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Nestin and WT1 expression in small-sized *vasa vasorum* from human normal arteries

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Summary. Introduction. Vasa vasorum (VV) neovasculogenic potential is now widely accepted, and possibly related to the presence of progenitor cells. We studied the morphology of VV in healthy arteries and their immunohistochemical (IHC) expression of Nestin and WT1, two markers of endothelial progenitor cells. Materials and Methods. Twenty arteries from 16 multiorgan donors were analyzed; IHC was performed manually (CD34, CD31, Nestin) or automatically (WT1). Microvessel positivity "density" for each antibody was calculated dividing vascular adventitia in 1-mm2 fields with an ocular micrometer. Double immunofluorescence was used to investigate Nestin and WT1 co-localization in VV. Results. The mean positivity "densities" for CD31, CD34, Nestin and WT1 were 13.63, 12.20, 8.90 and 7.98/mm^2 respectively. Mean Nestin/CD31 and WT1/CD31 ratios were 0.69 and 0.63 respectively. VV <50 μm in diameter showed a higher percentage of Nestin/WT1-positive cells than larger ones, especially in "hot spots", characterized by several small-sized arteriolar VV, often together with *nerva vasorum*. Immunofluorescence indentified Nestin and WT1 in the same endothelial cells. WT1 nuclear expression was mainly seen in <50 μm VV.

Discussion. We describe Nestin and WT1 in adult VV, especially <50 μm and gathered in "hot spots". The nuclear localization of WT1 could express an increasing transcriptional activity in progenitor-committed Nestin-positive cells. The "hot spot" could therefore represent a valid model for the vasculogenic niche in normal arteries and could potentially represent the main source for neovasculogenesis during atherosclerosis.

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Introduction

Vasa vasorum (VV) consist of small arteries present in the adventitia of vessels of major caliber. Their principal functions are nutrient and oxygen delivery and waste removal through the deeper vascular layers (Ritman and Lerman, 2007; Mulligan-Kehoe, 2010). In larger vessels the so-called first-order VV originate directly from other vessels and run longitudinally along the adventitia (Kwon et al., 1998). From a branching of the first-order VV derive the second-order VV, which reach the deeper layers of adventitia and media. In animal models the application of microscopic computed tomography demonstrated VV to form a plexus around the adventitia, with second-order VV generally lower in number, rather than first-order VV in healthy vessels, but dramatically rising in pathological conditions (Kwon et al., 1998). Neovascularization has been largely described in diseased arteries, and the trigger mechanism is vessel wall hypoxia. In these pathological conditions VV, and especially second-order VV, undergo a rapid proliferation, also regulated by local and circulating growth factors such as vascular endothelial growth factor, hypoxia inducible factor, etc. (Moreno et al., 2006).

This neovasculogenic potential has been largely studied in the last decades, and the existence of endothelial progenitor cells (EPCs) within the layers of the larger vessels has been widely accepted, although their location, phenotype and role in vascular damage and angiogenesis are still debated (Zengin et al., 2006; Pasquinelli et al., 2007; Torsney and Xu, 2011). Most authors agree that a population of progenitor cells is

localized in the adventitia, characterized by positivity for different markers of vascular immaturity/stem potential, such as CD34, c-Kit, CD105, Notch-1, SCA1, and KDR (Alessandri et al., 2001; Hu et al., 2004; Pfister et al., 2005; Zengin et al., 2006; Pasquinelli et al., 2010; Leri et al., 2011).

Nestin is an intermediate filament protein expressed in neural and mesenchymal stem cells, as well as in a variety of progenitors cells (Wagner et al., 2006). It was also seen that Nestin is an angiogenic marker for newly formed microvessels, and numerous Nestin-positive microvessels were described in tumors of control mice (Ramasamy et al. 2011). Moreover, a recent study showed that Nestin is expressed in newly forming blood vessels and also in a high number of endothelial cells after myocardial infarction (Wagner et al., 2006). A previous study showed that medial vascular smooth muscle cells in the developing arteries potently express Nestin, but its expression is abolished in adult arteries (Oikawa et al., 2010). It is therefore believed that Nestin expression, representing an early endothelial differentiation or an endothelial progenitor phenotype, could be a reliable marker of "progenitor-committed" endothelial cells and/or of a "young" endothelium.

