Autonomous xenogenic cell fusion of murine and chick skeletal muscle myoblasts

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Running Head: AUTONOMOUS XENOGENIC MYOBLAST FUSION

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ABSTRACT

Cell-cell fusion has been a great technology to generate valuable hybrid cells and organisms such as hybridomas. In this study, skeletal muscle myoblasts were utilized to establish a novel method for autonomous xenogenic cell fusion. Myoblasts are mononuclear myogenic precursor cells and fuse mutually to form multinuclear myotubes. We generated the murine myoblasts (mMBs) expressing green fluorescent protein (GFP) termed mMB-GFP, and the chick myoblasts (chMBs) expressing *Discosoma* red fluorescent protein (DsRed) termed chMB-DsRed. mMB-GFP and chMB-DsRed were cocultured and induced to differentiate. After 24 h, the multinuclear myotubes expressing both GFP and DsRed were observed, indicating that mMBs and chMBs fuse interspecfically. These $GFP^+/DsRed^+$ hybrid myotubes were able to survive and grew to hyper-multinucleated mature form. We also found that undifferentiated mMB-GFP efficiently fuse to the chMB-DsRed-derived myotubes. This is the first evidence for the autonomous xenogenic fusion of mammalian and avian cells. Myoblast-based fusogenic technique will open up an alternative direction to create novel hybrid products.

Key words: cell fusion, hybrid cell, myotube formation, skeletal muscle myoblast.

INTRODUCTION

Intercellular fusion of distinct types of cells enables to create the novel cells having remarkable characteristics. The most successful application is hybridomas, the fusion products of myelomas and antibody-producing B cells (Kohler & Milstein 1975). Artificial cell fusions can be induced by Sendai virus (Okada 1962), polyethylene glycol (PEG) (Pontecorvo 1976), or electroporation (Zimmermann & Scheurich 1981). Using these techniques, various xenogenic hybrid cells and organisms have been developed, *e.g.*, pomato (Melchers *et al.* 1978). The latest study reported the human-plant hybrid cells maintaining chromosomes from both species (Wada *et al.* 2016), indicating that xenogenic cell fusion is a powerful method to provide useful biological tools. However for economic mass-production of the hybrids, current fusogenic approaches are not convenient, efficient, or scalable. It is considered that the spontaneous phenomena occurred in multicellular organisms may help to solve these problems.

Animal development involves several cell-fusion processes such as fertilization, placenta development, giant cell formation, and myogenesis (Chen *et al.* 2005). Skeletal muscle is composed of myofibers that are hyper-multinucleated myocytes. During muscle development or regeneration, mononuclear myogenic precursor cells called myoblasts fuse to existing myofibers or fuse mutually to form primitive myotubes (Abmayr *et al.* 2012). Myoblast fusion is the well-regulated multistep event involving numerous molecules. It has been reported that a metalloprotease/disintegrin meltrin-α

(Yagami-Hiromasa *et al.* 1995), extracellular matrix receptors of β_1-integrin (Schwander *et al.* 2003), and the transmembrane glycoprotein M-cadherin which activates Rac1 (Charrasse *et al.* 2007) are implicated in myotube formation. In addition, recent studies revealed that a skeletal muscle-specific transmembrane protein, myomaker, is necessary for myoblast fusions in mouse (Millay *et al.* 2013), chicken (Luo *et al.* 2015), and zebrafish (Landemaine *et al.* 2014). Notably, forced expression of myomaker alone is sufficient for non-muscle cells to fuse to myoblasts. It strongly suggests that myomaker is essentially required to activate other fusogenic proteins for initiating myoblast fusion (Millay *et al.* 2013). Amino acid sequences of myomaker are evolutionarily conserved in vertebrates (Fig. 1A), prompting us to investigate the spontaneous xenogenic myoblast fusion between two animal species in distinct classes.

