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Anatomy and Physiology: Current Research

Short commentry

Use of D2-40 as an IHC marker is inappropriate for 3D Reconstruction of the Venous System

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ABSTRACT

The circulatory system plays an important role in performing life activities, but the mechanisms of embryologic distribution have not been understood clearly. This study **aimed to elucidate the venous vasculature in human fetuses with 3D Reconstruction of vessel and nerve localisation. According to this article, D2-40 was used as a venous marker, whereas we used α SMA instead of D2-40. D2-40: Podoplanin (PDPN) a lymph**atic and mesotheliomalmarker,**is not expressed in normal blood vessels. The aim of this paper is to report embryological evidence disputing the detectability of blood vessels using D2-40 and provide the correct blood vessel specific markers** that should be chosen instead. **Unfortunately, the research was not the first good report about the vessel-system of human fetus. This is the article which was submitted by one of co-authors without the approval of Kurobe.**

KEY WORDS

α SMA, D2-40

INTRODUCTION

This study (1) aims to observe development of the venous system in fetuses with three-dimentional reconstruction because anatomical studies are scare on embryological development of the vascularization process. However, it is a serious mistake to use D2-40 to observe blood vessels. D2-40 is a marker specific to lymphatic endothelial cells, not to blood vessels. The lymphatic vessels are located in close proximity to the venous vessels but it is different from the venous vessels. Markers for lymphatic vessels cannot be used as substitute markers for venous vessels. However, in the first experiment of the 14-week old fetus (W14), α SMA (SMA, M0851, Dako, Denmark) at pH6, a dilution of 1:300 was used. (Figure1). After Immunohistochemistry (IHC), the tissue slices are processed by the computer. Since the digitalized images are reffered to by their corresponding ID numbers thereafter (Figure2, Table1, Table 2), Dr.Uhl (3D reconstructor) should have confirmed the names of markers on the original slides. (Figure 1) I noticed his error and I asked him tocheck all slides of SMA and continue to use SMA. But he claimed « This is the good result of D2-40. D2-40 is for blood vessels, too. » This is the article which was submitted to Anatomical Record (AR) by him once and rejected because of the lack of the consent of all co-authors. Just after that, he rapidly submitted the same article to Surgical and Radiologic Anatomy (SRA) without the approval of the first author (Computer technician) and the second author (IHC technician, Hôpital Necker E.M.) and accepted.

TEXT

Blood and lymphatic vessels are fully developed during the fetal period and their network formation is thought to be mediated by angio-guiding nerves. From the viewpoint of centrifugal theory. I show here role of PDPN in explaining the process of Lymphvasculogenesis.The venous differentiation occurs with coinciding high expression of VEGFR2. Lymphvasculogenesis starts with LYVE-1 in the venous endothelial cells. Some venous endorhelial cells become positive for LYVE-1 and Sox18, and acquire the ability to differentiate into lymphatic vessels. Sox18 induces Prox1, a master control gene in the process of lymphatic endothelial cell differentiation. Prox1 induces its target genes, such as VEGFR3 (a receptor for VEGF-C), and PDPN, which increases cell mobility. Prox1 also temporally suppresses VE-cadherin, enabling free migration, so that the cells can detach