Nowadays the known transcription factors regulating Nestin are: Pou, Sox, TTF-1 and WT1 (Wagner et al., 2006; Pelizzoli et al., 2008). The Wilms tumor suppressor (WT1) was originally described in the paediatric Wilms' tumor of the kidney, but its involvement has been demonstrated in a variety of other tumors and tumor cell lines (Hohenstein and Hastie, 2006; Wagner et al., 2008). Some recent studies showed the involvement of WT1 as an activator of the Nestin gene (Hohenstein and Hastie, 2006), and the co-localization of WT1 and Nestin during the different development steps in embryogenesis (Wagner et al., 2006).

In this study we performed a histological and immunohistochemical analysis of VV in non-diseased arteries from healthy subjects, in order to verify Nestin and WT1 expression in endothelial cells of normal adult vessels, and VV in particular. The aims are to identify the morphology and the localization of those VV that express Nestin and WT1, two markers of vessel immaturity and endothelial cell progenitors, and to localize and quantify in normal arteries these microvessels that could be responsible for neo-vascularization in diseased arteries.

Materials and methods

Tissue specimens and histopathological analysis

Vascular specimens collected from multiorgan donors were kindly provided by the Cardiovascular Tissue Bank, Service of Transfusion Medicine, Policlinico S.Orsola-Malpighi of Bologna. At the end of organ procurement, a vascular surgeon is called to the Tissue Bank to grossly evaluate the status of the vascular specimens, and to exclude atheromasic plaques. Parts of those vascular segments without macroscopic lesions are therefore sent to our Pathology Unit for histopathological analysis, in order to verify their suitability for allograft transplant. Sixteen patients were prospectively selected and evaluated in one-year time length, 9 males and 7 females, mean age 36.27±16.27 (range 16-58). In four cases two different vascular segments were retrieved, with a final number of 20 collected arterial specimens, from femoral artery (12 cases, 60%), abdominal aorta (4 cases, 20%), iliac artery (2 cases, 10%) and renal artery (2 cases, 10%). Histopathological analysis with Haematoxylin and Eosin, Masson's trichrome stain and Weigert-van Gieson stain for elastic fibers, was performed in all cases by two dedicated pathologists to exclude tissue alterations due to bad preservation and/or misdiagnosed pathological conditions. Histology confirmed a normal architecture in all cases. In particular the adventitia was well preserved, and *vasa vasorum* were well identifiable in the adventitial fat tissue. Intimal endothelial cells were preserved and evaluable in 12 specimens.

Immunohistochemical assay

Formalin-fixed paraffin-embedded 5-um-thick tissue sections were processed following the non-biotin-amplified method (NovoLink Polymer Detection Kit, Novocastra, NewCastle, UK) as previously described (Faggioli et al., 2011). Sections were incubated with monoclonal antibodies against CD34, CD31 and Nestin (see also Table 1). WT1 antibody detection was automatically performed with BenchMark XT® (Ventana Medical Systems, Inc, Tucson, USA) following the manufacturer's instructions. Cell nuclei were stained with Mayer's haematoxylin. Sections with PBS instead of the primary antibody served as negative controls. Finally, the samples were viewed under a light

Table 1. Technical characteristics of the antibodies used for immunohistochemistry.

Antibodies	Type	Company	Clone	Dilution	Antigen unmasking treatment
CD31	Mouse Monoclonal	Dako A/S, Copenhagen, Denmark	JC70A	1:50	Citrate Buffer, heat mediated
CD34	Mouse Monoclonal	Dako A/S, Copenhagen Denmark	Q-BEnd-10	1:80	Citrate Buffer, heat mediated
Nestin	Mouse Monoclonal	Millipore USA	10C2	1:400	Citrate Buffer, heat mediated
WT1	Mouse Monoclonal	Cell Marque Corporation USA	6F-H2	Pre-diluted	EDTA Buffer

microscope (Leitz Wetzlak, Germany) connected with a CCD camera Olympus. Images were digitalized using Image-Pro Plus software (Media Cybernetics http://www.mediacy.com) and processed with ImageJ free software (http://rsbweb.nih.gov/ij/).

