



Published in final edited form as:

*Dev Cell*. 2009 November 17; 17(5): 649–661. doi:10.1016/j.devcel.2009.09.004.

## MOR23 promotes muscle regeneration and regulates cell adhesion and migration

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### Summary

Odorant receptors (ORs) in the olfactory epithelium bind to volatile small molecules leading to the perception of smell. ORs are expressed in many tissues but their functions are largely unknown. We show multiple ORs display distinct mRNA expression patterns during myogenesis *in vitro* and muscle regeneration *in vivo*. Mouse OR23 (MOR23) expression is induced during muscle regeneration when muscle cells are extensively fusing and plays a key role in regulating migration and adhesion of muscle cells *in vitro*, two processes common during tissue repair. A soluble ligand for MOR23 is secreted by muscle cells *in vitro* and muscle tissue *in vivo*. MOR23 is necessary for proper skeletal muscle regeneration as loss of MOR23 leads to increased myofiber branching, commonly associated with muscular dystrophy. Together these data identify a functional role for an OR outside of the nose and suggest a larger role for ORs during tissue repair.

### Keywords

fusion; migration; MOR23; Olfr16; myofiber branching; myofiber splitting; regeneration; adhesion; olfactory receptor

### Introduction

Odorant receptors (ORs) are G-protein-coupled receptors (GPCR) expressed within the olfactory epithelium of the nose where they function as chemosensors to detect small molecules we perceive as smell. ORs are the largest receptor family in mammals comprising 3-5% of all genes (Young and Trask, 2002), with approximately 913 potential OR genes in the mouse genome, and 390 in the human genome (Olender et al., 2008; Zhang and Firestein, 2002). Microarray analyses suggest ORs are expressed in many tissues (Feldmesser et al., 2006), but functions of these receptors are known for only three: chemosensing in olfactory epithelium, proliferation in prostate cancer and chemotaxis in sperm of humans and mice (Fukuda et al., 2004; Neuhaus et al., 2009; Spehr et al., 2003).

One of the ORs that regulates sperm chemotaxis is mouse OR23 (MOR23, Olfr16) (Fukuda et al., 2004). We unexpectedly observed an increase in MOR23 expression in a microarray

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analysis performed during myogenesis of primary cultured mouse muscle cells (unpublished data). This increase occurred when muscle cells were undergoing extensive cell migration, adhesion and fusion to form multinucleated cells. In adult skeletal muscle, myogenesis occurs to regenerate muscle after injury. This process is dependent on satellite cells, which are normally quiescent, but in response to injury proliferate, and their progeny myoblasts differentiate and fuse with each other or with existing myofibers to restore normal tissue architecture. Migration and cell-cell adhesion are necessary for muscle cell differentiation (Kang et al., 2004) and fusion *in vitro* (Jansen and Pavlath, 2006). Therefore, identifying the molecules that control muscle cell adhesion and migration may reveal potential molecular targets for improving muscle regeneration. As MOR23 regulates migration of sperm, we hypothesized that ORs may affect similar processes during myogenesis.

Here, we demonstrate that MOR23 is an important regulator of myogenesis *in vitro* and *in vivo* through affects on cell migration and adhesion, influencing downstream fusion events. In addition, our data suggest a MOR23 ligand is secreted by muscle cells *in vitro* and is also present in muscle tissue. Furthermore, a number of ORs were also expressed by regenerating muscle, which may suggest a larger role for ORs in tissue repair.

## Results

### Multiple ORs, with distinct patterns, are expressed during myogenesis

Multinucleated myofibers form through fusion of muscle cells, which requires cell migration and adhesion factors (Jansen and Pavlath, 2008). Myogenesis *in vitro* occurs in distinct phases (Fig. 1A): proliferating myoblasts terminally differentiate to become fusion-competent myocytes in differentiation media (DM). By 24 hrs in DM, myocytes fuse with one another to form small, nascent myotubes with few nuclei. Further rounds of fusion create mature myotubes with many nuclei, by 48 hrs in DM. By microarray analysis, MOR23 mRNA was upregulated at 24 hrs of myogenesis (unpublished data). To validate the expression of MOR23 and also to determine whether other ORs are expressed during myogenesis *in vitro* and *in vivo*, we performed Real Time RT-PCR. Given the large numbers of mouse ORs, we choose 18 ORs previously identified in microarray screens of skeletal muscle (Beggs et al., 2004; Melcon et al., 2006; Tseng et al., 2002; Zhao et al., 2002). Olfactory bulb RNA was used as a positive control for OR mRNA (Supp. Table 1). Thirteen ORs were expressed during myogenesis (Supp. Table 2 and 3, Fig. 1). The majority of ORs expressed *in vitro* were most highly expressed at 0 hrs, when the muscle cells are proliferating myoblasts (Supp. Table 2, Fig. 1B, C). A smaller number of ORs were increased with terminal differentiation, and most highly expressed at 24 hrs in DM, when myocytes are fusing. Interestingly, no ORs were upregulated or exclusively expressed at 48 hrs in DM, when most cells have already fused.

Expression of these ORs was also highly regulated during skeletal muscle regeneration (Supp. Table 3, Fig. 1D, E). Only one OR was most highly expressed in uninjured muscle tissue, whereas the majority of ORs were upregulated at day 5 of regeneration, when muscle cells are proliferating, differentiating and fusing into nascent myofibers. The ORs expressed at day 5 were those detected at both 0 and 24 hrs *in vitro*. Two ORs were most highly expressed at day 10 after regeneration, when most muscle cell fusion had already occurred. The mRNA for most ORs was expressed both *in vitro* and *in vivo*, except for two. Olfr70 was expressed at very low levels *in vitro* and was not apparent *in vivo*. This discrepancy may be due to problems with sensitivity of detection or time points chosen *in vivo* or may be related to cell culture conditions. Olfr49, expressed only *in vivo*, might be expressed by cells other than muscle, or in a sub-population of muscle cells that was not retained *in vitro*. Together, our results demonstrate multiple ORs display distinct expression patterns during myogenesis, suggesting that individual ORs may have non-redundant functions. ORs increased after differentiation and during fusion may have similar functions to ORs in sperm and olfactory neurons, regulating

migration or adhesion. This subset of ORs was of specific interest as they might have conserved functions in muscle.

### MOR23 is expressed during myogenesis and regulates myocyte migration

To investigate the role of a specific OR, MOR23 was selected, as its mRNA was upregulated during times of cell fusion and its expression was verified by use of a second primer pair (Supp. Table 2 and 3, Fig. 1B, D, 2A, SFig. 2); similarly immunoblot analysis revealed increased amounts of MOR23 protein at 24 hrs in DM (Fig. 2B). The MOR23 antibody likely cross-reacts with the closely related olfactory receptor, Olfr1403, which shares 85% homology to MOR23 and whose mRNA is most highly expressed in myoblasts (SFig. 1A, B). Based on the mRNA expression pattern for Olfr1403 most of the antibody reactive band at 24 hrs in DM likely reflects MOR23 and not Olfr1403.

In both olfactory neurons and sperm activation of ORs leads to similar downstream signaling events: activation of  $G_{\alpha_{olf}}$ , a G-protein specific to odorant receptors, initiates signal transduction through membrane adenylyl cyclase III (mACIII) (Spehr et al., 2004). We observed both isoforms of  $G_{\alpha_{olf}}$  and mACIII in muscle cells at 24 hrs in DM by immunoblotting (Fig. 2B) suggesting that canonical OR signaling may occur within muscle cells.

Given that MOR23 is upregulated when myocytes are migrating and fusing, we hypothesized that MOR23 may have a conserved migratory function in myocytes. Thus, we examined the migration of myocytes in which MOR23 expression was knocked down by siRNA. First, to determine efficiency of knockdown, RNA was isolated from cells retrovirally infected with control or one of two distinct MOR23 siRNAs at 24 hr in DM and RT-PCR was performed; no MOR23 mRNA was observed with either MOR23 siRNA (SFig. 2). Since both MOR23 siRNAs resulted in similar knockdown, subsequent experiments were carried out using MOR23 siRNA 3, unless otherwise indicated. To determine whether expression of other ORs was affected by MOR23 siRNA, RNA was isolated from cells infected with control or MOR23 siRNA and Real Time RT-PCR was performed; no affect on mRNAs for other ORs was observed with MOR23 siRNA (Supp. Table 4). By immunoblotting, cells infected with MOR23 siRNA exhibited at least a 58% decrease in MOR23 protein although some of the residual antibody reactive band could have been Olfr1403, and hence a greater decrease in MOR23 protein actually occurred (Fig. 2C). To determine if MOR23 signaling was abrogated due to MOR23 siRNA, control and MOR23 siRNA cells were exposed to the synthetic MOR23 ligand lylal (Fukuda et al., 2004), and the levels of cAMP were detected by ELISA (Fig. 2D). Levels of cAMP were increased 60% in control siRNA cells and MOR23 siRNA abrogated this effect, indicating MOR23 protein levels are significantly decreased in MOR23 siRNA cells as they do not increase cAMP in response to lylal.

