Published in final edited form as:

Genesis. 2007 April ; 45(4): 208–217. doi:10.1002/dvg.20293.

Eomes::GFP—A Tool for Live Imaging Cells of the Trophoblast, Primitive Streak, and Telencephalon in the Mouse Embryo

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Summary

Expression of T-box family member Eomesodermin (Tbr2) is spatiotemporally restricted in the mouse embryo; initially expressed in extraembryonic lineages in the sequential progression from the trophectoderm of the blastocyst, its derivatives the extraembryonic ectoderm, and thereafter the chorion, in addition to the visceral endoderm and primitive streak at gastrula stages, and the telencephalon at later stages. We describe the spatiotemporal expression of GFP in embryos of a *Tg(Eomes::GFP)* BAC transgenic strain, and have compared it with the localization of endogenous *Eomes* transcripts and protein. Our analysis reveals the following: (1) robust easily visualized reporter expression in live hemizygous transgenic embryos, (2) increased levels of expression in live homozygous transgenic embryos that are compatible with embryo viability, and (3) a close correlation between endogenous *Eomes* and GFP reporter expression in BAC transgenic embryos. These features establish the *Tg(Eomes::GFP)* BAC transgenic strain as a novel reagent for both live imaging and the isolation of Eomes expressing cells from specific locations within the embryo.

Keywords

mouse embryo; T-box; Eomesodermin; Tbr2; green fluorescent protein; live imaging; blastocyst; trophectoderm; extraembryonic ectoderm; primitive streak; telencephalon; limb; digit condensation; stomach; pancreas

Eomesodermin (Eomes or Tbr2) a member of the T-box family of transcriptional regulators was originally identified for its ability to specify mesoderm in *Xenopus* (Ryan *et al.*, 1996). Mouse *Eomes* mutants have uncovered its role in the regulation of trophoblast lineage specification and mesoderm migration during embryonic development and the development of cell-mediated immunity in the adult (Pearce *et al.*, 2003; Russ *et al.*, 2000). The tightly regulated spatiotemporal expression of *Eomes* (Bulfone *et al.*, 1999; Ciruna and Rossant, 1999; Hancock *et al.*, 1999) and the paucity of available transgenic mouse strains expressing GFP in a lineage-specific fashion in early mouse embryos led us to determine if an *Eomes::GFP* BAC transgenic mimicked endogenous gene expression, and could therefore be used as a reporter strain for live imaging (Lee *et al.*, 2001; Zhang *et al.*, 2004, 2005).

The *Tg(Eomes::GFP)* BAC transgene was obtained from a library generated by the GENSAT consortium (Gong *et al.*, 2003). The BAC covers ~225 kb of mouse genomic DNA, containing sequences spanning ~186 kb upstream and ~18 kb downstream of the *Eomes* locus. Enhanced

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green fluorescent protein (EGFP) and a pA sequence were inserted directly upstream of the *Eomes* coding region preserving gene structure while providing a readout of promoter activity (Fig. 1a). *Tg(Eomes::GFP)* BAC transgenic animals were generated through the GENSAT consortium (<http://www.gensat.org/index.html>). Hemizygous and homozygous transgenics were viable and fertile and indistinguishable from non-transgenic littermates. Hemizygous *Tg(Eomes::GFP)/+* embryos were used for all data presented. Homozygotes exhibited identical localization of GFP, but with increased levels of fluorescence.

INITIATION OF GFP EXPRESSION AT PERI-IMPLANTATION

Previous studies have documented *Eomes* gene expression and protein localization in mouse oocytes and preimplantation embryos (McConnell *et al.*, 2005). Since expression assayed by wholemount in situ hybridization or lacZ staining in a targeted allele initiates in the trophectoderm of the blastocyst, we imaged live *Tg(Eomes::GFP)* embryos from compacting morula to late blastocyst stages. We were unable to detect GFP expression in early blastocysts (~E3.5; Fig. 1b,c); however, weak expression within the trophectoderm could be seen to initiate in later hatched blastocysts (~E4.0; Fig. 1d–h). By peri-implantation (~E4.5) embryos exhibited an increased level of GFP fluorescence restricted to the trophectoderm (Fig. 1i–m). The localization of GFP expression in *Tg(Eomes::GFP)* embryos mirrored endogenous *Eomes*, albeit somewhat temporally delayed (Hancock *et al.*, 1999; Strumpf *et al.*, 2005). Any lag in GFP expression could in part be due to the fact that the routine detection of endogenous *Eomes* transcripts or protein utilizes an amplification step, whereas in the *Tg(Eomes::GFP)* transgenics the visualization of GFP is a direct and linear readout of promoter activity in living embryos, and thus sufficient levels of detectable protein must be present.