Sequential double immunofluorescence assay

To investigate the expression of Nestin and WT1 in the same cells, a double immunofluorescence procedure was used. All washings were performed with PBS solution. Formalin-fixed paraffin-embedded 5-um-thick tissue sections were dewaxed and rehydrated through graded steps of ethanol. (Xilol 30 min, 100% 10 min, 95% 5 min, 70% 5 min). Antigen retrieval was performed with a heat-mediated method at 1 atm, 120°C for 20 min in a citrate buffer solution (pH 6) and cooling for 20 min. Samples were then washed for 10 min. The sections were treated with goat serum (1:10, Sigma-Aldrich) in 1% BSA in PBS for 30 minutes at RT and incubated first with anti human-Nestin (1:400) in a moist chamber for 60 min, at 37°C. The samples were washed and labelled with a goat anti-mouse Alexa Fluor® 488 (1:250, Invitrogen Corporation, Camarillo, CA) in a moist chamber for 60 min, at 37°C in the dark. After incubation and washing, slides were treated with donkey serum (1:10, Sigma-Aldrich) in 1% BSA in PBS for 30 minutes at RT and incubated with anti WT1 (1:1). Then, the slides were incubated with donkey anti-mouse Alexa Fluor® 647 (1:250, Invitrogen Corporation, Camarillo, CA) in a moist chamber for 60 min, at 37°C in the dark. Finally, after washing, the slides were mounted and nuclei counterstained with DAPI (Pro long anti-fade reagent, Molecular Probes, Milano, Italy). Negative controls were done by omitting the primary antibodies. After 24 hours, samples were observed under a fluorescence microscope (Leica, DMI 6000 B) connected to a CCD camera to capture images.

Evaluation of vasa vasorum morphology and "density" at IHC

Microvessel density was calculated through CD34 staining as a marker of both EPCs and mature endothelial cells, and CD31 staining as a marker of fully differentiated vascular and lymphatic endothelial cells.

At 20 x magnification we divided vascular adventitia in every IHC slide in 1-mm^2 fields, using a Olympus® ocular micrometer (1 length unit=5 μm, which means that an area of 100x100 units is equal to 0.25 mm^2). The microvessel "density" per section was obtained by dividing the sum of all vascular structures observed by the number of counted fields in the section of the total areas analyzed in all fields (number of positive vessels per mm^2). After microvessel density was determined, we counted the number of vascular structures expressing Nestin and WT1 in each field of all sections. Single-cell positivity and structure without a visible lumen were excluded from the count.

Afterwards, we focused only on the "hot spots", i.e. those areas particularly rich in Nestin- and WT1-positive structures. By means of ocular micrometer we sorted VV by diameter, and we classified them in ≤50 μm, 50 to 100 μm and ≥100 μm, according to the classification used by Giannoni (Giannoni et al., 2009), which is very