Subsequently, Boyden chamber experiments were performed using control and MOR23 siRNA cells with conditioned media collected from muscle cells at 24 hrs in DM as the chemoattractant (Fig. 2E). A 55% decrease in migration to CM was observed with each MOR23 siRNA. In order to determine if MOR23 siRNA cells had a general motility defect that could account for these results, time-lapse microscopy was performed at 24 hrs in DM in regular culture dishes (Fig. 2F). MOR23 siRNA did not significantly affect cell velocity when compared to control siRNA cells. To determine if removal of any OR was sufficient to alter migration, we focused on the following two ORs: Olfr1508 and Olfr1403; Olfr1508 was the only OR to share a similar expression pattern to MOR23, both *in vivo* and *in vitro*, (SFig. 3A, B), and Olfr1403 has the highest homology to MOR23. Knockdown of either Olfr1403 (SFig. 1D) or Olfr1508 (SFig. 3C) did not affect migration to CM (SFig. 1E, SFig. 3D). Hence, decreased migration to CM was specific to loss of MOR23 and not simply due to loss of any OR.

Migration was also examined in cells expressing recombinant MOR23. First, to determine whether recombinant MOR23 was properly trafficked to the plasma membrane, cell surface biotinylation was used. The subsequent biotin pull-down was assayed for MOR23 by immunoblotting; a 3-fold increase in cell surface MOR23 was observed (Fig. 2G). We performed Boyden chamber experiments using diluted CM to determine if these MOR23 over-expressing (OE) cells were more sensitive to CM. As the CM was diluted, less migration occurred in both control and MOR23 OE cells; however, the MOR23 OE cells exhibited higher levels of migration at all dilutions (Fig. 2H). Thus, increasing MOR23 levels alone is sufficient to change the migratory behavior of myocytes. Together, these data indicate that MOR23 regulates myocyte migration and suggest that an endogenous MOR23 ligand is secreted by fusing muscle cultures.

To discern whether a MOR23 ligand is present in muscle tissue *in vivo*, Boyden chamber experiments were performed using crushed muscle extract. CME resulted in a 9-fold increase of myocyte migration, which was abrogated in MOR23 siRNA cells (Fig. 2I). In order to test the migration of MOR23 OE cells, a different optimal concentration of CME was used based on preliminary dose response curves. With MOR23 OE a 3-fold increase in myocyte migration occurred (Fig. 2J). These data suggest that a ligand(s) or its precursor is present within muscle tissue prior to induction of muscle regeneration.

### MOR23 regulates directed migration

The decreased migration of MOR23 siRNA cells to CM likely results from a defect in directed migration as no general motility defect was observed. To address this question, we used the Dunn chemotaxis chamber to determine if MOR23 siRNA cells respond to a gradient of lylal. Time-lapse microscopy was performed for 3 hrs, the paths of individual cells tracked and the final location of each cell in relation to its origin determined (Fig. 3A). Myocytes migrated toward lylal, yielding migration paths that ended on the lylal side of the chamber. To statistically analyze the pattern of migratory paths in response to lylal, directional data were summarized in circular histograms. Rayleigh's statistical test for clustering revealed that control cells (Fig. 3B) but not MOR23 siRNA cells (Fig. 3C) migrated toward lylal. A rescue experiment in which myocytes expressed both MOR23 siRNA and recombinant MOR23 (SFig. 4) demonstrated directed cell migration toward lylal (Fig. 3D), indicating that migration to lylal is specific to MOR23. To determine whether migration towards lylal requires mACIII, an inhibitor of membrane adenylyl cyclases, SQ22536, was used (SFig. 5). Addition of the mAC inhibitor abrogated migration towards lylal (Fig. 3E), indicating that canonical OR signaling is used in lylal-directed migration. As a control, to determine if lylal-dependent migration was specific to MOR23, myoblasts which express the highly homologous Olfr1403 and the lylal-responsive Olfr15 (Saito et al., 2009) but not MOR23, were tested in Dunn chambers. These cells did not undergo directed migration towards  $10^{-7}$ M lylal (Fig. 3F). In addition, knockdown of Olfr1403 or Olfr1508 in myocytes did not affect migration to lylal (SFig. 1F, SFig. 3E), revealing that migration of myocytes to lylal is specific to MOR23 and occurs in a directed manner.

### Loss of MOR23 alters cell-cell adhesion

Recent evidence suggests that ORs may regulate axon guidance of olfactory neurons via downstream changes in the expression of cell-cell adhesion molecules (Serizawa et al., 2006). To determine whether loss of MOR23 alters cell adhesion, a solution based cell adhesion assay was utilized in which control or MOR23 siRNA myocytes were suspended in media and aliquots taken over 60 min to count the number of adhered and un-adhered cells using phase-contrast microscopy. Myocytes infected with MOR23 siRNA did not efficiently adhere to one another (Fig. 4A). Although no difference in adhesion was observed at early time points, at later time points, a significant increase occurred in un-adhered MOR23 siRNA cells (Fig. 4B).

At later times MOR23 siRNA cells remained in small clusters compared to control siRNA cells (Fig. 4C). Cell viability was ~99% at 60 min in all conditions (data not shown). Canonical OR signaling is also necessary for axonal migration and adhesion (Imai et al., 2006), and to determine whether it regulates cell-cell adhesion of myocytes, cells were treated with mAC inhibitor SQ22536, or vehicle (Fig. 4D). At early time points, no difference was observed, however at later time points a significant increase occurred in un-adhered cells with SQ22536 (Fig. 4E). Furthermore, SQ22536-treated cells formed smaller clusters than vehicle (Fig. 4F). Knockdown of Olfr1403 or Olfr1508 did not affect adhesion (SFig. 1G, SFig. 3F). Hence, adhesion affects were specific to loss of MOR23 and not simply due to loss of any OR. Together, these data suggest that MOR23 signaling affects adhesion of myocytes to one another likely through a downstream adhesion molecule.

### Myotube formation is dependent on MOR23

To test whether MOR23-dependent migration and/or adhesion is involved in muscle cell differentiation or fusion, we examined myogenesis *in vitro* in cells containing one of two MOR23 siRNAs. After 24 or 48 hr in DM, cells were immunostained using an antibody against embryonic myosin heavy chain (eMyHC). After 24 hrs in DM, both MOR23 siRNA cultures contained fewer and smaller nascent myotubes compared to control (Fig. 5A). This defect in myotube formation was not due to an affect on differentiation, as measured by the percentage of nuclei found in eMyHC<sup>+</sup> cells (Fig. 5B), nor to a decrease in the total number of nuclei (data not shown). Rather, MOR23 siRNA myocytes exhibited a clear defect in cell fusion (Fig. 5C-E). The fusion index, as well as the number of myotubes, was transiently decreased 33% and 26% respectively in MOR23 siRNA cultures (Fig. 5C, D). A 15% decrease in myonuclear number was also noted at 24 hrs in DM in MOR23 siRNA cultures, which remained virtually unchanged at 48 hrs (Fig. 5E) and 72 hrs (data not shown). The transient fusion defects in MOR23 siRNA cultures may be due to compensation from other factors which regulate fusion.

To determine whether the impaired ability of MOR23 siRNA cultures to fuse is specific to the loss of MOR23; assays using MOR23 OE were also performed. MOR23 OE resulted in an increased number of nascent myotubes at 24 hrs in DM (Fig. 5F), with no change in differentiation (Fig. 5G). Fusion assays revealed a 30% increase in the fusion index and a 40% increase in the number of myotubes at 24 hrs in DM, with smaller increases in both parameters still noted at 48 hrs and 72 hrs (Fig. 5H, I and data not shown). Myonuclear number was also increased ~10% at both time-points in MOR23 OE cultures (Fig. 5J). Together these data suggest that MOR23-dependent migration and/or adhesion contributes to the fusion of myocytes to form nascent myotubes.

Changes in myonuclear number with MOR23 siRNA or OE at 48 hrs suggest that MOR23-dependent migration and/or adhesion may also regulate formation of mature myotubes from fusion of myocytes with nascent myotubes. Since the formation of nascent myotubes was affected in previous assays, we could not independently determine a role for MOR23 in this later stage of fusion. To analyze if MOR23 affects the fusion of myocytes with nascent myotubes (Fig. 1A), control and MOR23 siRNA nascent myotubes and myocytes were created separately, labeled with green or orange fluorescent dye, co-cultured for 24 hrs (Fig. 5K), and the number of dual labeled myotubes was quantified. With MOR23 siRNA in myocytes the percentage of myotubes with dual label was decreased 20% compared to control (Fig. 5L), whereas MOR23 siRNA in myotubes had no effect on dual labeling. To determine whether increased expression of MOR23 on myocytes could enhance fusion, control or MOR23 OE myocytes were co-cultured with control nascent myotubes (Fig. 5M). MOR23 OE in myocytes increased dual labeling 35% (Fig. 5N), indicating MOR23 expression on myocytes alone is sufficient to increase fusion of myocytes with nascent myotubes. Together, these data indicate

that MOR23 is functionally required on myocytes. Therefore, MOR23-dependent processes can affect myonuclear addition at later stages of myogenesis.

