**METHODS.** Using 20 human fetuses (12–25 weeks of gestation), semiserial horizontal or sagittal paraffin sections were prepared at intervals of 20 to 100 μm. In addition to routine histology, the authors performed silver staining as well as immunohistochemistry for alpha smooth-muscle actin (SMA), vimentin, S100 protein, and tyrosine hydroxylase.

**RESULTS.** Up to 12 weeks, the orbital muscle appeared as a plate-like mesenchymal condensation between the ciliary and sphenopalatine ganglia. Up to 15 weeks, the thick smooth-muscle layer provided an inferoposterior wall for the orbit. A notable feature was a difference in fatty tissue development between the ocular (anterior) and posterior sides of the orbital muscle. At 20 and 25 weeks, SMA immunoreactivity and the amount of smooth-muscle basal lamina were decreased, in contrast to an increase in the number of collagenous fiber bundles. Nerves for the orbital muscle are unlikely to contain sympathetic fibers until 15 weeks.

**CONCLUSIONS.** The authors hypothesize that, in the early stage, the orbital muscle separates the orbital content from the surrounding loose spaces to maintain conditions adequate for the development of orbital fat and other connective tissues. Later, the orbital muscle is replaced by collagenous fibers and seems to provide guidance for calcification of the inferoposterior bony orbital wall. Vimentin-positive osteoprogenitor cells appear to migrate from the perichondrium of the sphenoid and ethmoid. (Invest Ophthalmol Vis Sci. 2011;52:1501-1506) DOI:10.1167/iovs.10-6013

According to Gray’s Anatomy,1 the “orbitalis muscle of Müller” is a thin layer of smooth muscle spanning the inferior orbital fissure (for convenience, we employ the term “orbital muscle” below). The attached structures are the sphenoid bone anteromedially and the maxilla and zygoma posterolaterally.2 Although its function remains uncertain, the orbital muscle has been considered to cause exophthalmos and/or to regulate venous drainage under autonomic nerve control.3 The orbital muscle covers a large area in the human fetal orbit relative to the usual images provided from adult anatomy because, in fetuses, the large inferior orbital fissure continues to the widely opened infraorbital canal.4 According to de Haan et al.,5 almost 50% of the fetal orbital floor is made by the orbital muscle. Thus, without the orbital muscle, the fetal orbital space would open widely and continue freely to the large sphenopalatine and infratemporal spaces.

The fetal orbital muscle seems to be a limited smooth muscle extending between hard tissues: another distinct mass of smooth muscle outside the viscera is found in the rectourethralis muscle, but it connects between the pelvic viscera or between the organ and bone.6,7 Notably, according to figures in Koornneef,8 the architecture and differentiation of fatty tissues differ between the anterior (ocular) and posterior sides of the orbital muscle: the orbital fat displays a lobular architecture much earlier than that in the sphenopalatine fossa and infraorbital groove. Moreover, in these pictures, the orbital muscle decreases in thickness and adopts a membranous appearance after 20 weeks. Unfortunately, because morphology of the muscle was beyond the scope of Koornneef’s study, no description was provided in the text.

According to Poissonnet et al.,9 in human fetuses, development of fatty tissue starts in the head and neck at 14.5 weeks and in the trunk at 15 to 16 weeks. Poissonnet et al.10 classified the differentiation of fatty tissue into 4 stages or critical morphologies: the angiogenesis at stage 1; the lobule-like mesenchymal condensation (mesenchymal lobule) at stage 2; the fatty tissue lobule at stage 3; and the definite sponge-like fatty tissue at stage 4. However, they did not describe a difference between the orbital and sphenopalatine spaces. In the present study, we focused not only on the orbital muscle architecture but also the development of fatty tissue in the anterior and posterior sides of the muscle. Our group has recently demonstrated the expression of desmin and vimentin at the muscle insertion during the early development of skeletal muscles.11 In the present study, however, because desmin is specific for striated muscle, we used an antibody against alpha smooth-muscle actin. In addition, vimentin is known to be a marker of osteoprogenitor cells12–14 and is expressed in the cell body and processes of osteoblasts and osteocytes.12 Consequently, to provide a better understanding of the function of the orbital muscle, the aim of this study was to re-examine the fetal development of this muscle using collections of human fetus sections kept at the Universidad Complutense (Madrid, Spain).