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from each other and move towards VEGF-C to form a stable lymphatic vessel. PDPN is a mucin-type transmembrane protein and it has the function as a Platelet-aggregating factor. PDPN-mediated platelet aggregation enhances involutions of lymphatic vessels to the separation of venous and lymphatic vessels each other. Platelet activation is mediated by binding of PDPN to CLEC-2 expressed on the platelets in the venous vessels, from which lymphatic vessels arise, and the vasculature totally differs from the venous vessels. PDPN is specially expressed on the lymphatic vessels at the final stage of lymphvasculogenesis. Thus, the normal venous endothelial cells never express PDPN, indicating that it is impossible for D2-40, anti-PDPN monoclonal antibody to detect the venous vessels. Blood vessels are covered by SMCs which can be detected using the specific marker α SMA. Following separation from venous vessels, the lymphatic vessels continue to express PDPN. Most of the lymphatic vessels do not express α SMA due to a lack of mural cell coverage except downstream of lymphatic vessels and the plexus surrounding the lymphatic vessels. Although these lymphatic vessels express α SMA, they can be easily distinguished because of their limited localization. Immunohistological studies have shown that overlapping expression between α SMA and PDPN cannot be observed, proving that PDPN positive vessels are not blood vessels but lymphatic vessels. PDPN serves as a marker only for lymphatic vessels, and cannot detect normal blood vessels. Immunohistochemical evidence also indicates that the D2-40 positive vessels observed in this study, were lymphatic vessels. D2-40 cannot be used to detect the venous vessels. In 2018, Susana M Chuva de Sousa Lopes and her PhD students; Leiden University Medical Center (Department of Anatomy and Embryology) voluntarily performed two confirmatory experiments, using 2-kidney of W20, W15.2 and multiple antibodies:different combinations of DAPI, PECAM1, PDPN (D2-40), ACTA2 (α SMA), Sox17 and they could see the differences between D2-40 (PDPN), KIT and SMA. (11/01/2018, 01/02/2018) They claimed that D2-40 cannot be used to observe the venous system. BOSMAN TF declared " D2-40 should not be used in this experiment".The study contains a significant error in methodology. The correct methodology should be performed with the correct marker combination which includes the pan-endothelial marker e.g. CD31, lymphatic vessel marker e.g. LYVE-1, Prox-1, or PDPN, and venous vessel marker e.g. EphB4. In this combination, the venous vessels can be detected as $CD31^{+}$/$LYVE\text{-}1^{-}$, $Prox\text{-}1^{-}$, or $PDPN^{-}$/$EphB4^{+}$.

USE OF SMA

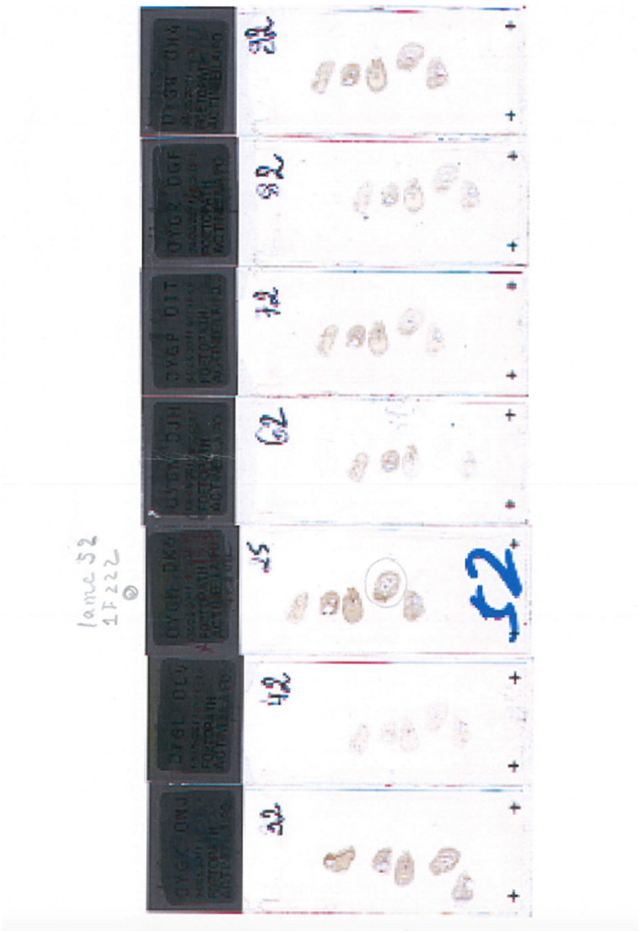


Figure 1:7 Actin-slide : No. 32, 42, 52, 62, 72, 82, 92 (Leila Hakkakian, 2011)