### MOR23 regulates myogenesis *in vivo*

To determine the detailed expression pattern of MOR23 during adult regenerative myogenesis, RNA was isolated from mouse gastrocnemius muscles at different times after injury. Using Real Time RT-PCR, MOR23 mRNA was induced 4-fold during days 3-7 post-injury (Fig. 6A), a time of extensive cell fusion. By 14 days post-injury, when regeneration is nearly complete, MOR23 mRNA levels had returned to uninjured control levels. MOR23 protein levels were increased at day 5 post-injury, a time when fusion is ongoing and nascent myofibers are present, as determined by immunoblot (Fig. 6B, G). Olfr1403 mRNA was not increased during muscle regeneration at these times (SFig. 1C). Sections from gastrocnemius muscles at day 5 post-injury were immunostained with an antibody against MOR23 (Fig. 6C). Regenerating myofibers, identified by their centrally located nuclei and small size, contained MOR23. To determine whether mononucleated muscle cells express MOR23 during regeneration, cells were isolated from regenerating muscle and myogenic cells ( $\alpha 7$ -integrin<sup>+</sup>CD31<sup>-</sup>CD45<sup>-</sup> (Kafadar et al., 2009)) were separated from immune and endothelial cells ( $\alpha 7$ -integrin<sup>-</sup>CD31<sup>+</sup>CD45<sup>+</sup>) by FACS (SFig. 6). Real Time RT-PCR revealed expression of MOR23 mRNA in  $\alpha 7$ -integrin<sup>+</sup>CD31<sup>-</sup>CD45<sup>-</sup> cells, which also expressed myogenin mRNA, confirming their myogenicity (Fig. 6D). MOR23 mRNA was not apparent in the  $\alpha 7$ -integrin<sup>-</sup>CD31<sup>+</sup>CD45<sup>+</sup> fraction; this fraction did express Mac1 mRNA, suggesting many of these cells are immune cells. Furthermore, immunostaining for MOR23 in  $\alpha 7$ -integrin<sup>+</sup>CD31<sup>-</sup>CD45<sup>-</sup> cells indicated ~86% of muscle cells express MOR23 (Fig. 6E). The myogenic purity of these cells was >99% as determined by the expression of Myf5. Thus, MOR23 expression is induced in myogenic cells during muscle regeneration when myofibers are forming. Overall these data suggest that the induction of MOR23 expression may be tied to migration and/or adhesion events that occur during cell fusion in muscle regeneration.

To analyze MOR23 function during muscle regeneration, gastrocnemius muscles were induced to regenerate with BaCl<sub>2</sub> two days prior to electroporation of either MOR23 siRNA or OE plasmids. Induction of regeneration allowed myoblasts to take up plasmid, as determined in preliminary experiments. Gastrocnemius muscles were isolated 5, 10 or 20 days post-electroporation (Fig. 6F). Knockdown of MOR23 protein was determined by immunoblot at day 5, and an 85% reduction of MOR23 was observed (Fig. 6G), suggesting that the majority of the immunoreactive band is MOR23 and not Olfr1403. Although little to no difference existed in proliferation or differentiation in muscles that received MOR23 siRNA compared to control (SFig. 7), abnormalities in muscle regeneration were noted in MOR23 siRNA muscles at all time points (Fig. 6H). The mean cross-sectional area (XSA) of regenerating myofibers in MOR23 siRNA muscles was decreased 26-38% (Fig. 6I) with a corresponding 30% increase in the number of myofibers smaller than 1500 $\mu\text{m}^2$  at day 20 (Fig. 6J). In addition, the number of regenerating myofibers per field increased ~50% with MOR23 siRNA (Fig. 6K). In contrast, MOR23 OE *in vivo* resulted in a 5-fold increase in MOR23 mRNA (SFig. 8), and increased myofiber XSA ~50% (Fig. 6L), with a corresponding 39% decrease in the number of myofibers smaller than 1500 $\mu\text{m}^2$  at day 20 (Fig. 6M). In addition, the number of regenerating myofibers per field decreased 21-36% with MOR23 OE (Fig. 6N). These data suggest that optimal muscle regeneration is dependent on MOR23.

### MOR23 affects myofiber branching *in vivo*

Small clusters of myofibers in MOR23 siRNA muscles were observed, which suggested that myofiber branching may have occurred with loss of MOR23. Myofiber branching is characterized by a single myofiber having a plasma membrane contiguous with several smaller myofibers (Fig. 7A). Myofiber branching is an important phenomenon as it is increased with

multiple injuries, aging, and muscular dystrophy (Ontell et al., 1982). The molecules which regulate this process are unknown, although cell-cell adhesion molecules are hypothesized to play a role fusing branched myofibers to form mature myofibers. We hypothesized that the increase in small myofibers observed in MOR23 siRNA muscles may be due to an increase in unresolved myofiber branches, yielding the appearance of many small myofibers rather than large myofibers apparent in control siRNA muscles. To determine whether MOR23 may regulate myofiber branching, cross-sections 170 $\mu$ m apart were isolated from gastrocnemius muscles 5 days after electroporation. Myofiber branching was quantified by determining the number of regenerating myofibers that were a single myofiber in one section and multiple fibers occupying the same area in a second section (Fig. 7B). Muscles with MOR23 siRNA exhibited a 40% increase in the percentage of branching myofibers (Fig. 7C), and more branches per myofiber (Fig. 7D). MOR23 OE did not result in changes in myofiber branching at day 5 (Fig. 7E). In order to visualize the entire myofiber, single myofibers were isolated to determine the extent of branching. We were unable to isolate intact regenerating myofibers at day 5; however myofiber branches could be visualized at day 10 and 20 using phase-contrast microscopy and DAPI-staining was used to identify regenerating myofibers by the presence of centrally located nuclei (Fig. 7F). Myofibers with multiple tracks of central nuclei, but unbranched cytoplasm, are mature regenerated myofibers (Fig. 7F, arrowheads). Muscles electroporated with MOR23 siRNA exhibited a 60% increase in regenerating myofibers with branches (Fig. 7G) and 90% more branches per myofiber (Fig. 7H). In contrast, a 50% decrease in the number of branched myofibers (Fig. 7I) occurred in MOR23 OE muscles at day 20 with 70% fewer branches per myofiber (Fig. 7J). These data suggest that myofiber branching is regulated by MOR23, possibly through its effects on migration and adhesion of muscle cells.

## Discussion

In many systems, tissue repair requires cell migration and adhesion. Our results suggest these processes are also necessary for proper regeneration of muscle tissue. We show multiple ORs are expressed by muscle cells during myogenesis *in vitro* and muscle regeneration *in vivo*. In depth studies on one of these ORs, MOR23, revealed a role in regulating migration and adhesion of muscle cells, which are critical for cell fusion. Importantly, these studies identify an unexpected functional role for MOR23 in skeletal muscle regeneration, demonstrating a novel function for an OR in tissue repair.

MOR23 was expressed by terminally differentiated myocytes *in vitro*, functioning to regulate their migration. Interestingly, MOR23 also regulates migration of mouse sperm *in vitro* (Fukuda et al., 2004) and ORs are hypothesized to play a role in axonal growth cone migration (Feinstein et al., 2004), suggesting that MOR23 has a conserved function in migration of multiple cell types. Although synthetic ligands are known for many odorant receptors, endogenous ligands have not been identified. Both crushed muscle extract and conditioned media contained a ligand that stimulated myocyte migration in a MOR23-dependent manner. CME was prepared from uninjured muscle tissue, which indicates that the MOR23 ligand is present prior to the onset of muscle injury. The ligand may be present in an inactive form in normal muscle tissue and become activated by the “crushing” used in preparing CME. *In vivo*, HGF is expressed in an inactive form, which is expressed by muscle cells and then bound by the extracellular matrix until matrix metalloproteases cleave and release the active form (Allen et al., 2003). Alternatively, the ligand may be stored within myofibers and released upon myofiber degeneration. CM collected from differentiating muscle cultures *in vitro* also contained the MOR23 ligand, demonstrating that muscle cells themselves can be the source of the ligand. These results do not rule out that other cell types may also express the MOR23 ligand *in vivo*. Preliminary data indicate the MOR23 ligand is likely not sensitive to heat or Proteinase K digestion (data not shown). Identification of sources for the MOR23 ligand will allow future purification of an endogenous OR ligand from a readily available source, as

opposed to follicular fluid, which is the only other hypothesized source of an endogenous OR ligand (Spehr and Hatt, 2004). If the ligand for MOR23 can be isolated, it may give insight into the nature of endogenous ligands for other odorant receptors, including the human odorant receptor found in sperm.

MOR23 also regulated cell-cell adhesion, indicating another conserved function for ORs between muscle and olfactory neurons. In olfactory neurons, OR signaling is required for expression of adhesion molecules, which correlates with altered axon guidance into specific glomeruli (Imai et al., 2006). However, changes in the adhesiveness of the olfactory neurons were not assayed. In contrast, we performed adhesion assays which demonstrated that loss of MOR23 or OR signaling decreased cell-cell adhesion, suggesting that MOR23 regulates adhesion indirectly, possibly through regulating expression of specific downstream adhesion molecules. Further studies are needed to determine which adhesion molecules may be affected by MOR23 signaling.

MOR23 expression was induced with muscle regeneration and manipulating the levels of MOR23 affected several aspects of muscle regeneration, including myofiber cross-sectional area, number and branching. Although branched myofibers have been noted in the literature for 100 years (Schmalbruch, 1976; Volkmann, 1893), no molecule is known to regulate their formation. We show that loss of MOR23 increased myofiber branching. Expression of MOR23 by mononucleated muscle cells and regenerating myofibers *in vivo* suggests both migration and adhesion could contribute to these effects. If myocyte migration is required for fusion with existing regenerating myofibers, then defects in migration would increase the formation of *de novo* myofibers. Alternatively, formation of small myofibers may normally occur, which adhere to one another, and then fuse to form larger myofibers, and when adhesion is disrupted, branched myofibers occur. Therefore, loss of MOR23 may disrupt myofiber formation on several levels. Since muscles containing unbranched myofibers are stronger than those with extensive branching (Chan et al., 2007) and MOR23 OE can decrease the occurrence of branching, MOR23 may be an important target for therapies directed at increasing strength of dystrophic muscle.