GFP EXPRESSION AT GASTRULA STAGES

By the pre-streak stage, robust fluorescence was detected exclusively in the extraembryonic ectoderm, a derivative of the polar trophectoderm (Fig. 2a,b). Subsequently, by the early-streak stage GFP expression was maintained in extraembryonic regions, but also had initiated within the primitive streak concomitant with its formation (Fig. 2d,e). Visualization of endogenous *Eomes* transcripts by in situ hybridization of the same embryos live imaged for GFP established the correlation between endogenous *Eomes* expression and reporter expression at these stages (Fig. 2c,f). In addition, GFP fluorescence in the extraembryonic ectoderm and primitive streak closely correlated with the distribution of *GFP* transcripts (Fig. 2m–p). These observations imply that the cis-acting regulatory elements necessary for *Eomes* activation within the trophoblast, extraembryonic ectoderm, and primitive streak are present within the BAC. Previous work on the dissection of the *Xenopus Eomes* promoter led to the identification of an activin responsive element (ARE) and two FAST2 binding sites necessary for mesodermal expression that were positioned upstream of the transcriptional start site (Ryan *et al.*, 2000). Database searches revealed that both ARE and FAST2 sites were present within the region located immediately upstream of the first exon of the mouse *Eomes* locus and were contained within the BAC insert (data not shown).

Knock-in strains with primitive streak-specific fluorescent protein expression have been reported (Hart *et al.*, 2002; Huber *et al.*, 2004). In these cases a GFP reporter was introduced into an endogenous locus by gene targeting in embryonic stem cells. However, since knock-in alleles perturb wild type gene function, these strains are not favored as neutral indicators for analyzing mutants due to the possibility of genetic interactions and epistasis effects. The *Tg(Eomes::GFP)* strain therefore provides a solution, since it is the first transgenic strain reported to exhibit robust GFP expression within the early primitive streak of the mouse embryo concomitant with the onset of gastrulation.

By the late-streak stage (~E7.5) when *Eomes* was localized to the chorion and primitive streak (Fig. 2i), GFP expression was downregulated within the extraembryonic ectoderm, while expression within the epiblast was observed but at reduced intensity, encompassing an expanded domain including nascent mesoderm cells emanating from the primitive streak, likely resulting from the perdurance of the fluorescent protein in cells emanating from the primitive streak. (Fig. 2g,h). By early headfold stages (E7.75) when *Eomes* was maintained in the chorion, but downregulated in the primitive streak (Fig. 2l), GFP fluorescence was undetectable in all regions of the embryo (Fig. 2j,k). These observations reveal that GFP fluorescence was extinguished prior to downregulation of endogenous *Eomes*, suggesting the following: (1) Distinct cis-acting regulatory elements are necessary for maintenance of *Eomes* expression. Such cis-regulatory elements might reside at, or co-operate with, sites distant to the *Eomes* locus not contained within the BAC, alternatively the introduction of EGFP into the *Eomes* locus may have perturbed the spacing and thus function of such elements. (2) Perdurance of GFP does not extend over more than a period of a few hours. (3) The *Tg(Eomes::GFP)* strain can be used to visualize, and thus discriminate, early vs. later primitive streak.

GFP EXPRESSION WITHIN THE CNS

Live imaging of midgestation embryos revealed fluorescence in two locations: the central nervous system (CNS) and developing limbs which correlated with previously reported endogenous *Eomes* and persisted until perinatal stages when it was downregulated (Bulfone *et al.*, 1999; Ciruna and Rossant, 1999; Hancock *et al.*, 1999; Russ *et al.*, 2000).