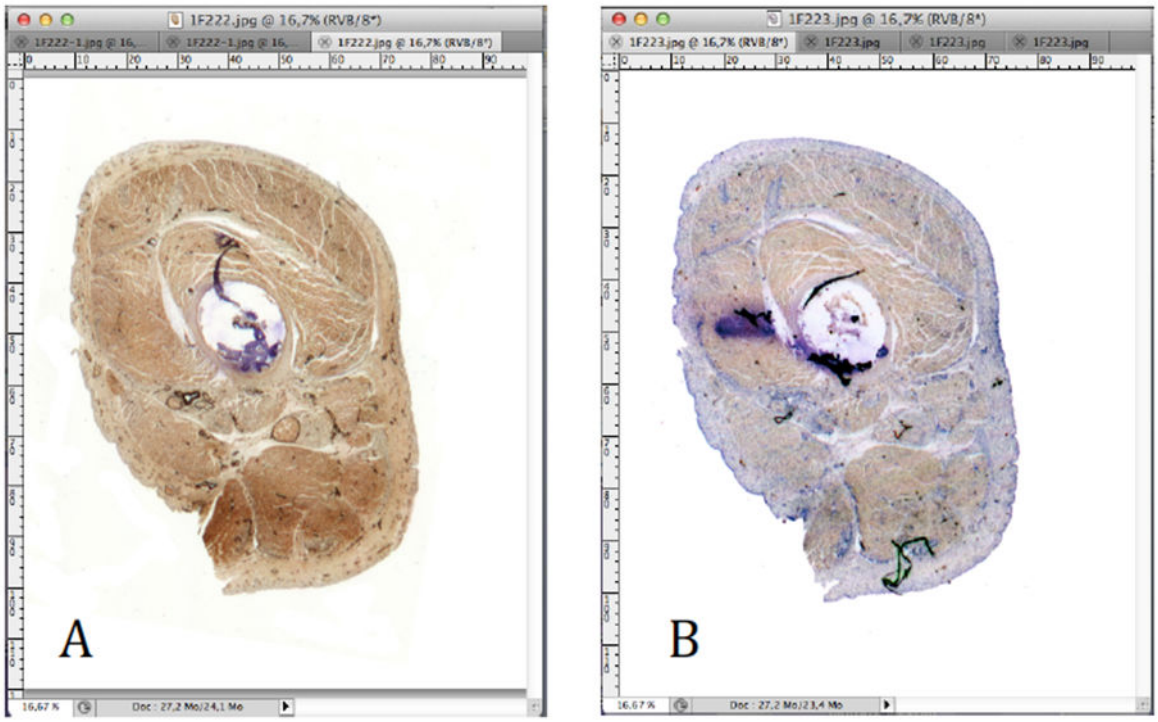
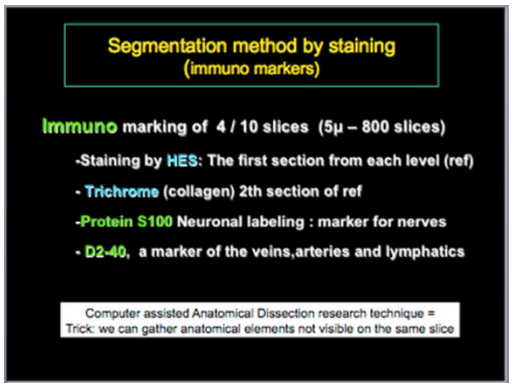


Figure2: Expression of IHC marker: 1F222 and 1F223 (Kurobe, 2011).

A: 1F222, SMA
Positive: Artery, Vein
femoral artery, great saphenous vein, axial vein, femoral plexiform vein
B: 1F223, D2-40
Negative: Artery, Vein
* FFPE
* Leica BONDMAX, Bond Refine DAB Detection KIT

1



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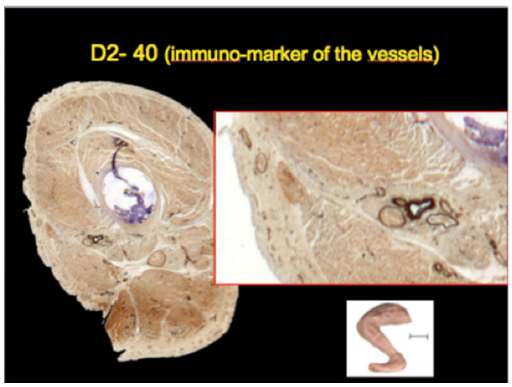


Figure 3 : PPT 1-2 (J-F. Uhl2011)