As MOR23 functions in the repair of skeletal muscle, and multiple ORs are expressed during myogenesis, other ORs may regulate similar or distinct processes. Recent evidence suggests that activation of an OR inhibits proliferation of prostate cancer cells (Neuhaus et al., 2009), therefore, ORs that were upregulated in myoblasts may regulate proliferation. Also, as muscle cells must interact directly with one another for terminal differentiation to occur (Krauss et al., 2005), ORs in addition to MOR23 may play a role in the migration or adhesion of these cells. As Olfr49 was expressed only in muscle tissue, other cell types present in muscle, such as immune cells, endothelial cells, fibroblasts, or neurons may also express ORs. Finally, ORs present in uninjured muscle or at later time points in regeneration may be necessary for quiescent satellite cells, either to keep these cells in their niche or to affect their proliferation state. Our data suggest that multiple ORs may regulate myogenesis, and therefore, may be necessary for proper tissue repair. Additional studies will be necessary to determine the specific effects of each of these ORs both *in vitro* and *in vivo*.

Interestingly, in the olfactory neuron field, only one OR is expressed in each cell (Touhara et al., 1999). As multiple ORs were expressed by muscle cultures, sub-populations of muscle cells may each express a single OR. Potentially the “one cell, one OR” hypothesis may not hold true for muscle cells, and several ORs are expressed by each muscle cell. A redundant system may exist, so that even if one OR signal is somehow blocked, other ORs may substitute. Alternatively, cells expressing multiple ORs may utilize specific ORs in a spatial-temporal manner. Additional studies may elucidate whether multiple populations of muscle cells each express a single OR, or whether each muscle cell expresses several ORs.



In summary, MOR23 is a key regulator of myogenesis through its actions on cell migration and adhesion, affecting downstream fusion *in vitro* and muscle regeneration *in vivo*. Cell migration within muscle is a major issue in transplantation of cells for treatment of muscular dystrophy (Skuk and Tremblay, 2003; Smythe et al., 2001). Study of the receptor-ligand pairs that regulate migration and/or adhesion of muscle cells may allow for more efficient therapeutic strategies. In addition, further studies on ORs may reveal additional unexpected functions in various tissues.

## Methods

### Animals and muscle injuries

Adult mice between 8–24 weeks of age were used and handled in accordance with the IACUC guidelines of Emory University. To induce regeneration, gastrocnemius muscles of male C57BL/6 mice were injected with BaCl<sub>2</sub> (O'Connor et al., 2007) and collected as described (Abbott et al., 1998).

### Primary muscle cell culture, differentiation and fusion assays

Primary myoblasts were derived from the hindlimb muscles of Balb/C mice and cultures were >99% myogenic as assessed by MyoD immunostaining (Jansen and Pavlath, 2006). To induce differentiation, cells were switched to differentiation media (DM), immunostained with an eMyHC antibody (F1.652; Developmental Studies Hybridoma Bank) and analyzed as described (Jansen and Pavlath, 2006). At least 500 nuclei per condition were analyzed for each assay. Cell mixing experiments were performed as described (Jansen and Pavlath, 2006). The presence of dual label was analyzed in 50–100 myotubes with  $\geq 3$  nuclei in each experiment. For all experiments 3–6 independent isolates were analyzed.

### Plasmid production and retroviral infection

Oligonucleotides (see Supp. Methods) encoding siRNA to MOR23 were cloned into the retroviral plasmid pSUPER.retro.puro according to OligoEngine protocol. The control plasmid contained a scrambled siRNA sequence. Retroviral production and infection were performed as described (Abbott et al., 1998) and cells were used 48 hrs later in experiments. The efficiency of retroviral-mediated gene transfer was >95%, based on cell survival in the presence of puromycin following two rounds of retroviral infection.

To generate a MOR23 retroviral expression vector, a cDNA fragment encoding a FLAG-Rho-MOR23 was subcloned from the pME18S construct (Katada et al., 2003) into the retroviral vector pTJ84 using the AvrII and SalI/XhoI sites (Abbott et al., 2000). pTJ84 was used as the control vector.

### Real-time RT-PCR

Real-time RT-PCR was performed on DNase treated total RNA, reverse transcribed to cDNA, amplified using primers from SABiosciences, and analyzed using the iCycler iQ Real-Time Detection System and software (Bio-Rad) as described (Bondesen et al., 2004). For conditions and controls, see Supp. Methods. MOR23 mRNA was quantified in reference to MOR23-pME18S plasmid standard and normalized to 18S rRNA levels.

### Immunoblotting

Cells were harvested in RIPA-2 (O'Connor et al., 2007) with protease inhibitors (Mini-Complete; Roche) and immunoblots were performed as described (Friday and Pavlath, 2001) with primary antibodies ( $\alpha$ -MOR23, GeneTex;  $\alpha$ -G<sub>olf</sub>,  $\alpha$ -mACIII and  $\alpha$ -tubulin, Abcam, Inc.)

and detected with appropriate secondary HRP-conjugated antibodies (Jackson ImmunoResearch Lab., Inc.).

Cell surface biotinylation was performed as described (Salazar and Gonzalez, 2002). Sulfo-NHS-SS-Biotin was used at 500 $\mu$ M (Pierce) and protein was isolated in RIPA-2. Immunoprecipitation was performed with NeutrAvidin Agarose Resins (Pierce) with 300 $\mu$ g of protein at 4°C overnight.

### **cAMP assay**

cAMP was detected using the CatchPoint kit from Molecular Probes. Myocytes were generated as for Boyden chambers and plated in 96-well plates at  $1 \times 10^5$  cells/well. Cells were treated with either buffer or  $10^{-4}$ M lyral (International Flavors and Fragrances) for 15 min in triplicate.

### **Cell migration and adhesion assays**

Migration of muscle cells was quantified using time-lapse microscopy as described (Jansen and Pavlath, 2006). Images were recorded (QImaging Camera and OpenLab 3.1.4 software) every 5 min for 3 hours. Cell velocities were calculated in  $\mu$ m/hr using ImageJ software by tracking the paths of mononucleated cells. Approximately 20 mononucleated cells were tracked for each experiment. Boyden chambers were performed as described (Mylona et al., 2006). Primary myoblasts were seeded on 150-mm plates at low density ( $9 \times 10^5$  cells/plate) and switched to DM for 24 hrs to generate myocytes in the absence of myotube formation. Cells ( $7.5 \times 10^4$  cells in 200 $\mu$ L DM) were loaded in the upper wells of the Boyden chamber and incubated at 37°C for 5 hrs. Migrated cells were fixed, stained and counted. To prepare CM, myoblasts were incubated in DM for 24 hrs; the media, which had been “conditioned” with secreted factors, was then collected, filtered (0.45  $\mu$ m), flash frozen, and stored at -80°C until use. Crushed muscle extract was created as described (Chen and Quinn, 1992) using gastrocnemius muscles from C57BL/6 mice (N=10) and stored at -80°C.

Dunn chambers were performed as described (Jansen and Pavlath, 2006). The outer well of the Dunn chamber was filled with DM or  $10^{-7}$ M lyral in DM. SQ22536 (Sigma) was used at 2.5mM. Directional analyses were performed using at least 15 cells per assay.

Cell-cell adhesion in suspension was analyzed by incubating myocytes at  $2 \times 10^5$  cells in 2mL of DM after lifting using Cell Dissociation Buffer Enzyme-Free PBS-Based (Invitrogen). SQ22536 was used at 2.5mM. Duplicate 50 $\mu$ L aliquots of cells were taken at regular intervals for 60 min. This cell concentration was used to permit counting of individual cells within clusters using phase-contrast microscopy. Trypan blue staining was used at 60 min to determine cell viability.

### **Flow cytometry**

FACS was performed as described (Kafadar et al., 2009) using cells isolated from day 5 regenerating gastrocnemius muscles. Cells were sorted using a BD FACSVantage SE and placed into Trizol Reagent (Invitrogen) for RNA isolation or plated for immunostaining. For detailed methods, see supplement.

### **Immunostaining**

MOR23 immunostaining was performed on muscle sections or muscle cells isolated by flow cytometry from gastrocnemius muscles 5 days post-injury. After blocking, sections and cells were incubated overnight at 4°C with MOR23 antiserum or purified rabbit IgG (Genetex Inc.) diluted 1:100 in blocking buffer. Immunostaining was visualized using the TSA Rhodamine Tyramide Signal Amplification kit (PerkinElmer). Nuclei were counterstained with 25  $\mu$ M 4,6-diamidino-2-phenylindole in 0.1% BSA. For detailed methods, see supplement.