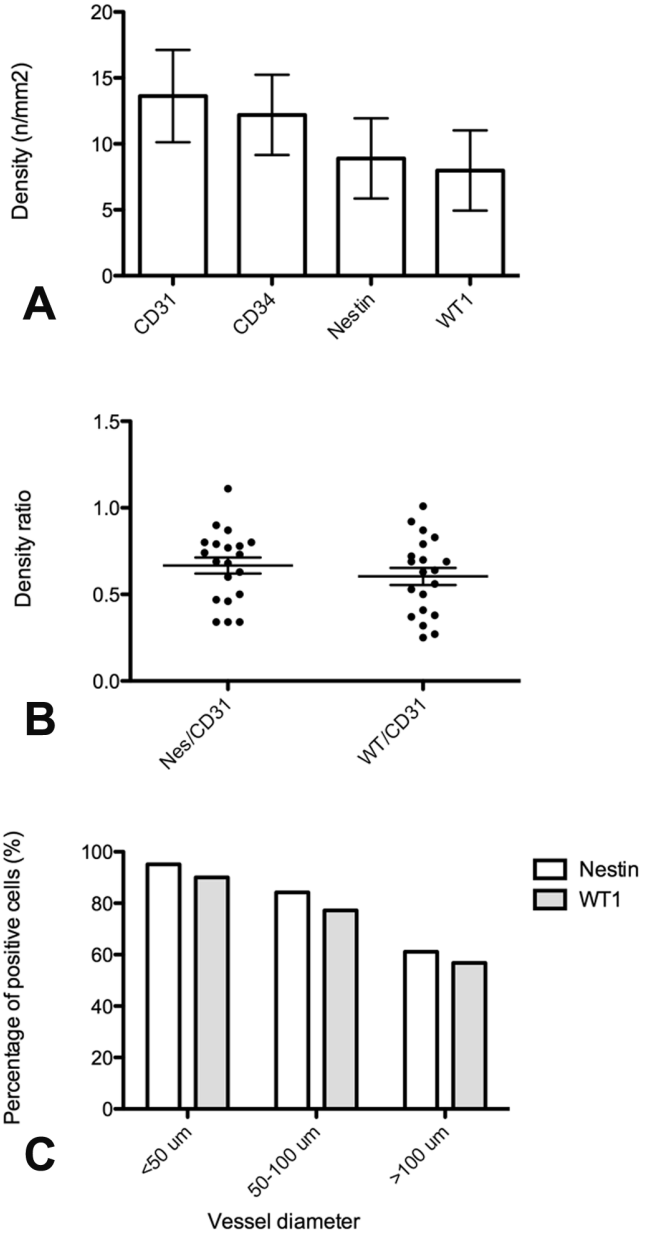

Fig. 1. Immunohistochemistry results. **A.** Histogram representing mean densities of positivity expressed as number of positive vessels / mm^2 of CD31, CD34, Nestin and WT1. **B.** Scatter plot illustrating the distribution of the Nestin/CD31 and WT1/CD31 ratios in all cases (see text for details). **C.** Histogram with the percentage of Nestin- and WT1-positive cells in the three groups of vasa vasorum sorted by diameter.

similar to the definition of "first order" and "second order" VV used by other authors (Kwon et al., 1998; Mulligan-Kehoe et al., 2010). Finally, the different percentages of Nestin- and WT1-positive cells in the "hot spots" were counted in the three different groups of VV.

Results

CD31 and CD34

The mean number of CD31-positive vascular structures in the adventitia of the 20 cases was 181.60±94.52 (median 152, range 67-425), the mean counted fields were 12.86±4.23 mm^2 (median 12, range 6-21 mm^2), the mean final "density" of CD31-positive structures was 13.63±3.50/mm^2 (range 8.11-22.67/mm^2, Fig. 1A).

The mean number of CD34-positive vascular adventitial structures was 146.30±72.27 (median 133, range 34-329), the mean counted fields were 11.53±3.98 mm^2 (median 11, range 4-18 mm^2), the mean final "density" of CD34-positive structures was 12.20±3.04/mm^2 (range 7.68-20.89/mm^2, Fig. 1A).

The mean ratio between CD34 and CD31 "densities" was 0.94±0.24, very close to 1. Although CD31 and CD34 show quite similar positivity and densities, the former includes a wider spectrum of micro-vessels (including lymphatics). On the other hand CD34 is less specific, and often shows reactions of variable degree with some smooth muscle cells and interstitial cells, making it sometimes difficult to count small-sized vessels. For those reasons we have chosen CD31 for further analyses.