Table1: List of 1F. Coloration and IHC (Kurobe, 2011) 7 Actin -slide : No. 32, 42, 52, 62, 72, 82, 92

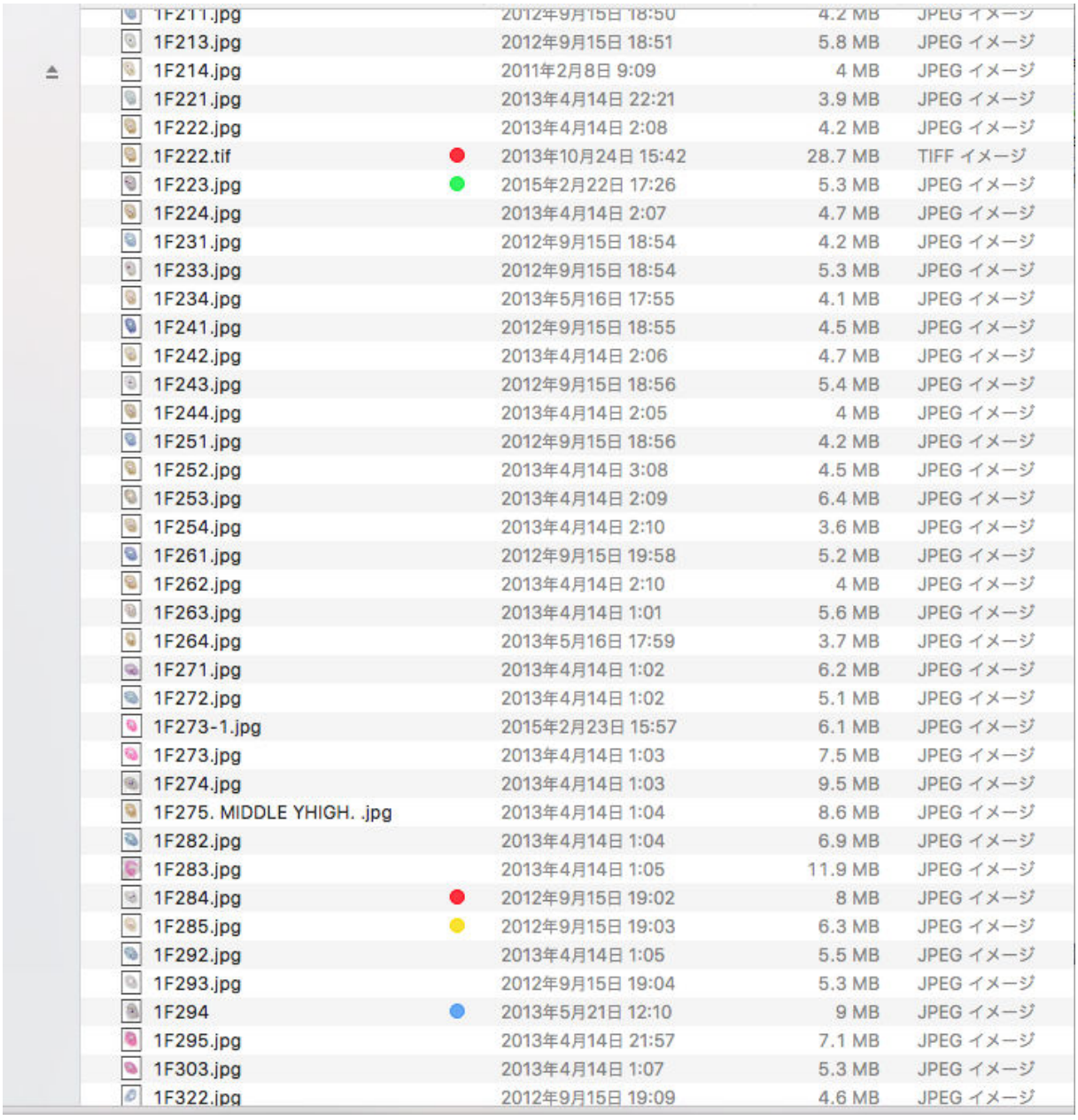
1F Slide No.	COLORATION. et IHC	SERIE 1	SERIE 2	SERIE 3	SERIE 4	SERIE 5
00	HES	1F00	1F170	1F340	1F510	1F680