### ***In vivo* electroporation**

Gastrocnemius muscles of C57BL/6 mice were injected with BaCl<sub>2</sub> to induce muscle regeneration. Two days later the muscles were injected with the indicated plasmid (25µg DNA; 1µg/µL in PBS) and electroporated as described (O'Connor et al., 2007). Post-electroporation, muscles were collected and homogenized in RIPA-2 for protein isolation or frozen and sectioned as described (Abbott et al., 1998). Single myofibers were isolated from previously injured and electroporated gastrocnemius muscles as described (Kafadar et al., 2009). Serial 14µm cross-sections were stained with hematoxylin and eosin and analyzed by quantifying myofiber number and cross-sectional area (XSA) for three 307,200µm<sup>2</sup> fields in the core of each regenerating muscle (O'Connor et al., 2007). In preliminary experiments, electroporation of a lacZ plasmid was utilized to determine efficiency of gene delivery to MyoD<sup>+</sup> cells in the gastrocnemius muscle; 70% of adherent cells were lacZ<sup>+</sup>, 80% were MyoD<sup>+</sup>. All analysis were performed blinded with N=4-8 mice per condition and 90-100 myofibers per mouse.

### **Statistics**

To determine significance between two groups, comparisons were made using Student's *t*-tests or the Mann Whitney test (*in vivo*). Analyses of multiple groups were performed using a one-way or two-way analysis of variance with Bonferroni's posttest as appropriate. Statistical analyses were performed using GraphPad Prism 4.0 (GraphPad). For all statistical tests, a confidence interval of  $p < 0.05$  was accepted for statistical significance.

### **Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

### **Acknowledgments**

We thank Drs. K. Touhara, S. Traynelis, V. Faundez and F. Rossi for reagents and technical help. GKP was supported by grants AR-047314, AR-051372, and AR-052730 from the National Institutes of Health and the Muscular Dystrophy Association. CAG was supported by National Institutes of Health training grant T32-GM08367. KAK was supported by an MDA Development grant.

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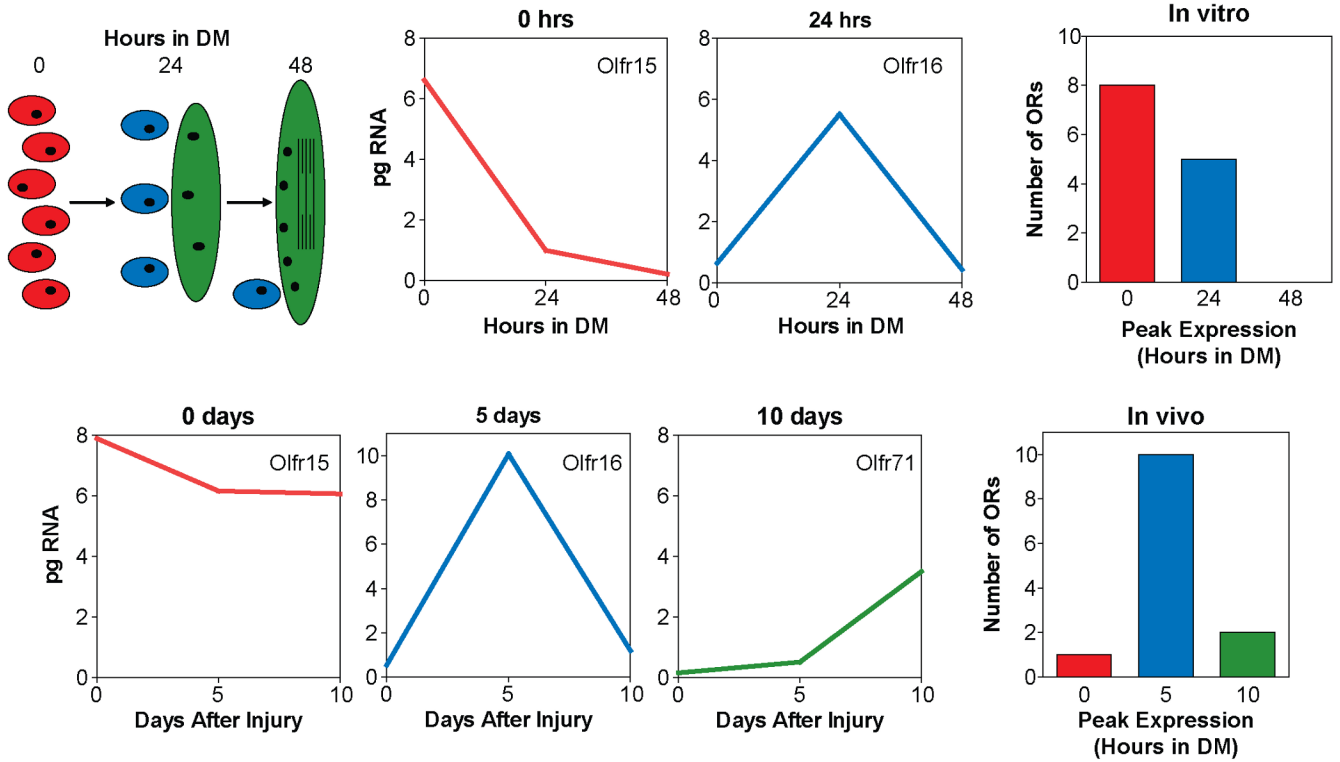
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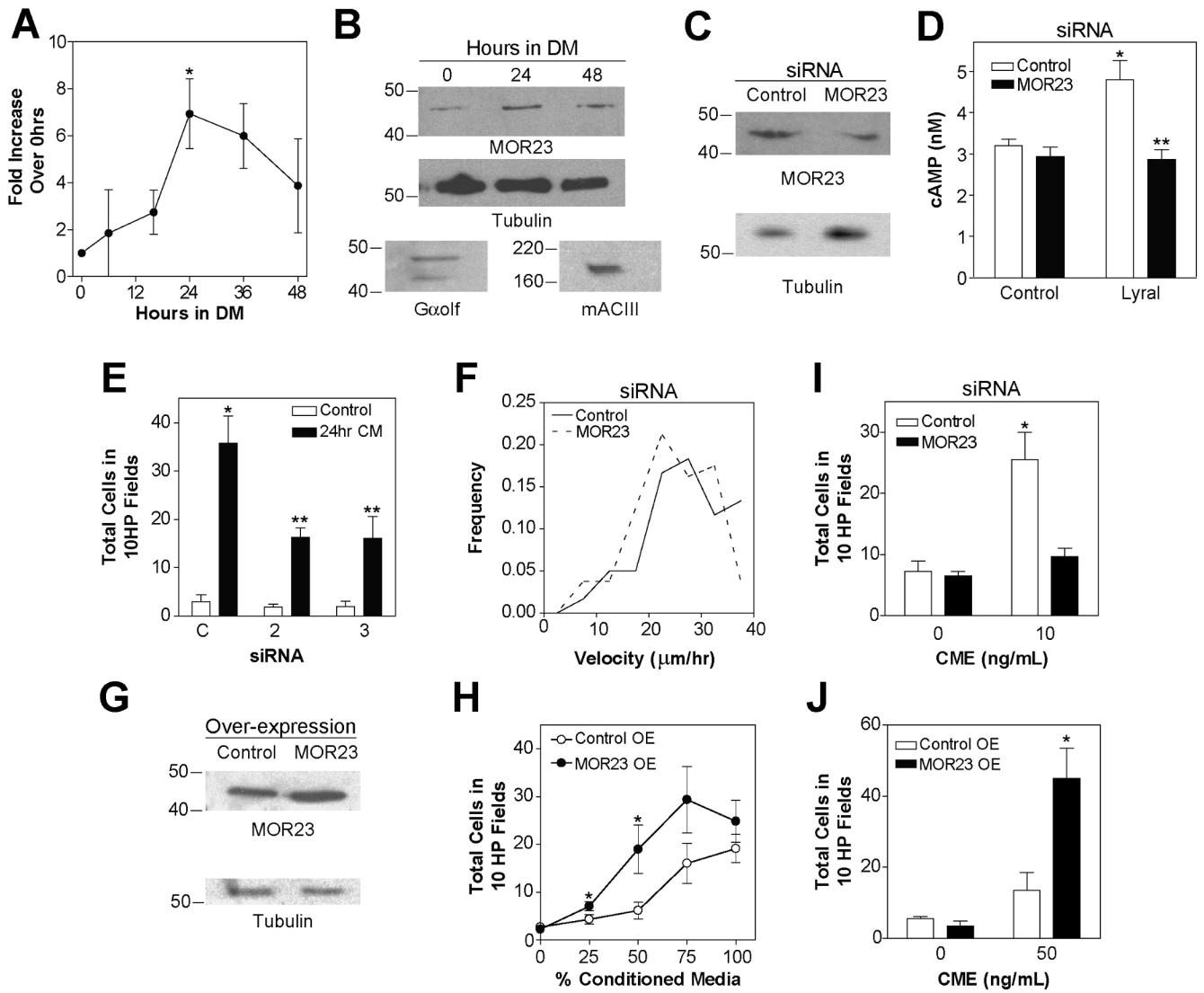
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## Abbreviations

<b>OR</b>	odorant receptor
<b>MOR23</b>	mouse odorant receptor 23
<b>hOR17-4</b>	human odorant receptor 17-4
<b>GM</b>	growth media
<b>DM</b>	differentiation media
<b>CM</b>	conditioned media
<b>CME</b>	crushed muscle extract
<b>eMyHC</b>	embryonic myosin heavy chain
<b>OE</b>	over-expression
<b>XSA</b>	cross-sectional area



**Figure 1. Multiple ORs are expressed during *in vitro* myogenesis and muscle regeneration**  
 Real Time RT-PCR was used to analyze the time-course of expression both *in vitro* and *in vivo* for 19 ORs. A) During myotube formation the majority of myoblasts (red) differentiate into myocytes (blue). Myocytes fuse with one another to form small nascent myotubes with few nuclei. Subsequently, nascent myotubes fuse with myocytes to form large myotubes with many nuclei. B) Two patterns of expression were observed *in vitro*: peak expression either during proliferation or after terminal differentiation. Olfr15 and Olfr16 (MOR23) are shown. C) The number of ORs with highest expression levels at each time point *in vitro*. The majority of ORs are expressed in proliferating cells at 0 hrs in DM; however several ORs are also expressed at 24 hrs in DM, during the fusion process. D) Three patterns were observed *in vivo*: peak expression in uninjured muscle and increased expression day 5 or day 10 after injury. Olfr15, Olfr16 (MOR23) and Olfr71 are shown. E) The number of ORs with highest expression levels at each time point *in vivo*. The majority of ORs demonstrated peak expression at day 5 after injury, with few ORs showing peak expression either earlier or later. All data are mean  $\pm$  SEM.