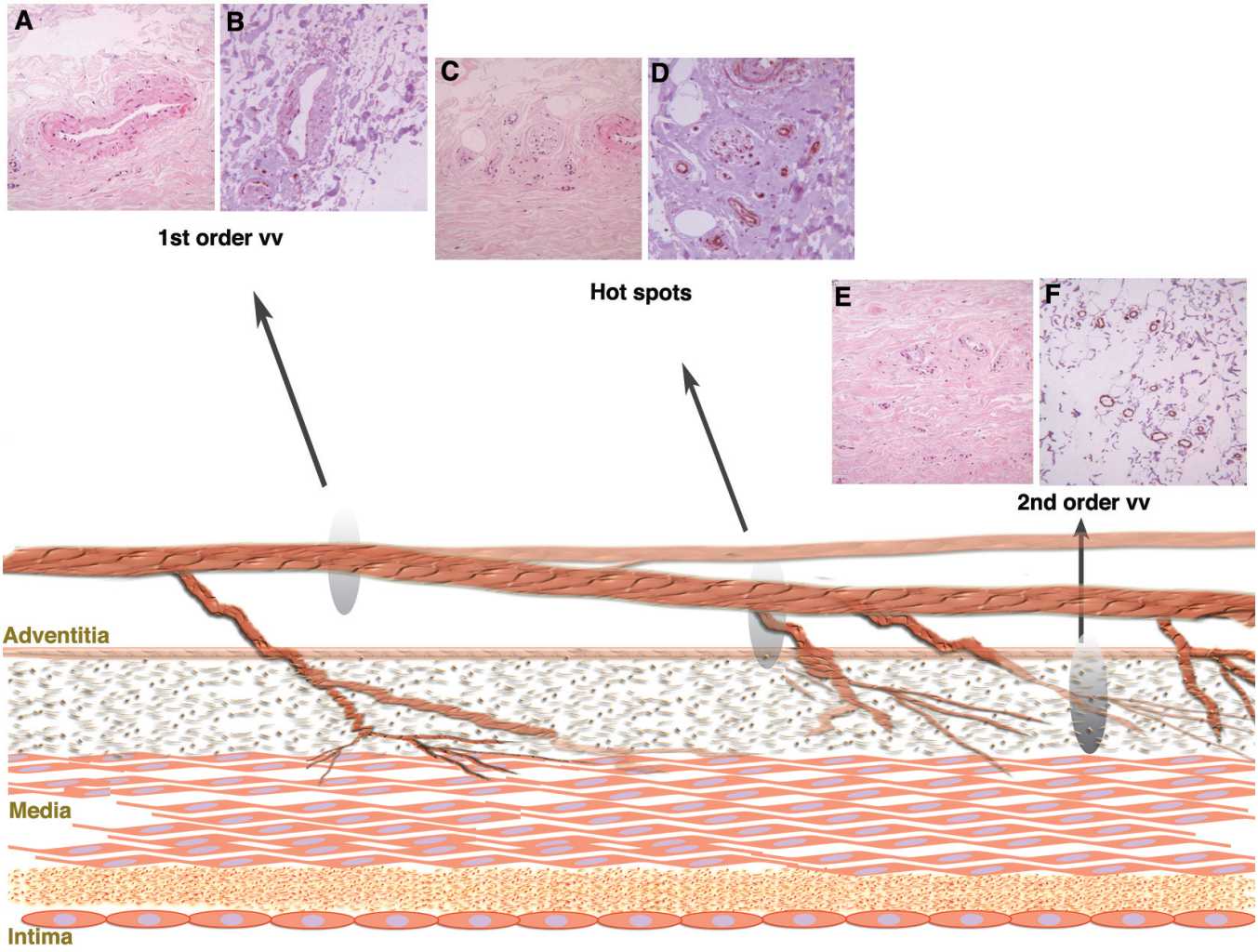

Fig. 2. A graphic representation of a first-order vasa vasorum, its second-order vasa vasorum, and a hypothetical "hot spot" in the branching. Inserts **A, C, E** show the histological appearance of each level (Haematoxylin-Eosin stain). Inserts **B, D, F** show Nestin immunoreactivity. x 10

Nestin and WT1

The mean number of Nestin-positive vascular structures in the adventitia of the 20 cases was 117.10±59.13 (median 114, range 19-242), the mean counted fields were 12.83±4.20 mm^2 (median 14, range 5-19 mm^2), the mean final density of Nestin-positive structures was 8.90±3.04/mm^2 (range 3.04-15.87/mm^2, Fig. 1A). The ratio between Nestin density and CD31 density ranged from 0.34 to 1.29, with a mean Nestin/CD31 ratio of 0.69±0.26, which briefly means that a mean of 69% of CD31-positive vessels showed concomitant positivity for Nestin as well (Fig. 1B).

The mean number of WT1-positive adventitial vessels was 105.85±51.63 (median 95, range 23-209), the mean counted fields were 13.24±3.82 mm^2 (median 14, range 2-20 mm^2), the mean final density of Nestin-positive structures was 7.98±3.04/mm^2 (range 2.88-14.73/mm^2, Fig. 1A). The ratio between WT1 density and CD31 density ranged from 0.25 to 1.27, with a mean WT1/CD31 ratio of 0.63±0.27, which means that a mean of 63% of VV showed concomitant positivity for WT1 and CD31 (Fig. 1B).

As expected, the ratio between Nestin and WT1 "densities" was very close to 1:1. Indeed the mean Nestin/WT1 ratio in all 20 cases was 1.15±0.25.

Comparison between IHC and vessel morphology

Since not all VV showed positivity for Nestin and WT1, we focused on the "hot spots", i.e. the areas of major positivity. Of note, Nestin-positive and WT1-positive vascular structures of ≤50 μm in diameter were positive for those antibodies in the large majority of cells (95% and 90% of cells respectively for Nestin and WT1). Positive VV with diameter ranging from 50 to 100 μm had a mean of 84% and 77% of Nestin- and WT1-positive cells respectively. Positive vessels ≥100 μm in diameter expressed only 61% and 57% of Nestin and WT1 respectively (see also Fig. 1C).