In the CNS, fluorescence was first observed at E10.0 within the telencephalon (Fig. 3a), and became more robust by E12.5–E14.5 (Fig. 3b,c), then diminished by P0 (Fig. 3d). We investigated the colocalization of GFP and endogenous *Eomes* protein (Fig. 3e–h) and determined that GFP was expressed in a larger population of cells. High magnification views of sectioned brains at E14.5 revealed that GFP was detectable in a subpopulation of cells expressing and colocalized with *Eomes* protein predominantly within the subventricular zone (SVZ), but GFP expressing cells were also present in the intermediate zone (IZ) to marginal zone (MZ) (Fig. 3i–l). This absence of GFP expression in *Eomes* +ve cells of the SVZ is likely to be analogous to the delay in onset of detectable fluorescence observed in blastocyst stage embryos (discussed previously). We also noted that GFP fluorescence extended to more differentiated regions of the neocortex where *Eomes* protein was not present, likely due to perdurance of the fluorescent protein.

We next determined colocalization of the GFP reporter with molecular markers representing the different cell types present in the telecephalon, including both distinct classes of neural progenitors and postmitotic cells. Pax6 marks a population of radial glial progenitor cells (Gotz *et al.*, 1998) fated for glutamatergic differentiation (Schuurmans *et al.*, 2004). Pax6 antibody staining revealed no overlap between Pax6 expression in the VZ and GFP expression in the SVZ and MZ, suggesting that the transgene was not prematurely activated (Fig. 3m–p). However, staining with T-brain1 (Tbr1) antibody revealed a subpopulation of cells co-expressing GFP and Tbr1 (Fig. 3q–t). Since *Eomes* (Tbr2) and Tbr1 are expressed in mutually exclusive populations of cells (Englund *et al.*, 2005), this result suggests that GFP fluorescence extended into regions containing postmitotic neurons and that this could result from perdurance of the fluorescent protein or downregulation of the reporter. Furthermore, staining with the M-phase marker phosphohistone-H3 revealed colocalization with a subpopulation of GFP expressing cells representing a non-surface dividing intermediate progenitor cell population (Fig. 3u–x).

To establish if the expanded domain of GFP fluorescence observed in *Tg(Eomes::GFP)* brains was due to perdurance of GFP protein, we processed sequential sections to determine and

compare the localization of GFP protein, *GFP* transcripts, and *Eomes* transcripts. *GFP* transcripts were more restricted than GFP protein (Fig. 4a,b,d–e), and were localized to an identical domain as *Eomes* transcripts, residing predominantly within the subventricular and intermediate zones at E14.5 (Fig. 4c), and the hippocampus at P0 (Fig. 4f). Thus perdurance of the fluorescent protein results in the expanded domain of GFP fluorescence, such that the GFP reporter acts as a lineage tracer for postmitotic neurons that have recently extinguished *Eomes* having migrated into the cortical plate (cp).

Within the developing limb, a focus of GFP fluorescence was observed at the base of digit IV (Fig. 3b–c, Fig. 4i). Sections through the forelimb at E12.5 revealed that GFP was localized in a ring of cells around the condensations associated with the fourth digit (Fig. 4j–n). In addition, *Eomes* transcripts within the forelimb at this stage were also restricted to the same ring of cells at the base of the fourth digit, and thus closely correlated with transgene expression (Fig. 4g–h).

ADDITIONAL SITES OF GFP EXPRESSION AT MIDGESTATION THROUGH FETAL STAGES

At midgestation through fetal stages additional sites of readily detectable GFP expression included discrete populations of cells within the stomach, pancreas, and placenta (Fig. 5). Within the E14.5 placenta, a small population of fluorescent cells were observed within the parietal yolk sac (Fig. 5a,b). These cells were present in the endodermal layer of the parietal yolk sac, as at this stage no GFP expression was observed in trophoblast giant cells, which are derivatives of the trophoblast. This population persisted until birth.

Transgene expression was also observed in the pylorus of the stomach (Fig. 5h–k), with increased magnification revealing the elongated cell morphology of GFP-positive cells (Fig. 5l). E-cadherin antibody staining revealed that these GFP positive cells represented a subpopulation of cells within the mucosa (Fig. 5s–u). GFP fluorescence was also observed within the developing pancreas (Fig. 5m–r) at E14.5. Thus, to better define the identity of these cells, an antibody against pancreatic duodenal homeobox-1 (Pdx1) was used to stain sections through the pancreas. The staining revealed that there was no colocalization of Pdx1 and GFP (Fig. 5v–x). Since Pdx1 is present in endocrine progenitor cells at E14.5, and is later involved in β -cell maturation, the GFP positive cells observed within the pancreas are unlikely to represent the endocrine cells of the islets of Langerhans, including the alpha-(glucagon-secreting) and beta-(insulin-secreting) cells, but more likely to represent a subpopulation of pancreatic exocrine cells.