01	TRIC	1F01	1F171	1F341	1F511	1F681
02	D2-40	1F02	1F172	1F342	1F512	1F682
03	PS100	1F03	1F173	1F343	1F513	1F683
10	HES	1F10	1F180	1F350	1F520	1F690
11	TRIC	1F11	1F181	1F351	1F521	1F691
12	D2-40	1F12	1F182	1F352	1F522	1F692
13	PS100	1F13	1F183	1F353	1F523	1F693
20	HES	1F20	1F190	1F360	1F530	1F700
21	TRIC	1F21	1F191	1F361	1F531	1F701
22	D2-40	1F22	1F192	1F362	1F532	1F702
23	PS100	1F23	1F193	1F363	1F533	1F703
30	HES	1F30	1F200	1F370	1F540	1F710
31	TRIC	1F31	1F201	1F371	1F541	1F711
32	ACTIN	1F32	1F202	1F372	1F542	1F712
33	D2-40	1F33	1F203	1F373	1F543	1F713
34	PS100	1F34	1F204	1F374	1F544	1F714
41	TRIC	1F41	1F211	1F381	1F551	1F721
42	ACTIN	1F42	1F212	1F382	1F552	1F722
43	D2-40	1F43	1F213	1F383	1F553	1F723
44	PS100	1F44	1F214	1F1384	1F554	1F724
51	TRIC	1F51	1F221	1F391	1F561	1F731
52	ACTIN	1F52	1F222	1F392	1F562	1F732
53	D2-40	1F53	1F223	1F393	1F563	1F733
54	PS100	1F54	1F224	1F394	1F564	1F734
61	TRIC	1F61	1F231	1F401	1F571	1F741

62	ACTIN	1F62	1F232	1F402	1F572	1F742
63	D2-40	1F63	1F233	1F403	1F573	1F743
64	PS100	1F64	1F234	1F404	1F574	1F744
71	TRIC	1F71	1F241	1F411	1F581	1F751
72	ACTIN	1F72	1F242	1F412	1F582	1F752
73	D2-40	1F73	1F243	1F413	1F583	1F753
74	PS100	1F74	1F244	1F414	1F584	1F754
81	TRIC	1F81	1F251	1F421	1F591	1F761
82	ACTIN	1F82	1F252	1F422	1F592	11762
83	D2-40	1F83	1F253	1F423	1F593	1F763
84	PS100	1F84	1F254	1F424	1F594	1F764
91	TRIC	1F91	1F261	1F431	1F601	1F771
92	ACTIN	1F92	1F262	1F432	1F602	1F772
93	D2-40	1F93	1F263	1F433	1F603	1F773
94	PS100	1F94	1F264	1F434	1F604	1F774
101	HES	1F101	1F271	1F441	1F611	1F781
102	TRIC	1F102	1F272	1F442	1F612	1F782
103	PERLS	1F103	1F273	1F443	1F613	1F783
104	D2-40	1F104	1F274	1F444	1F614	1F784
105	PS100	1F105	1F275	1F445	1F615	1F785
111	HES	1F111	1F281	1F451	1F621	1F791
112	TRIC	1F112	1F282	1F452	1F622	1F792
113	PERLS	1F113	1F283	1F453	1F623	1F793
114	D2-40	1F114	1F284	1F454	1F624	1F794
115	PS100	1F115	1F285	1F455	1F625	1F795