There is an increase in Nestin and WT1 expression, in terms of number of positive cells, and it is inversely correlated to the size and calibre of the micro-vessels. In particular in this study we observed higher positivity for Nestin and WT1 in small arteries (recognizable by the muscular layer), less than 50 μm in diameter, especially in "nests", relatively distant from vascular media, in which several small arteries were visible together with small veins of similar size and peripheral nervous structures (Figs. 2, 3). Conversely, vessels of more than 100 μm in diameter, both arteries and veins, were generally negative for Nestin and WT-1, or showed only few scattered positive cells.

Localization of Nestin and WT1 at immunofluorescence

Since in the IHC assay we noticed that the Nestin expression pattern closely resembled the pattern of WT1 and the ratio between Nestin and WT1 "densities" is

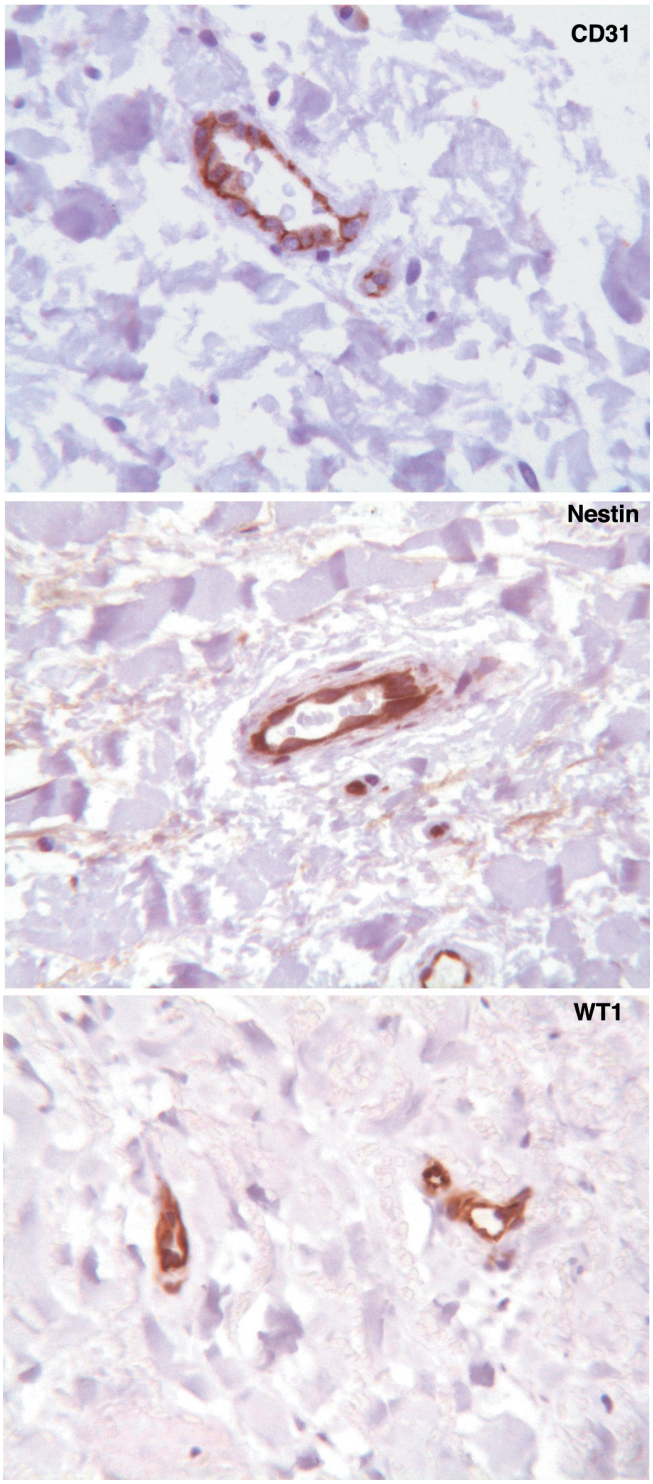

Fig. 3. An example of immunohistochemical pattern of positivity for CD31 (upper), Nestin (center), and WT1 (lower) in small-sized vessels. x 25

very close to 1:1, we investigated by double immunofluorescence whether these two proteins had an overlapping localization. Nestin and WT1 signals co-localized in the endothelial cells from adventitial VV, including the "hot spots". WT1 signal was strongly expressed by endothelial cells mainly in vessels of ≤50 μm in diameter. The expression was confined in the perinuclear and nuclear area (Fig. 4A), but a cytoplasmic pattern was also observed (Fig. 4D). Nestin expression was restricted to the cytoplasm (Fig. 4B,C). The merge of WT1 and Nestin revealed that they were mostly expressed in the same cell population (Fig. 4E) and, surprisingly, cytoplasmic expression of WT1 overlaps nestin expression. This phenomenon was mainly observed in endothelial cells from vessels of >100 μm in diameter.