In summary we have demonstrated that GFP expression in the GENSAT *Tg(Eomes::GFP)* BAC transgenic strain closely correlates with endogenous mouse *Eomes* expression during embryonic development. This strain therefore represents a potentially useful tool for live imaging cell behavior in the trophoblast and its derivative extraembryonic ectoderm, the early primitive streak at gastrulation, as well as cell populations within the telencephalon during neurogenesis in addition to populations of cells within the limb, stomach, and pancreas during later fetal stages (Hadjantonakis *et al.*, 2003). Furthermore lineage specific GFP expression also permits the isolation of *Eomes* expressing cells from specific locations within the embryo (Hadjantonakis and Nagy, 2000). The *Eomes* BAC represents a reagent that can be retrofitted to replace the native EGFP cassette with spectral variant or subcellularly localized fluorescent proteins for higher resolution live imaging, or site specific recombinase variants for lineage-specific gene modification (Branda and Dymecki, 2004; Hadjantonakis and Papaioannou, 2004; Rhee *et al.*, 2006).

METHODS

Embryo Collection

Preimplantation embryos were recovered in M2 medium and cultured in KSOM medium (Chemicon Specialty Media, Temecula, CA) in an organ culture dish (BD Falcon, cat no. 353037) or, for live imaging, under mineral oil in a MatTek glass bottom dish (cat. no. P35G-1.5-14C) at 37°C, 5% CO₂. Postimplantation embryos and organs were dissected in modified PB-1 (Papaioannou and West, 1981; Whittingham and Wales, 1969) medium containing 10% fetal bovine serum (FBS). Live embryos were counterstained with 7.5 mM DRAQ5 (DAKO, Carpinteria, CA).

Vibrating Microtome Sectioning and Counterstaining

Embryos were fixed in 4% PFA/PBS for 2–12 h after dissection, washed in PBS, and embedded in 4% low-melt agarose, 5% sucrose in PBS. Blocks were cut out of embedding molds, trimmed using a razor blade, and then mounted onto a vibrating microtome chuck (Leica VT1000S) using superglue. Sections were cut at a thickness of 25–200 µm. Embryos were counterstained with Hoechst (cat no. H3570, Invitrogen) and AlexaFluor633 phalloidin (cat no. A22284, Invitrogen).

In Situ Hybridization and Immunocytochemistry

In situ hybridizations were performed according to standard protocols (Nagy *et al.*, 2003). For immunohistochemistry, brains were cut into 70 µm-thick sections, blocked for 1 h at RT in PBSMT, and then incubated with one of the following antibodies diluted in PBSMT at 4° overnight: anti-Tbr2 (1:200; cat. no. AB9618, Chemicon International), anti-Tbr1 (1:500; Hsueh *et al.*, 2000), anti-Pax6 (1:200; Developmental Studies Hybridoma Bank), anti-pHH3 (1:500; cat. no. 06-570, Upstate Biotechnology), anti-E-cadherin (1:200; cat. no. U-3254, Sigma), or anti-Pdx1 (1:200; cat. no. ab47267, Abcam). The next day, the sections were washed with PBSMT and then incubated with Vectastain blocking solution (Vector Labs, Burlingame, CA) for 1 h at RT, followed by incubation with a biotinylated secondary antibody at 4° overnight. The sections were then washed with PBSMT and rinsed in PBT before being incubated with anti-rabbit Streptavidin-AlexaFluor546 (Molecular Probes, Eugene, OR) for 1 h at RT in the dark. Finally, sections were washed in PBT before being imaged.

Image Acquisition

Laser scanning confocal data were acquired using a Zeiss LSM510 META laser scanning confocal mounted on a Zeiss Axiovert 200M microscope. Whole embryos were kept in PBS in MatTek glass bottom dishes during imaging. Widefield images were acquired using a Zeiss AxioCam MRc or MRm camera mounted on a Leica MZ16FA microscope.