**MATERIALS AND METHODS**

The study was performed in accordance with the provisions of the Declaration of Helsinki 1995 (as revised in Edinburgh in 2000). We examined the paraffin-embedded histology of 20 mid-term fetuses at 12
to 25 weeks of gestation: four fetuses each at 12 weeks (71–92 mm crown-rump length or CRL), 15 weeks (102–118 mm CRL), 18 weeks (145–158 mm CRL), 20 weeks (170–180 mm CRL), and 25 weeks (205–225 mm CRL) according to estimated ovulation age. Twelve weeks correspond to a stage just after the formation of the primordial orbit.5 The present paraffin blocks contained the neck, oral floor, and most parts of the mandible. The sectional planes were horizontal (most of the specimens) or sagittal (two specimens at 12 weeks). All specimens were part of the large collection kept at the Embryology Institute of the Universidad Complutense, Madrid, being the products of miscarriages and ectopic pregnancies managed at the Department of Obstetrics of the University. Approval for the study was granted by the ethics committee of the university.

After routine procedures for paraffin-embedded histology, sections were cut horizontally with a thickness of 5 (fetuses earlier than 15 weeks) or 10 (fetuses later than 20 weeks) μm, at intervals of 50 (early stage) or 100 (late stage) μm. There were around 50 sections for each of the fetuses. Most sections were stained with hematoxylin and eosin (HE), while some (5–6 sections per early-stage fetus) were subjected to immunohistochemical staining and silver staining. The primary antibodies used were rabbit polyclonal anti-human α-smooth-muscle actin (dilution, 1:100; Dako Cytomation, Kyoto, Japan), mouse monoclonal anti-human vimentin (dilution, 1:10; Dako), mouse monoclonal anti-human S100 protein (dilution, 1:100; Dako Cytomation), and rabbit polyclonal anti-human tyrosine hydroxylase (dilution, 1:100; Chemicon, Temecula, CA). Pretreatment in an autoclave was not conducted because of the fragile nature of the fetal tissues. The secondary antibody (Dako Chem Mate Envision Kit; Dako) was labeled with horseradish peroxidase (HRP), and antigen-antibody reactions were detected by an HRP-catalyzed reaction with diaminobenzidine (with hematoxylin counterstaining). The present silver staining (so-called gitter staining) is based on Lillie et al.15 and allows discrimination of basement membrane type-I collagen (colored black) from usual connective tissue fibers (type-I collagen; colored violet-brown). However, for simplicity, the latter type-I fibers are referred to as ‘collagenous fibers’ in the text.

RESULTS

At 12 weeks, there was a variation in development of the orbital muscle: in three of the four specimens at this stage, the orbital muscle appeared as a plate-like mesenchymal condensation that extended along the mediolateral axis, and had not yet attached to the cranial base cartilage anlagen. It was located immediately posterior to the ciliary ganglion, and a loose mesenchyme was interposed between the ciliary and sphenopalatine ganglia. However, in another specimen (92 mm CRL), the orbital muscle was identified as a fibrous band connecting between the maxilla and sphenoid. Notably, this fetus is the smallest specimen in which a difference in fatty tissue development was seen (Fig. 1): abundant vessel-like structures appeared in the orbital space in contrast to the sphenopalatine fossa. The orbital muscle was strongly positive for smooth-muscle actin (SMA; Fig. 2B). The smooth-muscle fibers had an accompanying basal lamina, between which wavy, fragmented collagenous fibers were scattered (Fig. 2C). Until 15 weeks, the orbital muscle exhibited a dense innervation (Fig. 2D) but these nerves were negative for tyrosine hydroxylase (Figs. 2E, 2F).

At 15 weeks, the sphenopalatine fossa still continued to the infratemporal fossa as well as the parasellar area (cranial cavity) without demarcation (Fig. 2A). In the orbit, the presence of abundant thin vessels in loose tissues suggested the initiation of fatty tissue differentiation (Fig. 2A; stage 1 after Poissonnet et al.9,10; see Introduction in current study). Immunohistochemistry showed that the orbital muscle was strongly positive for smooth-muscle actin (SMA; Fig. 2B). The smooth-muscle fibers had an accompanying basal lamina, between which wavy, fragmented collagenous fibers were scattered (Fig. 2C). Until 15 weeks, the orbital muscle exhibited a dense innervation (Fig. 2D) but these nerves were negative for tyrosine hydroxylase (Figs. 2E, 2F).