Discussion

VV are not all the same. A "classic" model describes the first-order VV to derive from major arteries, run longitudinally along the vessel wall and giving rise to

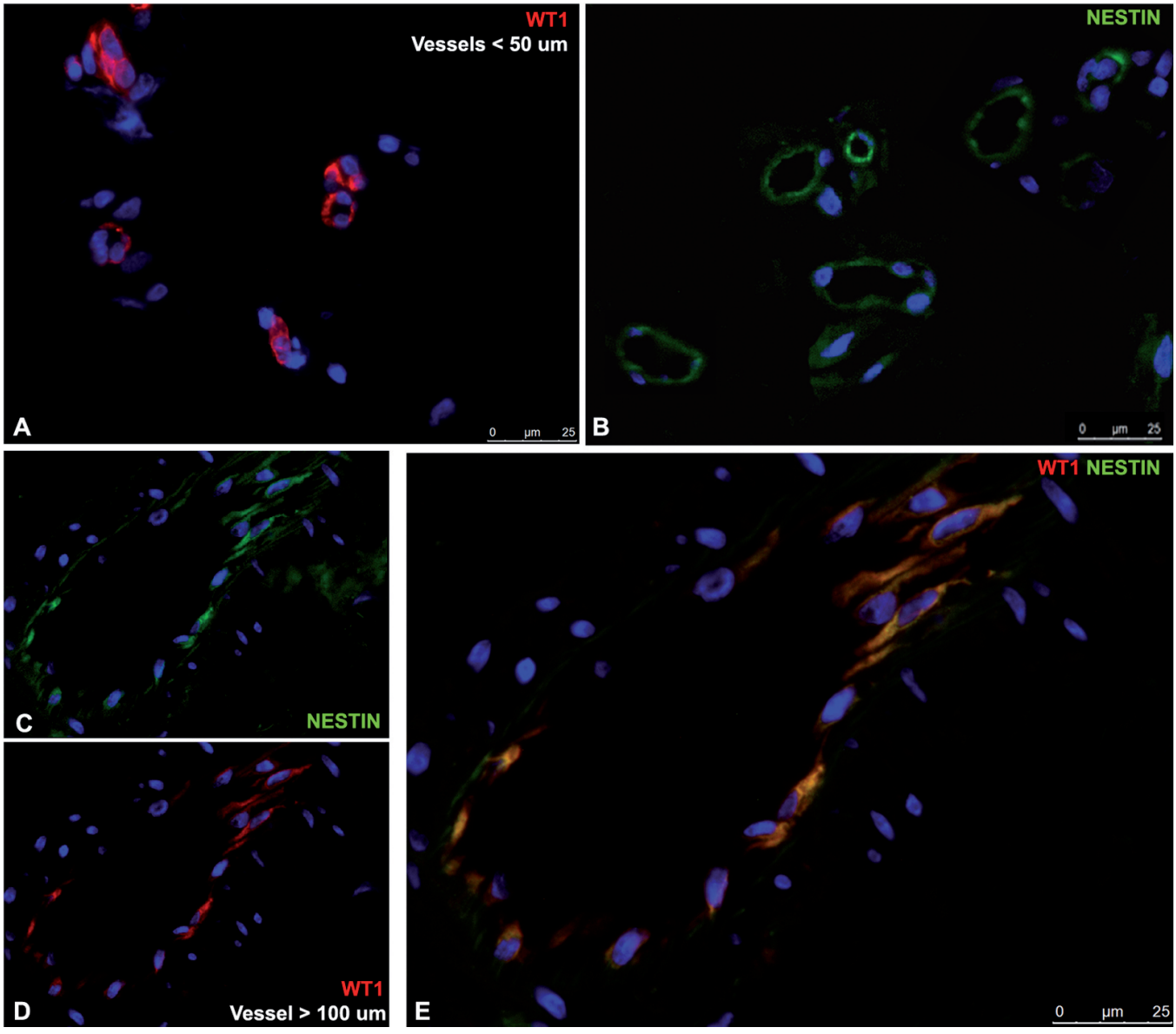

Fig. 4. Double-label immunofluorescence micrographs representing the Wilms' tumor suppressor Wt1 (red) detectable in the nucleus of vessels <50 um **(A)** or in the cytoplasm of vessels with a diameter >100 um **(D)** and Nestin (green), detectable only in the cytoplasm **(B, C)**. The merged image **(E)** shows the colocalization in the cytoplasm of Nestin and WT1 proteins in a vessel of >100um. Nuclei (blue) were counterstained with DAPI. Scale bars: 25 um.

