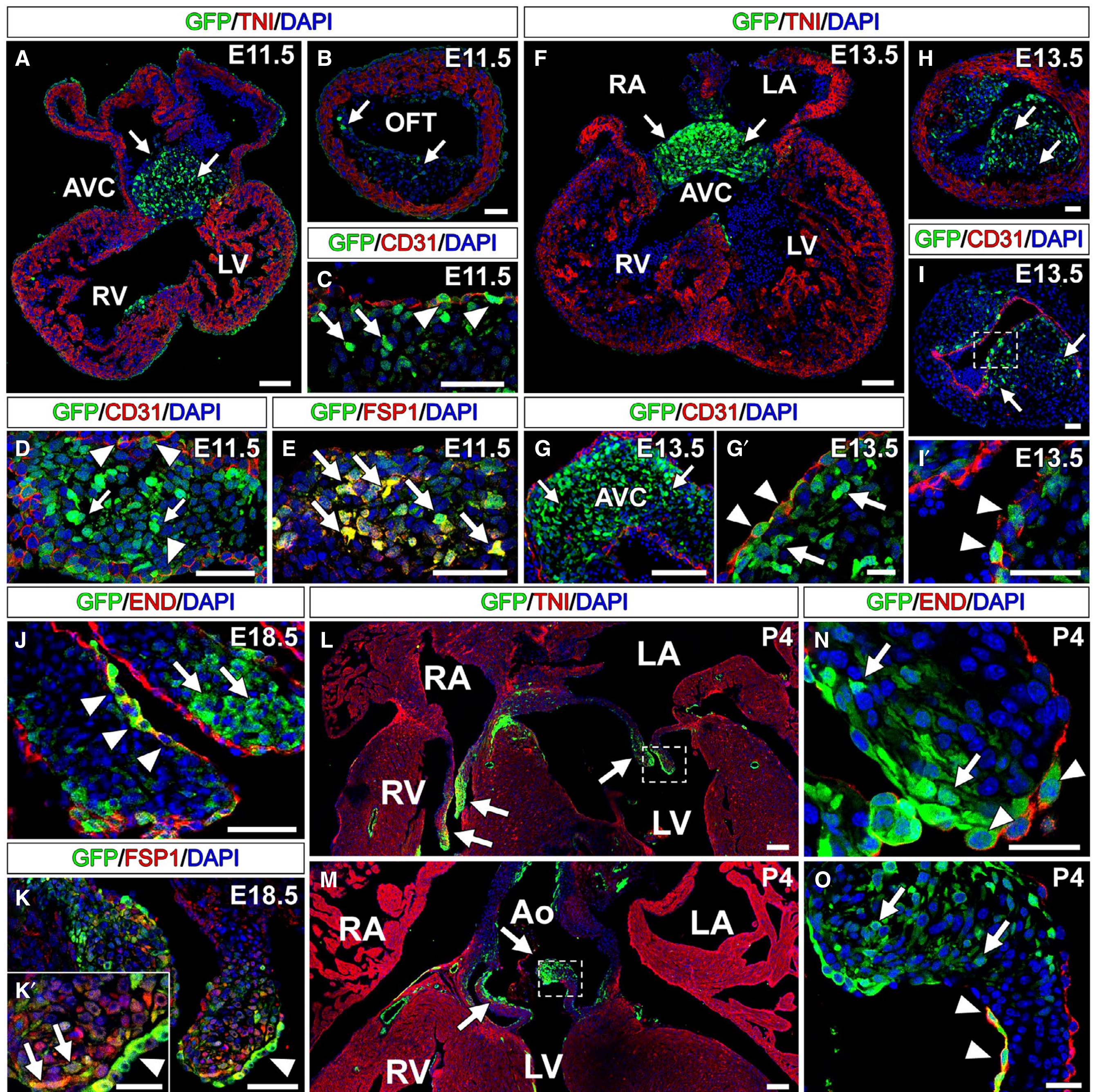

FIGURE 1 Fsp1 in developing and adult atrioventricular valves. (a, b) GFP expression was first observed the developing atrio-ventricular (a, arrows) and cardiac outflow tract (b, arrows) valve primordia/cushions of ED11.5 embryos. (c, d) Both endocardial and mesenchymal cells in the OFT (c, arrowheads and arrows, respectively) and AV (d, arrowheads and arrows, respectively) were GFP$^+$. (e) GFP and Fsp1 protein extensively colocalize in these tissues (arrows). (f, g) At ED13.5, GFP expression was robustly expressed in cardiac AV cells (arrows). Both endocardial and cushion mesenchymal cells were GFP$^+$ (g', arrows and arrowheads, respectively). (h, i) ED13.5 OFT cushions also are GFP$^+$ (arrows); GFP expression is evident in endocardial and cushion mesenchymal cells (i, arrows; i', arrowheads). (j–k') At ED18.5, Fsp1 protein strongly colocalized with the GFP reporter in endocardium and mesenchyme of the forming AV leaflets (j, arrowheads and arrows, respectively). Fsp1 protein and GFP expressions overlapped in both the endocardium (k, arrowhead) and the cushion mesenchyme (k', arrows). (l, m) Postnatal (P4) AV valves (l, arrows) and arterial valves (m, arrows) strongly expressed GFP. Boxed areas in l and m are magnified in n and o, respectively, to show GFP expression in maintained in both endocardial (End$^+$, arrowheads) and mesenchymal (End$^-$, arrows) cell subsets. Scale bars: a, f, g, l, m = 100 μm; b, c, d, e, h, i, i', j, k = 50 μm; k', n, o = 25 μm; g'=10 μm. AV, atrioventricular; AVC, atrioventricular canal; LA, left atrium; LV, left ventricle; OFT, cardiac outflow tract; RA, right atrium; RV, right ventricle