Acknowledgments

We thank Shiao-ching Gong and Mark Tomishima for advice on GENSAT BAC construction; Terry Capellini for discussions on limb expression; Stewart Anderson for antibodies and advice on transgene expression in the telencephalon; Sonja Nowotschin for comments on the manuscript; Ginny Papaioannou for in situ probes, assistance, and insightful discussions pertaining to transgene expression and comments on the manuscript.

Contract grant sponsor: NIH, Contract grant number: RO1-HD052115

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FIG. 1.

Schematic representation of *Tg(Eomes::GFP)* BAC transgene and onset of GFP expression in live peri-implantation mouse embryos. **(a)** Map of the GENSAT *Tg(Eomes::GFP)* BAC transgene spanning 186 kb upstream and 18 kb downstream of the mouse *Eomes* locus. Gray boxes depict exons encoding untranslated regions, green box depicts EGFP cassette with pA in blue. Orange boxes represent exons. **(b,c)** Absence of GFP fluorescence in early blastocysts (~E3.5) with DRAQ5 live nuclear counterstain (shown in red). **(d–g)** Onset of GFP fluorescence in later blastocysts in both polar trophectoderm (PTE; white arrowheads) and mural trophectoderm (MTE; yellow arrowheads). **(h)** 3D rendered confocal image of ~E4.0 embryo depicting GFP localization to the trophectoderm. **(i–l)** Peri-implantation stage (~E4.5) embryo. Differentiating giant cells at the implantation site (blue arrowheads). **(m)** 3D rendered confocal image (red and green channel merge). Scale bars = 50 μm. ICM, inner cell mass; PTE, polar trophectoderm; MTE, mural trophectoderm; 2D, two-dimensions; 3D, three-dimensions.

FIG. 2.

GFP expression in the extraembryonic ectoderm and early primitive streak of live gastrula stage embryos. (**a,b,d,e**) Pre-streak stage (~E5.75) to early streak stages (~E6.5). (**c,f**) In situ hybridization of the same embryos to detect *Eomes*. (**g,h**) Late-streak stage (~E7.5) embryos exhibit no fluorescence in extraembryonic ectoderm, while GFP fluorescence within the epiblast was reduced in intensity, encompassing a broad domain. High magnification inset of a transverse section through a late-streak stage embryo, depicting GFP fluorescence in cells of the nascent mesoderm as well as a subpopulation of cells in the visceral endoderm. Nuclear counterstain reveals all cells in the section, including cells within the epiblast and primitive streak that do not exhibit GFP fluorescence. (**j,k**) By early headfold stages (~E7.75) GFP fluorescence was undetectable. (**i,l**) *Eomes* expression in late-streak stage embryos (~at E7.5) within the chorion (red arrowhead) and primitive streak (i; blue arrowhead). By the early headfold stage (~E7.75), *Eomes* transcripts are only detected in the chorion (l; red arrowhead). (**m–p**) GFP fluorescence in the extraembryonic ectoderm and primitive streak at E6.5 (m, n, o) closely correlates with the distribution of *GFP* transcripts as assayed by in situ hybridization to the same embryo (p). Scale bars = 100 μ m. bf, brightfield; df, darkfield.

FIG. 3.

GFP expression within the telencephalon of *Tg(Eomes::GFP)* embryos at midgestation and early postnatal stages. (a) Onset of GFP fluorescence in the central nervous system (CNS) at E10.0. (b,c) Wholemount brightfield and GFP overlays depicting GFP fluorescence in the developing telencephalon at E12.5 (b) and E14.5 (c). Discrete foci of GFP fluorescence in both forelimbs and hindlimbs (white arrowheads). (d) By postnatal day zero (P0), GFP fluorescence was markedly reduced. (e-h) Fluorescent staining with Eomes antibody on vibratome sections revealed colocalization of GFP with Eomes protein, although the domain of GFP fluorescence was broader than that of the endogenous protein. (i-l) High magnification rendered confocal images of sectioned brains at E14.5 (boxed region in g) showing nuclear counterstain (i), GFP