At 18 weeks, due to an increase in the size of the lesser wing of the sphenoid, the opening of the infratemporal fossa to the orbital space became narrow. However, the orbital muscle still faced the temporalis muscle, and thus separated the orbit from the infratemporal fossa containing the temporalis muscle (Fig. 3A). The mesenchymal lobules were scattered through the entire orbit. The density was especially high in the retroglobal area (Figs. 3A, 3B; stage 2 after Poissonnet et al.9,10; see Introduction in current study). The mesenchymal lobule was also evident in a limited portion of the sphenopalatine-infratemporal space complex near the temporalis muscle (Fig. 3A). The orbital muscle contained an abundant basal lamina for smooth muscles, but collagenous fibers were increased in number and arrayed irregularly between the basal lamina (Fig. 3C). SMA immunoreactivity was decreased in parts of the smooth muscles (Fig. 3D).

At 20 weeks, the orbital muscle still faced the sphenopalatine fossa as well as the infratemporal fossa, but the lesser wing of the sphenoid became interposed between these two spaces.
fibers occupied the medial and lateral 1/4 of the orbital muscle near aggregation around the eyeball (Fig. 4A). Notably, collagenous fibers surrounded by the septa, constituted a continuous characterizing the orbital fat (Fig. 4A). Lobule-like fat tissues, each other than the orbital muscle, vessels exhibit positive reactivity because of the specific character of this antibody. Inset DE in (B) will be shown in (D) and (E). In (C), short wavy collagenous fibers (violet-brown) are scattered between the basal lamina of smooth muscles (black). (D) S100 immunostaining; pan-neuronal marker displays dense innervations of the orbital muscle. However, these nerves are unlikely to be sympathetic. (E) tyrosine hydroxylase immunostaining (sympathetic nerve marker). (F) A positive control of the tyrosine hydroxylase immunostaining (maxillary nerve branches). (D-F) Sections near (A), prepared at the same magnification as in (C). ET, ethmoid bone; IO, inferior oblique muscle; NLD, nasolacrimal duct; SPG, sphenopalatine ganglion. Other abbreviations are the same as in Figure 1.

Fatty tissue was evident not only in the orbital space but also in the sphenopalatine fossa (Figs. 4A, 4B; stage 3 or 4 after Poissonnet et al.9,10; see Introduction). However, a marked condensation of collagenous fibers became evident in the orbital space. Thus, in contrast to the homogeneous structures in the sphenopalatine fossa, net-like connective tissue septa4 characterized the orbital fat (Fig. 4A). Lobule-like fat tissues, each of which was surrounded by the septa, constituted a continuous aggregation around the eyeball (Fig. 4A). Notably, collagenous fibers occupied the medial and lateral 1/4 of the orbital muscle near the bony attachments (Fig. 4B). Even in the middle part, black staining of the basal lamina became pale (Fig. 4B) and, simultaneously, SMA immunoreactivity became significantly decreased (Fig. 4C). In the lateral and medial parts, thick collagenous fiber bundles replaced the basal lamina (Fig. 4D). In the middle part, collagenous fibers bundled and fragmented the smooth-muscle fibers (Fig. 4E).

Between 15 and 20 weeks, the eyeball more than doubled its maximum diameter. In contrast, the maximum length of smooth-muscle fibers of the orbital muscle appeared to increase <1.5-fold. This meant that the ratio of the orbital muscle occupying in the posterior wall of the orbit decreased considerably, and that the wall became occupied by other fibrous tissue, especially in the medial and lateral areas. The latter collagenous tissue was continuous with the periostium of the sphenoid and ethmoid bones. Up to 25 weeks, in the orbital muscle, most of the smooth muscle became replaced by collagenous fiber bundles.

Figure 5 summarizes the results of vimentin immunohistochemistry. Vimentin-positive cells appeared around sites of ossification in the sphenoid and ethmoid up to 12 weeks. However, at 12 and 15 weeks, they were absent in the orbital muscle. At 18 weeks, the orbital muscle, extra-ocular striated muscles, temporalis muscle, and orbital fatty tissue contained vimentin-positive cells. These were located along the vessels in the orbital muscle, but at the insertion and origin in the extra-ocular striated muscles. Their density was especially high in the fibrous tissues connecting between the orbital muscle and the periostium of the sphenoid and ethmoid bones. Thus, we had no evidence that smooth-muscle fibers express vimentin.