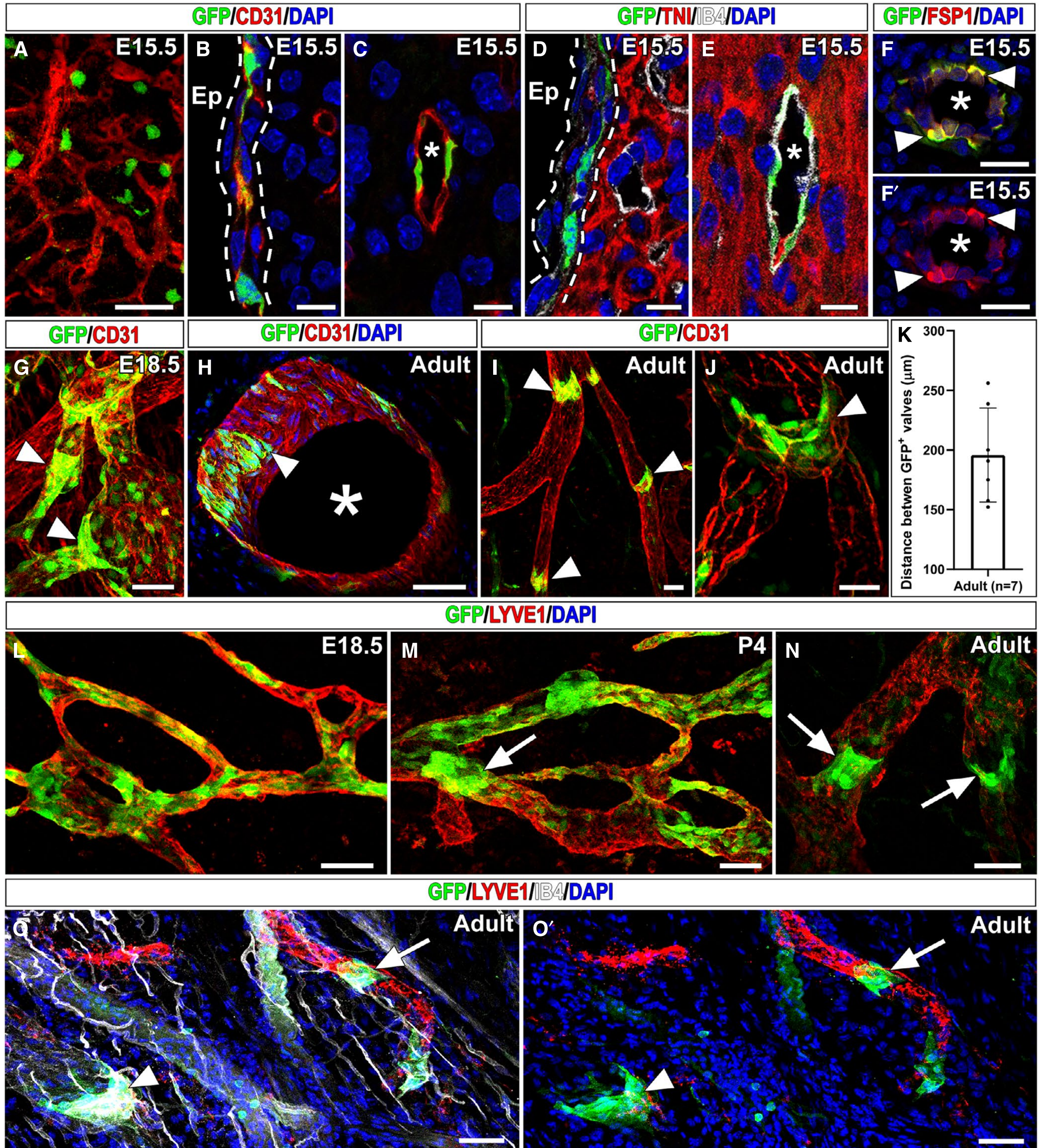

FIGURE 2 Fsp1 in developing and adult coronary and lymphatic vascular systems. (a) At E15.5, most cardiac GFP$^+$ cells were found associated to but not integrated into the CD31$^+$ developing coronary endothelium (arrowheads). (b–f′) First GFP$^+$ vascular endothelial cells (either CD31$^+$ or IB4$^+$) were found in the subepicardial (b, d; the subepicardial space is marked by dashed lines) and intramyocardial (c, e) coronary vessels of the AV and interventricular groove asterisks mark the vascular lumen); colocation of GFP and Fsp1 was obvious in these cells (f, f′, arrowheads). (g) At E18.5, GFP expression was widely observed along the CD31$^+$ coronary endothelium. (h–j) Vibratome sectioning of adult hearts revealed a patterned distribution of these GFP$^+$ cells in discrete CD31$^+$ venous endothelial domains (h, arrowhead; the asterisk marks the vessel lumen) and structures that had the unambiguous 3D morphology of venous valves (i, j, arrowheads). (k) These GFP$^+$ endothelial domains were spatially patterned (k, arrowheads). (l) At E18.5, GFP signal was evident in the developing Lyve1$^+$ lymphatic endothelium. (m–o′) Postnatal (m) and adult (n) cardiac Lyve1$^+$ lymphatics, showed GFP signal restricted to lymphatic valves (m, n, o, o′ arrows) and IB4$^+$/Lyve1$^-$ venous valves (o, o′, arrowhead). Scale bars: a, g, h, i, l, m, n, o, o′ = 50 µm; f, f′, j = 25 µm; b, c, d, e = 10 µm. Ep, epicardium

endothelium) in adult hearts showed that both lymphatic and blood vessels simultaneously expressed the GFP reporter (Figure 2o-o').

Our results show the expression of Fsp1 in different subsets of endocardial and endothelial cells, including cardiac veins and lymphatic valves. We suggest that Fsp1 role in the development of these structures could be related with the initiation of venous and lymphatic endothelial cell protrusion towards the vessel lumen, an event marking the first steps of venous and lymphatic valve leaflet morphogenesis (Bazigou *et al*., 2011). The molecular basis of this process are unknown, but is reasonable to speculate that Fsp1 could contribute to maintain the integrity of these delicate structures through the modulation of Liprin ß1 (Kriajevska *et al*., 2002). Interestingly, Liprin forms protein complexes with CASK, also known to interact with connexins (Márquez-Rosado *et al*., 2012). This idea would fit the characteristic expression of connexins in lymphatic vessels, and disrupted valve development in lymphatic-specific Connexin43 deletion (Munger *et al*., 2017).

4 | CONCLUSIONS

Our data show that Fsp1 is expressed in the endocardium, the coronary and the lymphatic endothelium in a characteristic spatio-temporal manner. Fsp1 expression in the endocardium and mesenchyme of cardiac AV and OFT valve primordia is compatible with a role for this protein in the endocardial EMT that mediates the cellularization of these structures. Moreover, the unique *Fsp1* coronary endothelial and lymphendothelial expression pattern, with a marked restriction of expression in coronary venous and lymphatic valves, strongly suggest a function for Fsp1 in the morphogenesis and maintenance of these structures from perinatal embryonic stages to adult life.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

AUTHOR CONTRIBUTIONS

S. Cano-Ballesteros acquired data and contributed to data analysis and interpretation; Paul Palmquist-Gomes and Ernesto Marín-Sedeño acquired data and contributed to data analysis; J.A. Guadix contributed to the design of the work, acquired and interpreted data and provided financial support; J.M. Pérez-Pomares conceived the paper concept, drafted and designed the manuscript and provided financial support. All authors revised the final version of the manuscript and approved it.

DATA AVAILABILITY STATEMENT

All data supporting the findings of this study are available from the corresponding author upon reasonable request.

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