fluorescence (j), and Eomes protein expression (k). Three channel merge (l) reveals that GFP is not expressed in some cells with Eomes protein (yellow arrowhead) while other cells have both GFP and Eomes protein expression (white arrowhead). **(m–p)** Staining with T-brain1 (Tbr1) antibody on similar sections reveals colocalization of GFP and Tbr1, illustrating that GFP fluorescence is extended into regions of postmitotic neurons. **(q–t)** Pax6 antibody staining reveals minimal overlap between Pax6 expression in ventricular zone (vz) and GFP expression in subventricular zone (svz) to marginal zone (mz). **(u–x)** Staining with the phosphohistone-H3 shows colocalization with some GFP expressing cells (white arrowheads). Scale bars = 100 μm . vz, ventricular zone; svz, subventricular zone; iz, intermediate zone; sp, subplate; cp, cortical plate; mz, marginal zone; fl, forelimb; hl, hindlimb.

FIG. 4.

The expanded domain of GFP fluorescence within the telencephalon of *Tg(Eomes::GFP)* embryos is due to perdurance of GFP protein. (a) Brightfield and GFP overlay of vibratome sections of E14.5 brains illustrating GFP localization. (b,c) *GFP* expression by in situ hybridization (b) reveals that *GFP* transcript has a more restricted expression domain that appears to be identical to the expression of *Eomes* transcripts (c). High magnification insets depict the localization of *GFP* and *Eomes* transcripts largely within the subventricular and intermediate zones. (d) By postnatal day 0 (P0), GFP fluorescence is reduced to regions of the olfactory bulb (not shown) and the hippocampus. High magnification inset illustrates relatively restricted domain of GFP fluorescence. (e,f) In situ hybridization reveals that both *GFP* and *Eomes* expression is localized to identical regions of the hippocampus and is complementary with observed GFP fluorescence (d). High magnification insets depicting expression of *GFP* and *Eomes* in early postnatal hippocampus. (g,h) In situ hybridization depicting *Eomes* transcript localization at the base of the developing digit IV (g) in a ring of cells surrounding the fourth condensation (h). (i,l) GFP fluorescence is also localized to the base of digit IV and correlates closely with endogenous *Eomes* expression. (k-n) High magnification confocal images depicting the ring of GFP expression (m) around a more densely populated condensation. Scale bars = 100 μm. nctx, neocortex; lge, lateral ganglionic eminence; mge, medial ganglionic eminence; ctx, cortex; hp, hippocampus; DG, dentate gyrus; th, thalamus; hy, hypothalamus.

FIG. 5.

Additional sites of GFP fluorescence in *Tg(Eomes::GFP)* embryos at midgestation to fetal stages of development. **(a,b)** A population of fluorescent cells present in the placenta of E14.5 embryos. **(c–e)** Rendered confocal images depicting nuclear counterstain (c), GFP (d), and two channel merge (e). **(f)** Highmagnification rendered image (boxed region in e) showing cell morphology and localization of GFP-positive cells. **(g)** Brightfield and GFP overlay showing fluorescence in the pylorus and pyloric sphincter region of the stomach. **(h–k)** Rendered confocal images of nuclei (h), GFP (i), F-actin (j), and three channel merge (k). **(l)** High magnification image depicting the elongated cell morphology of GFP-positive cells. **(m)** Brightfield and GFP overlay of GFP fluorescence in the developing pancreas. **(n–q)** Rendered confocal images of nuclei (n), GFP (o), F-actin (p), and merge (q). **(r)** High magnification image showing localization and morphology of GFP-positive cells. **(s–u)** Rendered confocal images of GFP (s), E-cadherin (t), and two channel merge with Hoechst nuclear counterstain (depicted in blue) (u), within the pylorus of the stomach. High magnification inset demonstrating that GFP positive cells, representing a subpopulation of cells within the mucosa, are part of an epithelium and stain positive for E-cadherin. **(v–x)** Rendered confocal images of GFP (v), Pdx-1 (pancreatic duodenum homeobox-1) (w), and two channel merge with Hoechst nuclear counterstain (x). High magnification inset illustrating that GFP fluorescence does not colocalize with differentiating Pdx1-positive pancreatic beta cells. Scale bars = 100 μ m. umb, site of attachment of the umbilicus; lab, labyrinthine layer; sp, spongiotrophoblast; mp, muscularis propria; sm, submucosa; m, mucosa.