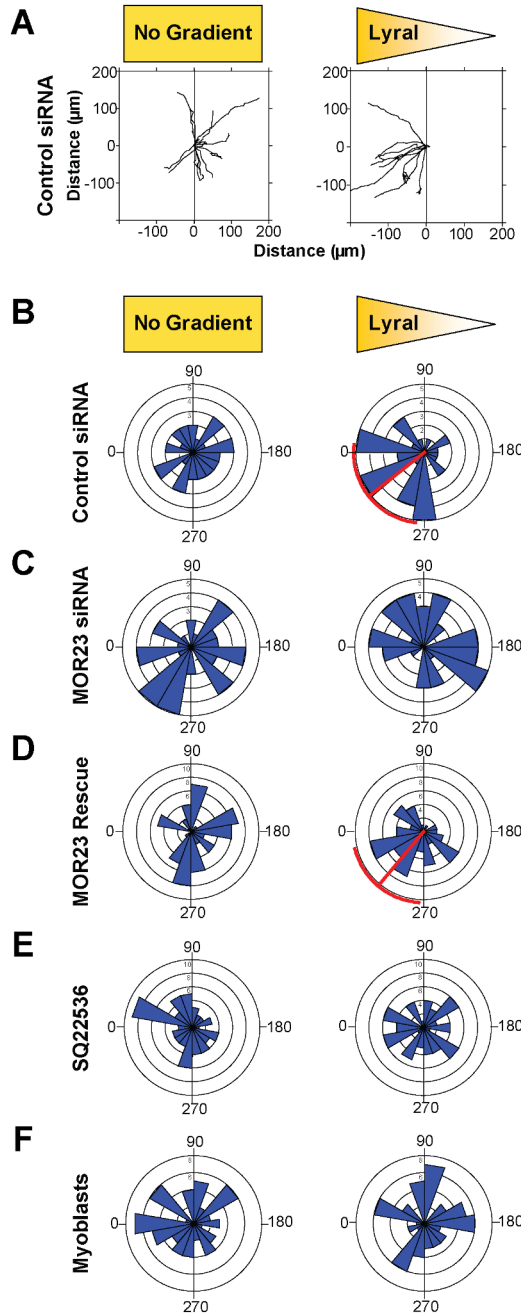


**Figure 2. MOR23 regulates myocyte migration during myogenesis**

A) Real Time RT-PCR indicates MOR23 mRNA was significantly increased at 24 hrs in DM. Data were normalized to 18S rRNA (\* $p < 0.05$  from 0). B) Immunoblots for MOR23 and OR signaling proteins; G $\alpha_{olf}$  and membrane adenylyl cyclase III (mACIII) at 24 hrs in DM. Tubulin was used as a loading control. C) Immunoblots indicate MOR23 protein was decreased at least 58% by MOR23 siRNA. D) Myocytes from control or MOR23 siRNA cultures were treated with lyril and levels of cAMP were determined (\*  $p < 0.05$  from control; \*\*  $p < 0.05$  from lyril). E) Myocytes from control or MOR23 siRNA cultures were allowed to migrate to conditioned media. MOR23 siRNA decreased migration by 55% compared to control (\*  $p < 0.05$ ; \*\*  $p < 0.01$  from control). F) Myocyte velocity was calculated from 3 hrs of time-lapse microscopy with 20 cells analyzed in each of 3 isolates. Minor decreases in velocity were observed in MOR23 siRNA cultures at 24 hrs in DM. G) MOR23 OE cells at 24 hr in DM demonstrated increased levels of cell surface MOR23 compared to control; immunoblots were performed after cell surface biotinylation and biotin pull-down. H) Myocytes from control or MOR23 OE cells were allowed to migrate to dilutions of CM. MOR23 OE myocytes exhibited increased migration at all concentrations of CM compared to control (\*  $p < 0.05$ ). I) Control or MOR23 siRNA myocytes were allowed to migrate in Boyden chambers to crushed muscle extract.

Control siRNA myocytes increased migration to CME 2.5-fold, but MOR23 siRNA myocytes did not. J) Control or MOR23 OE myocytes were allowed to migrate in Boyden chambers to CME. Migration of MOR23 OE myocytes to CME was increased 3-fold compared to control myocytes (\*  $p < 0.05$ ). All data are mean  $\pm$  SEM.