the second-order VV, which form a plexus around the adventitia and the vascular media. These two orders of VV are different in size, functions and anatomic sites: the VV <50 μm in diameter that we described in this study are likely to represent the second-order VV (described in animal models as vessels of 60-70 μm in size), while the vessels ≥100 μm should represent the first-order VV (more than 150 μm in pig coronary arteries) (Kwon et al., 1998; Moreno et al., 2006; Ritman and Lerman, 2007; Mulligan-Kehoe et al., 2010). We have chosen to maintain a "grey zone", from 50 to 100 μm in diameter, because it is sometimes difficult to evaluate vascular size, due to cut effects which can induce an overestimation of capillary diameter. The results we obtained seem to support this view, as the 50-100 μm VV showed intermediate IHC expressions, but were more similar to the <50 μm VV group.

Nestin is a progenitor cell and angiogenic marker, also involved in early development steps in embryogenesis (Wagner et al., 2006; Green et al., 2009) and WT1, is a transcription factor of Nestin, which acts also as critical regulator of organogenesis (Hohenstein and Hastie, 2006). Here we saw that both proteins are expressed in adult arteries as well. The variability among VV is reflected by the different expression of nestin and WT1 that we found analysing the adventitia of healthy arteries. Indeed a more pronounced expression of Nestin and WT1 was found in VV <50 μm in diameter (which show more than 90 % of positive cells) rather than VV >100 μm in diameter (which are often negative and, when positive, show a mean of 60 % of positive cells). Furthermore, those small-sized VV positive for Nestin and WT1 are recognizable in areas (that we called "hot spots"), characterized by several small-sized VV, arteriolar in morphology, often together with peripheral nervous structures (identifiable with the *nerva vasorum*). Due to their location (deeper layer of adventitia, relatively far from media) and morphology ("network" of small vessels with nervous structures), we can hypothesize that the "hot spots" may represent points where second-order VV originate from a branching of first-order VV (see Fig. 2). The "hot spot" could therefore represent a valid model for the vasculogenic niche, i.e. a specific domain with a specific architecture and a specific location, containing several Nestin- and WT1-positive vascular structures.

As previously said, Nestin gene transcription is regulated by WT1 (Wagner et al., 2006; Pelizzoli et al., 2008). Our results show that Nestin and WT1 are expressed in the same cell population. Interestingly, WT1 is both expressed in the cytoplasm and in the nucleus. For a long time the presence of WT1 in the cytoplasm was attributed to an antibody-staining artefact (Hohenstein and Hastie, 2006) and there was no evidence of WT1 cytoplasmic expression at IHC. Our finding correlates with recent studies showing the presence of WT1 in the cytoplasm. The cytoplasmic isoform WT1 (+KTS) has a high affinity for RNA, while the nuclear isoform (–KTS) binds DNA. These and other observations have led to the proposal that the –KTS form of WT1 acts as a transcriptional regulator, while the +KTS form, in the cytoplasm, plays a role in RNA processing (Green et al., 2009). The cytoplasmic colocalization of WT1 and Nestin strengthens the role of WT1 as a post-transcriptional activator of Nestin protein. It would be interesting to compare WT1 sub-cellular localization in normal arteries versus pathological arteries to assess a correlation with neovascularization during atherogenesis. Indeed recent studies showed how an increase of WT1 in the cytoplasm is associated with different processes in cell differentiation (Wagner et al., 2008). In our series WT1 was mainly cytoplasmic in the larger vessels, while nuclear expression increased in the smaller vessels ("second-order" VV).

According to some authors, the main source for neovasculogenesis in diseased arteries is the proliferation of VV, and in particular second-order VV located in adventitia (Kwon et al., 1998; Mulligan-Kehoe et al., 2010). From this point of view our "hot spots" could represent the main manifestation of this proliferative potential: Nestin positivity is to be considered as a characteristic of a "young" endothelium, still progenitor committed, and not expression of real stem cell potential. Furthermore, the nuclear localization of WT1 could be associated to an increasing transcriptional activity in the "hot spots". These committed progenitor cells are likely to play a key role in neovascularization during atherogenesis and atheromasic disease.

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