Consequently, in the early stage, the fetal orbital muscle provided posterior and inferior demarcations of the orbit. On the anterior side of the orbital muscle, connective tissues, including fatty tissue, developed a specific architecture. The major content of the inferoposterior wall of the orbit changed from smooth muscle (orbital muscle) to periostium-like connective tissue comprising collagenous fibers. The smooth muscle itself was also replaced by collagenous fibers, and finally changed to periostium-like tissue. However, without transition from smooth muscle to hard tissue, vimentin-positive osteo-
genitor cells appeared to migrate from the perichondrium or periosteum of the sphenoid and ethmoid.

DISCUSSION

The present study demonstrated a difference in the development of fatty tissue between the orbital and sphenopalatine-infratemporal spaces. The orbital muscle served as a limited structure providing clear demarcation between the anterior and posterior fields of fatty tissue differentiation (Fig. 6). What is responsible for this difference in fatty tissue differentiation? Incomplete septation, such as that provided by the orbital muscle, is unlikely to result in a difference of chemical induction. Therefore, we hypothesize that a difference in mechanical stress exists between these spaces. Astersisks in (A) and (B) indicate postmortem damage before fixation. In (B), the middle part of the orbital muscle is black, whereas it is violet-brown in the medial and lateral parts (upper and lower sides of the figure). (D) and (E) Higher magnification views (silver staining) of the insets in (B). In (E), the basal lamina of smooth muscles (black) are bundled and fragmented by collagenous fibers (violet-brown). Abbreviations are the same as in Figure 1.

Figure 4. Horizontal sections of a 20-week fetus. The left-hand side of the figure corresponds to the anterior side of the body. (A) HE staining and (B) silver staining show adjacent sections, while (C) (SMA immunostaining of the middle part of the orbital muscle) displays a section 0.5 mm inferior to (A) and (B). In (A) and (B), the temporalis muscle (T) is located near the orbital content. Note the difference in fatty tissue architecture between the anterior and posterior sides of the orbital muscle. Asterisks in (A) and (B) indicate postmortem damage before fixation. In (B), the middle part of the orbital muscle is black, whereas it is violet-brown in the medial and lateral parts (upper and lower sides of the figure). (D) and (E) Higher magnification views (silver staining) of the insets in (B). In (E), the basal lamina of smooth muscles (black) are bundled and fragmented by collagenous fibers (violet-brown). Abbreviations are the same as in Figure 1.

Figure 5. Vimentin immunohistochemistry. (A) and (B) show a section near Figure 1 (15 weeks), while (C) and (D) display a section near Figure 3 (20 weeks). (A) corresponds to the left-hand side of Figure 1B, and (C) to the lowest part of Figure 3B. (B) and (D) are higher-magnification views of the insets in (A) and (C), respectively. In (A), vimentin-positive immunoreactivity is evident around ossification sites in the sphenoid and striated muscles (T, temporalis; IO, inferior oblique). However, in the periosteum (B), positive cells are still scarce. In (C) and (D), positive cells are distributed widely in the fibrous tissue between the orbital muscle and the sphenoid. The orbital fatty tissue also contains positive cells (C, asterisks).
However, as described in detail by Bergen, a remodelling of the orbital muscle. The present findings did not suggest any differentiation from smooth muscle to hard tissue, but vimentin-positive osteoprogenitor cells appeared to migrate from the peripherally located perichondrium or periosteum into the area of smooth muscle. To our knowledge, the present study is the first to have revealed a physiological transition of smooth muscle to bone, although there may be many pathologic examples, such as calcification in myoma uteri.

Overall, in view of the developmental histology, the orbital muscle is a very unique smooth-muscle mass in the human body: it connects between bones and is replaced by collagenous fibers even in fetuses. Moreover, the orbital muscle is likely to play a critical role as a septum to maintain a mechanical condition within the orbital space for the normal development of extra-ocular structures.

We cannot measure the intraorbital pressure of the human orbit to compare with that in the sphenopalatine space. Thus, we cannot directly prove our hypothesis. However, a study plan using experimental animals is also difficult because of the suggested differences in the anatomy and orbital connective tissue differentiation (see the second paragraph of the Discussion). In addition, we did not follow the later process of osteogenesis in the inferior wall of the orbit using materials from newborns, neonates, and children.

References

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