121	HES	1F121	1F291	1F461	1F631	1F801
122	TRIC	1F122	1F292	1F462	1F632	1F802
123	D2-40	1F123	1F293	1F463	1F633	1F803
124	PS100	1F124	1F294	1F464	1F634	1F804
125	PERLS	1F125	1F295	1F465	1F635	1F805
131	HES	1F131	1F301	1F471	1F641	1F811
132	TRIC	1F132	1F302	1F472	1F642	1F812
133	PERLS	1F133	1F303	1F473	1F643	1F813
134	D2-40	1F134	1F304	1F474	1F644	1F814
135	PS100	1F135	1F305	1F475	1F645	1F815
141	HES	1F141	1F311	1F481	1F651	1F821
142	TRIC	1F142	1F312	1F482	1F652	1F822
143	PERLS	1F143	1F313	1F483	1F1653	1F823
144	D2-40	1F144	1F314	1F484	1F654	1F824
145	PS100	1F145	1F315	1F485	1F655	1F825
151	HES	1F151	1F321	1F491	1F661	1F831
152	TRIC	1F152	1F322	1F492	1F662	1F832
153	PERLS	1F153	1F323	1F493	1F663	1F833
154	D2-40	1F154	1F324	1F494	1F664	1F834
155	PS100	1F155	1F325	1F495	1F665	1F835
161	HES	1F161	1F331	1F501	1F671	1F841
162	TRIC	1F162	1F332	1F502	1F672	1F842
163	PERLS	1F163	1F333	1F503	1F673	1F843
164	D2-40	1F164	1F334	1F504	1F674	1F844

165	PS100	1F165	1F335	1F505	1F675	1F845



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1F214.jpg	2011年2月8日 9:09	4 MB	JPEG イメージ	
1F221.jpg	2013年4月14日 22:21	3.9 MB	JPEG イメージ	
1F222.jpg	2013年4月14日 2:08	4.2 MB	JPEG イメージ	
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Table 2 : List of 1F, including 1F222, 1F223(Kurobe 2011 : original : 2011)

CONCLUSION

Because this article contained many scientific and ethical mistakes and questionable points, I sent a letter to SRA detailing the mistakes. I received the following reply from Fabrice DUPARC (Editor in chief of SRA): « After careful assessment, the referees concluded that the data published in the original article is scientifically sound. Therefore, I am afraid to say, we disagree with your point of view that a correction of the article is necessary. I am sorry to disappoint you on this occasion. » And he requested me to sign the attached contribution list and return an electronic copy to him. (21/04/2017). It is completely against the rule to sign after the publication of the article. By the way, I do not know the third author whose name was not in the list in AR. The fifth author (Prof. Hôpital Necker. E.M.)who presented us 10 speciments (Embryons) (04/2011), had thought to the last that they were used for our study. But in fact, Uhl changed the speciments. He decided to use the fetuses given by his friend. On the other hand, as the experimental study by UhlPhlebolymphology2015 ; 22(2) :59-60] which also had deceptive data, was published, I sent a letter to this journal and received the following e-mail of Françoise PITSCH: « We will leave the reply to Uhl. » (19/10/2016). However, I received no response from him. In this paper, the image (p.59, Figure9, Panel B) which was actually for SMA, 1F222, was shown as an image for D2-40. The following note was included: Vessel-specific immune marker

(Slice B with D2-40). This expression indicates that all 3D blood vessels were the result of D2-40.The background was too strong, and muscles and smooth muscles were stained, which is suggestive of SMA. SMA is a specific marker of vascular smooth muscle, but not expresses in the lymphatic vessels. Thus, D2-40 as a specific lymphatic vessel marker is inappropriate of venous system observation in this study. Uhl regarded the image for SMA in this experiment as that for D2-40, and is still continuing to report inaccurate study results. As I have adequate scientific evidence, I formally declare that the paper published in SRA and other reports by him in several fields contain major mistakes. The unfavorable effects of these mistakes on blood vessel studies in the future area of concern. We can not leave Uhl's various inaccurate publications and presentations. In case of meeting or congress, Uhl prepared PPT (Figure 3) and ordered me to report this study, saying that D2-40 is a marker of arteries, veins and lymphatics. I rejected that because D2-40 is not a marker for blood vessels. Even now, he never admit the use of SMA in our first experiment. But 07/07/2016, the second author confessed finally in front of Kurobe and URATA Ryoichi (President of AARJF, Paris), saying « I used SMA (DAKO, Denmark) for the first experiment. »(Figure1) She is the technician who gave me the protocol of SMA, 04/2011. She began to say, « I did not use SMA. » after Uhl finished his 3D work. She did not confess the fact for a long time. The research was not the first good report about the vessel-system of human fetus. We did not observe the vessel-system of the normal human fetus using IHC marker: D2-40.

ACKNOWLEDGEMENT

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