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# Mesenchymal calcium waves underpin intestinal smooth muscle differentiation

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## Abstract

Intestinal smooth muscle differentiation is a complex physico-biological process involving several different pathways. Here, we reveal using GCamp6f expressing mouse embryos that calcium waves propagate in the mouse intestinal mesenchyme one day prior to the emergence of  $\alpha$ -smooth muscle actin in the presumptive circular and longitudinal smooth muscle layers. We find that L-type calcium channel blockers and myosine light-chain kinase inhibitors halt these waves, inhibit smooth muscle differentiation and intestinal growth. We demonstrate that the effect of the calcium waves on differentiation are not mediated via mechanobiological pathways related to smooth muscle contractions. We recapitulate most of these results in the avian embryo, and show that early guts treated with L-type channel blockers up-regulate the LIX1 factor characteristic of mesenchymal stemness, whereas older guts display KIT expressing cells characteristic of Interstitial Cells of Cajal. We conclude that electrical connectivity of the mesenchyme precedes contractile function in intestinal development and that calcium waves are required for the differentiation of mesenchymal progenitors into contractile smooth muscle cells.

## Introduction

Gut motility is characterized by coordinated contractions of the intestinal smooth muscle cells (SMCs). Digestive SMCs derive from the embryonic mesoderm that gives rise to the mesenchyme, which in turn differentiates into submucosa, KIT-positive interstitial cells of Cajal (ICCs), and smooth muscle cells (SMCs) (Le Guen et al., 2015; Wallace and Burns, 2005). Digestive mesenchymal progenitors are defined by the expression of the LIX1 gene (McKey et al., 2016). Their differentiation into SMCs

requires the induction of *MYOCD*, a master regulator of SMC-restricted gene expression. Determined SMCs are defined by the early expression of the alpha and gamma isoforms of smooth muscle actin (respectively  $\alpha$ SMA and  $\gamma$ SMA). Later during development, SMCs organize in smooth muscle layers and express proteins involved in contractility, such as CALPONIN and SM22. Smooth Muscle organization in the gut is largely conserved in species as phylogenetically distant as hydra and humans (Shimizu et al., 2004). The SM is comprised of an inner circular smooth muscle (CSM) layer, an outer longitudinal smooth muscle (LSM) layer and, depending on species, a further thin circular and/or longitudinal layer located below the epithelium called the muscularis mucosae. The CSM and LSM are the essential effectors of bulk bolus motion by peristalsis (Amedzrovi Agbesi and Chevalier, 2021) or segmentation (Huizinga et al., 2014), while the muscularis mucosae drives smaller scale motion of epithelial villi and of the lymph vessels circulating within them.

The most important signaling pathways involved in CSM differentiation are the Sonic Hedgehog (Shh) and Bone Morphogenetic Protein pathways (BMP). Modulation of these pathways have been shown to result in strong alterations of CSM genesis, from complete disappearance (Mao et al., 2010; Ramalho-Santos et al., 2000) to hypertrophy (de Santa Barbara et al., 2005; Huycke et al., 2019) and misalignment (Sicard et al., 2022; Yang et al., 2021). Concerning the LSM, the Platelet-Derived Growth Factor (PDGF) pathway is critical because its inhibition leads to disappearance of the LSM in mice (Kurahashi et al., 2008).

It is now well understood that the emergence of smooth muscle depends not only on intrinsic factors, but also on mechanical tensile stress acting within the gut tissue: internal circumferential tension due to rapid proliferation of the epithelium causes the first SM layer to orient circularly (Huycke et al., 2019). Spontaneous contractions of this first layer generate cyclic circumferential compression and longitudinal tension that orients the second layer longitudinally (Chevalier, 2022; Chevalier et al., 2021a): halting contractions during CSM layer formation inhibits alignment of the LSM layer in the chicken embryo (Huycke et al., 2019).

Recently, the bioelectrical events underlying differentiation and morphogenesis of organs has attracted much attention (George and Bates, 2022). It is becoming clear that the polarization state of the cell (membrane potential), the electric connectivity between groups of cells (gap junctions), and their ability to propagate coordinated signals like calcium waves (Uhlén and Fritz, 2010), all play important roles in development. Thus the epithelium of the wing of the *D. Melanogaster* embryo spontaneously generates calcium waves and disruption of this wave pattern leads to abnormal wing development (Brodskiy et al., 2019). Calcium oscillations have been detected in the developing chicken feather buds and found to play a critical role in their morphogenesis (Li et al., 2018). Chondrogenesis in the mouse limb bud has been shown (Atsuta et al., 2019) to be preceded by a depolarization event that induces calcium transients mediated by the L-type calcium channel  $Ca_v1.2$ ; perturbations of the physiological electric pattern led to abnormal limb growth.

Here, we show using a genetically modified mouse line expressing the fluorescent reporter GCamp6f that L-type calcium channel sensitive calcium waves propagate in the mesenchyme one day prior to smooth muscle differentiation. L-type channel antagonists block smooth muscle differentiation and gut growth. We demonstrate that the effect of the calcium waves on differentiation is not mediated by the potential contractile force it can exert on the tissue. We recapitulate most of these results in the avian embryo, and show that early guts treated with L-type channel blockers up-regulate the LIX1 factor characteristic of mesenchymal stemness, whereas older guts display KIT expressing cells characteristic of Interstitial Cells of Cajal.