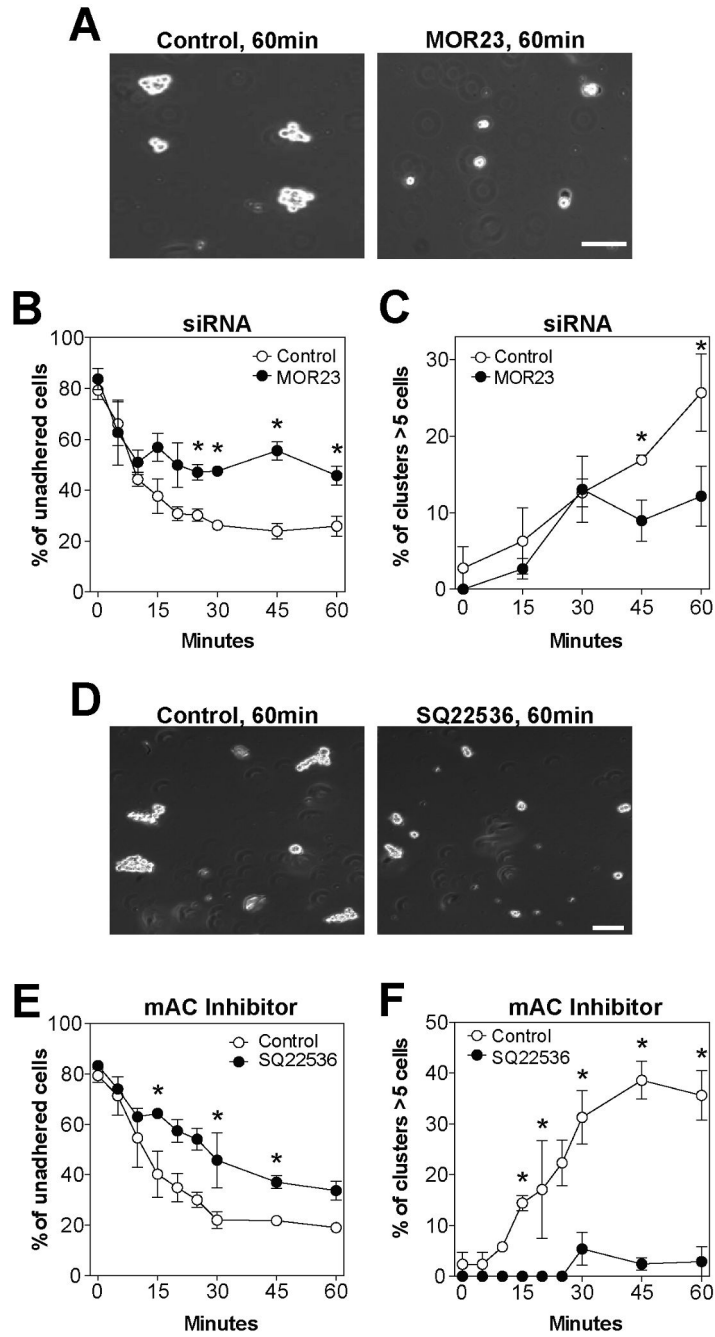




**Figure 3. MOR23 regulates directed migration of myocytes to lyral**

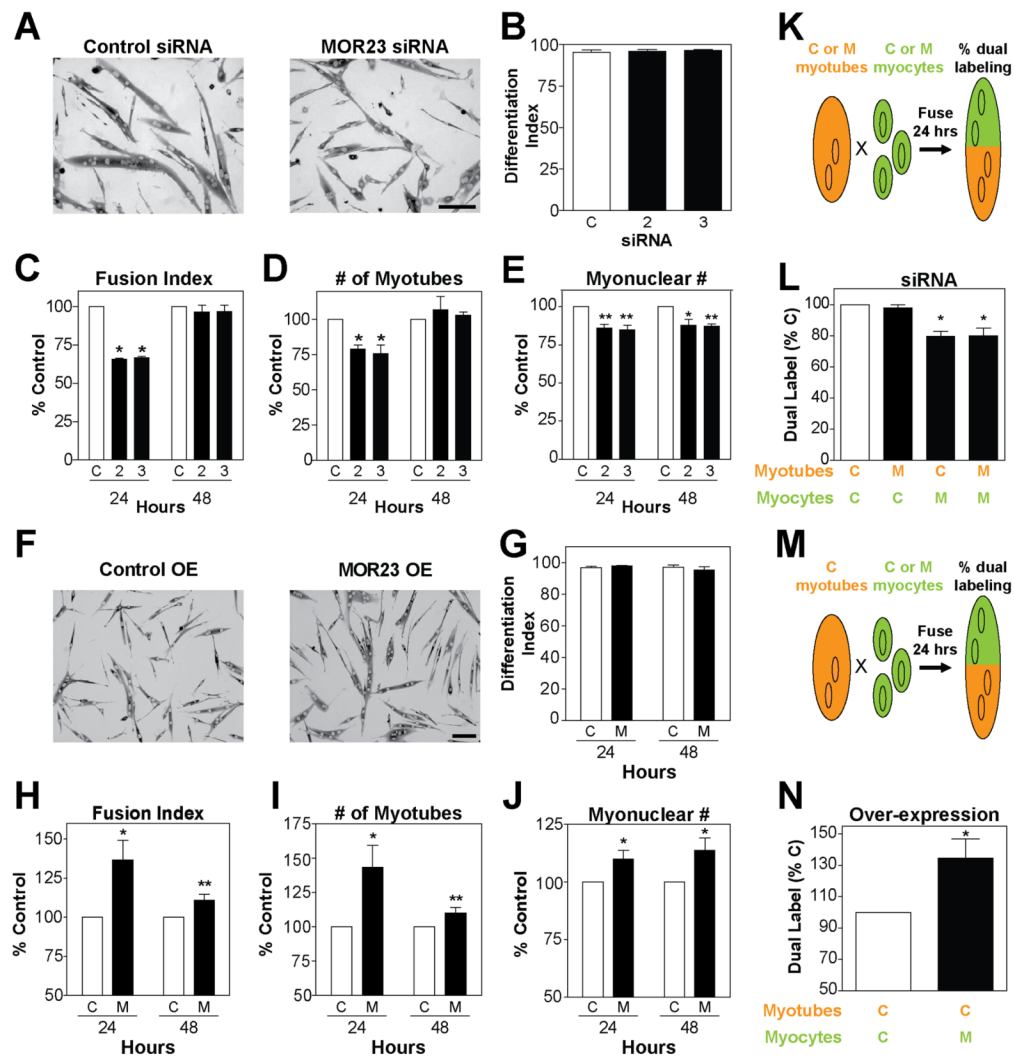
A) Migratory paths of myocytes tracked for 3 hrs with pictures every 5 min in a representative Dunn chamber experiment with 15-20 cells in each graph. Lyral gradient was highest at left side. B-F) Circular histogram plots summarizing Dunn chamber data from 3-5 independent cell isolates with 15-20 cells analyzed in each experiment. Lyral gradient was highest at left side. Red line and arc indicate the mean direction and 99% confidence interval for conditions in which significant clustering of cell migration occurred. B, C) Control cells exhibited directed migration to lyral which was abolished in MOR23 siRNA cells. D) Migration to lyral is MOR23-specific as MOR23 siRNA cells rescued by MOR23 OE exhibited directed migration. E) Migration is dependent on membrane adenylyl cyclase function as inhibitor SQ22536

abrogated directed migration to lylal. F) Myoblasts, which express Olfr1403 but not MOR23, did not exhibit directed migration towards lylal.



**Figure 4. Loss of MOR23 or OR signaling alters cell-cell adhesion**

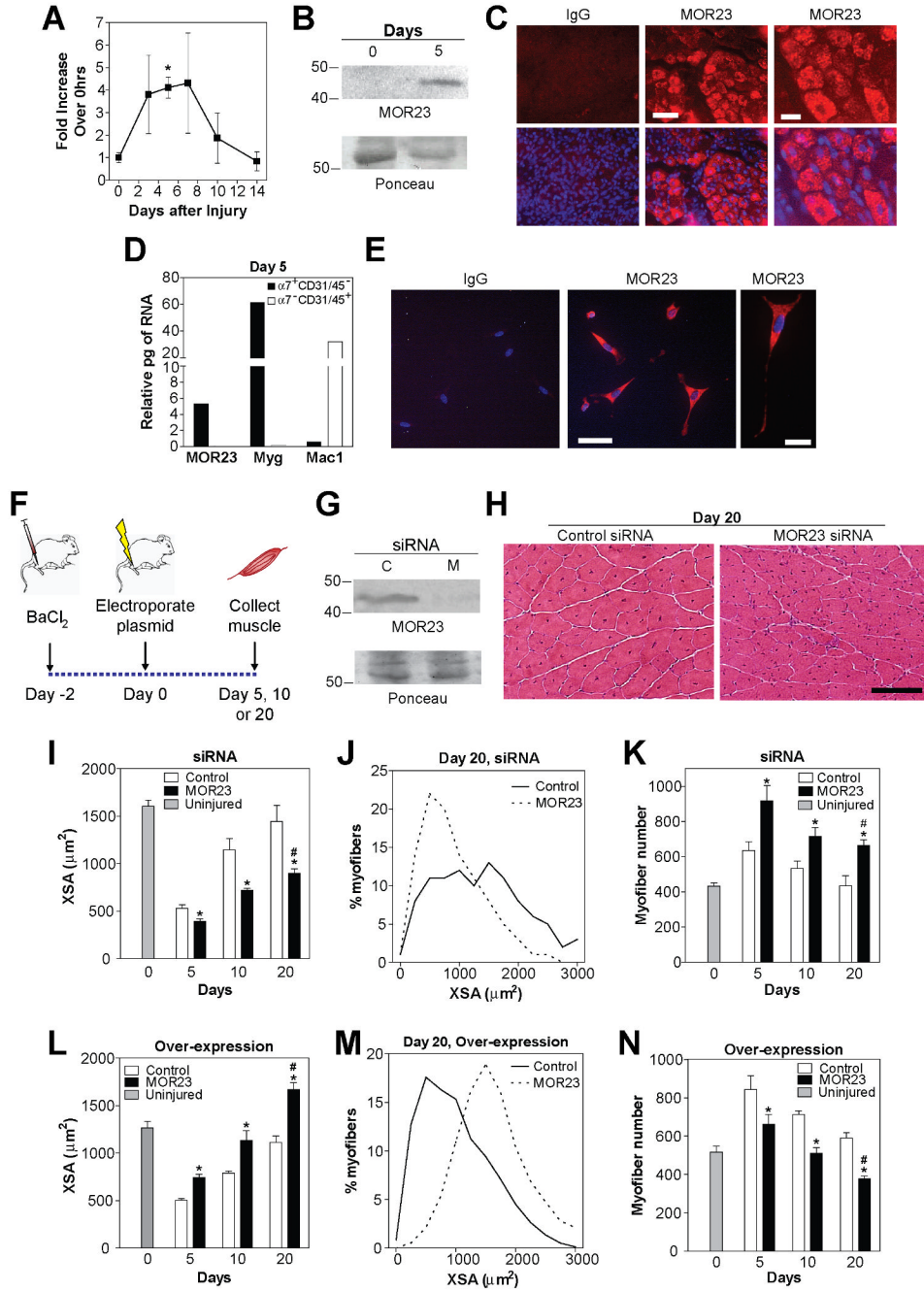
A) Myocytes infected with control or MOR23 siRNA were incubated in suspension for 60 min with aliquots taken regularly, to determine the percentage of un-adhered and adhered cells by phase-contrast microscopy (Bar=50 $\mu$ m). B) MOR23 siRNA cells displayed a significantly higher percentage of un-adhered cells at later time points than control (\*  $p < 0.05$ ). C) MOR23 siRNA cells exhibited fewer clusters with > 5 cells (\*  $p < 0.05$ ). D) Myocytes were suspended in media containing a mAC inhibitor, SQ22536, or vehicle (Bar=50 $\mu$ m). E) SQ22536-treated cells displayed a significantly higher percentage of un-adhered cells at later time points (\*  $p < 0.05$ ). F) SQ22536-treated cells formed small clusters (\*  $p < 0.05$ ). All data are mean  $\pm$  SEM.



**Figure 5. Myotube formation is regulated by MOR23**

A) Control or MOR23 siRNA 2 and 3 cells immunostained for eMyHC at 24 hrs in DM (Bar=50µm). B) No difference was observed in the percentage of nuclei in eMyHC<sup>+</sup> cells at 24 hrs in DM (differentiation index). C) The fusion index in MOR23 siRNA cultures was transiently decreased at 24 hrs in DM (\* p<0.001). D) The number of myotubes in MOR23 siRNA cultures was transiently decreased at 24 hrs in DM (\* p<0.001). E) Myonuclear number in MOR23 siRNA cultures was decreased at both 24 and 48 hrs in DM (\* p<0.05; \*\* p<0.01). F) Control (C) or MOR23 OE (M) cells were immunostained for eMyHC at 24 hrs in DM (Bar=50µm). G) No difference was observed in the percentage of nuclei in eMyHC<sup>+</sup> cells at 24 or 48 hrs in DM. H) The fusion index in MOR23 OE cultures was increased at 24 and 48 hours in DM (\* p<0.05; \*\* p<0.01). I) The number of myotubes in MOR23 OE cultures was increased at 24 and 48 hrs in DM (\* p<0.05; \*\* p<0.01). J) Myonuclear number in MOR23 OE cultures was increased at 24 and 48 hrs in DM (\* p<0.01). K) Nascent myotubes infected with control or MOR23 siRNA were labeled orange and mixed with control or MOR23 siRNA myocytes labeled green. After 24 hrs of co-culture, cultures were fixed and myotubes analyzed for dual labeling. L) With MOR23 siRNA in myocytes the percentage of myotubes with dual label was decreased 20% relative to control; however, MOR23 siRNA in myotubes had no affect (\* p<0.001). M) Control nascent myotubes were mixed with control or MOR23 OE

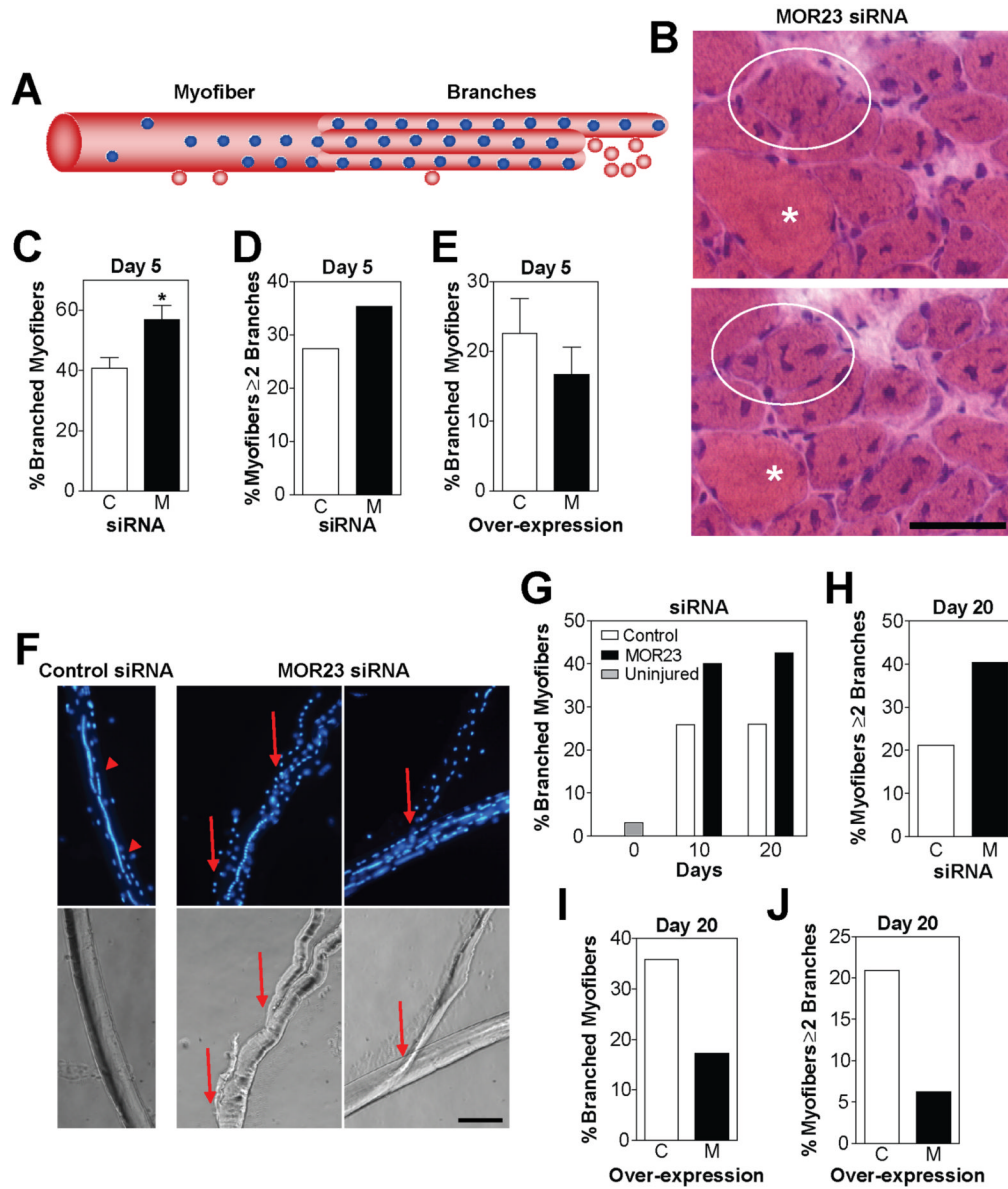
myocytes and analyzed as in K. N) With MOR23 OE in myocytes, the percentage of myotubes with dual label was increased 35% relative to control (\*  $p < 0.05$ ). All data are mean  $\pm$  SEM.



**Figure 6. Changes in MOR23 expression affect muscle regeneration**

A) Real Time RT-PCR for MOR23 mRNA in gastrocnemius muscles at different times post-injury. All days are normalized to 18S rRNA and expressed as fold increase over 0 days (\* p<0.01). B) Immunoblot for MOR23 at 0 and 5 days with a portion of the Ponceau-stained blot shown as control. C) Muscles 5 days post-injury immunostained for MOR23 or control IgG (Bar=25 $\mu\text{m}$ ). MOR23<sup>+</sup> regenerating myofibers are shown at higher magnification in last panels (Bar=10 $\mu\text{m}$ ). Overlay in bottom panels indicates DAPI-stained nuclei. D) Mononucleated cells were isolated from gastrocnemius muscles 5 days post-injury and immunostained with antibodies to CD31 (FITC), CD45 (FITC) and  $\alpha7$ -integrin (PE): CD31<sup>+</sup>CD45<sup>+</sup>, to identify endothelial and immune cells and  $\alpha7$ -integrin<sup>+</sup>CD31<sup>-</sup>CD45<sup>-</sup> for myogenic cells. Real Time

RT-PCR for MOR23, myogenin (Myg), and Mac1 mRNA in  $\alpha 7$ -integrin<sup>+</sup>CD31<sup>-</sup>CD45<sup>-</sup> cells or  $\alpha 7$ -integrin<sup>-</sup>CD31<sup>+</sup>CD45<sup>+</sup> cells. All genes are normalized to 18S rRNA and expressed as mean pg of RNA from N=2; 7 mice each. E) Mononucleated  $\alpha 7$ -integrin<sup>+</sup>CD31<sup>-</sup>CD45<sup>-</sup> muscle cells isolated from gastrocnemius muscles 5 days post-injury, were plated and immunostained for MOR23. A single MOR23<sup>+</sup> cells is shown at higher magnification in right panel (Bars=10 $\mu$ m). F) Gastrocnemius muscles were induced to regenerate using BaCl<sub>2</sub> 2 days prior to electroporation of control or MOR23 plasmids. Muscles were isolated 5, 10 or 20 days post-electroporation; uninjured muscles were collected from contra-lateral day 20 legs. G) Immunoblot demonstrating knockdown of MOR23 protein at day 5 with section of Ponceau-stained blot shown as control. H) Control or MOR23 siRNA muscles stained with hematoxylin and eosin (Bar=100 $\mu$ m). I) The cross-sectional area (XSA) of regenerating myofibers was decreased with MOR23 siRNA (\* p<0.05 from control, # p<0.05 from uninjured). J) The frequency of small regenerating myofibers increased with MOR23 siRNA. K) The number of regenerating myofibers increased with MOR23 siRNA (\* p<0.05 from control, # p<0.05 from uninjured). L) The cross-sectional area (XSA) of regenerating myofibers was increased with MOR23 OE (\* p<0.05 from control, # p<0.05 from uninjured). M) The frequency of small regenerating myofibers decreased with MOR23 OE. N) The number of regenerating myofibers decreased with MOR23 OE (\* p<0.05 from control, # p<0.05 from uninjured). All data are mean  $\pm$  SEM unless stated otherwise.



**Figure 7. MOR23 regulates myofiber branching**

A) Schematic of myofiber branching in which a single myofiber is contiguous with several smaller myofibers. Nuclei are blue and mono-nucleated cells are small red circles. B) Gastrocnemius muscles isolated 5 days after electroporation of plasmid were analyzed for myofibers that were a single myofiber in one section and multiple myofibers occupying the same area in a second section, 170 $\mu$ m away. Stars indicate the same myofiber in both sections, a branched myofiber is circled (Bar=50 $\mu$ m). C) Myofiber branching was increased with MOR23 siRNA. D) The number of branches per myofiber increased with MOR23 siRNA. Data are percentage of total myofibers. E) Myofiber branching was not affected at day 5 with MOR23 OE. F) Myofibers were isolated from gastrocnemius muscles 10 or 20 days after electroporation of plasmid. DAPI-stained centrally located nuclei and phase-contrast microscopy determined if regenerating myofibers exhibited branching. Arrows indicate a branched regenerating myofiber, arrow-head an unbranched regenerating myofiber (Bar=50 $\mu$ m). G) Muscles with MOR23 siRNA contained more branched regenerating



myofibers. H) The number of branches per myofiber increased with MOR23 siRNA. I) Muscles with MOR23 OE contained fewer branched regenerating myofibers. Data are percentage of total myofibers. J) The number of branches per myofiber decreased with MOR23 OE. All data are mean  $\pm$  SEM unless stated otherwise.