Skeletal muscle is the largest tissue in animals. Particularly, meat-type chickens such as broilers form well-developed muscles in a short period of time with high feed efficiency (Griffin & Goddard 1994). Xenogenic myoblast fusion may enable to utilize chick muscle as a biological factory to produce valuable proteins of small or rare mammals. Here we report the hybrid myotubes generated by the fusion of murine myoblasts (mMBs) and chick myoblasts (chMBs). This is the first evidence for autonomous xenogenic fusion of mammalian and avian cells.

MATERIALS AND METHODS

Myoblast culture

mMBs and chMBs were respectively isolated from skeletal muscle of 4-week-old C57BL/6J mice (Motohashi *et al.* 2014) and leg muscle of E10 Barred Plymouth Rock chicken embryos (Luo *et al.* 2015). All experimental procedures were conducted in accordance with the Regulations for Animal Experimentation of Shinshu University, and the animal protocol was approved by the Committee for Animal Experiments of Shinshu University.

Myoblasts were cultured on collagen-coated dishes at 37°C with 5% CO_2 throughout experiments. Undifferentiated myoblasts were maintained in growth medium (GM). GM for mMBs (mGM) consists of DMEM, 20% fetal bovine serum (FBS), and 2 ng/mL basic fibroblast growth factor (bFGF). GM for chMBs (chGM) consists of RPMI1640, 20% FBS, 1% non-essential amino acids, 1% chicken embryo extract, and 2 ng/mL bFGF. Undifferentiated myoblasts in GM were defined as day 0. For myotube formation, myoblasts were completely dissociated by treating 0.25% Trypsin with 1 mM EDTA (Wako) for 5 min at 37°C. Then 2.0×10^5 of mMBs and/or 1.0×10^5 of chMBs were seeded on the 30-mm dishes (VIOLAMO; VTC-D35) coated by collagen type I-C (Nitta Gelatin; Cellmatrix). The MBs were induced to differentiate in differentiation medium (DM) consisting of DMEM and 5% FBS.

Histocytochemistry

Cell nuclei were visualized by Hoechst 33342 (SIGMA). For myosin heavy chain (MHC) staining, myoblasts were fixed with 2% paraformaldehyde, permeabilize by 0.2% triton, and immunostained with mouse monoclonal anti-MHC antibody MF20 (R&D Systems). Phase-contrast and fluorescent images were taken and layered by EVOS FL Auto microscope (Life Technologies; AMAFD1000). The ratio of MHC^+ cells were defined as the number nuclei in all MHC^+ cells divided by the total number of nuclei. Fusion indexes were defined as the number of nuclei in multinuclear MHC^+ myotubes divided by the total number of nuclei. The numbers of $Hoechst^+$ nuclei were counted using ImageJ software (National Institutes of Health).

Quantitative real-time RT-PCR (qPCR)

Total RNAs from myoblasts were isolated using TRIzol Reagent (Thermo Fisher Scientific) and reverse transcribed using ReverTra Ace qPCR RT Master Mix (TOYOBO). qPCR were performed using GoTaq qPCR Master Mix (Promega) with StepOne Real -Time PCR System (Applied Biosystems). Primer sequences for murine myomaker (Millay *et al.* 2013), murine 18S ribosomal RNA (rRNA) (Hirai *et al.* 2010), chick myomaker (Luo *et al.* 2015), and chick 16S rRNA (Fujimura *et al.* 2008) were previously described.

Retroviral infection

Retroviral vectors pMX-GFP (Cell Biolabs) and pMXs-DsRed Express (Hong *et al.* 2009) which is a gift from Dr. Shinya Yamanaka (Addgene plasmid #22724) were transfected into retroviral packaging cell lines Platinum-E and

-GP (Cell Biolabs) to produce retroviruses for mMBs and chMBs, respectively. After 48 h of transfection, retrovirus-containing medium was harvested and filtered through a 0.45-μm filter. Undifferentiated myoblasts were infected with retrovirus by replacing with virus-containing medium for 6 h.

RESULTS

First, we confirmed myotube formation and myomaker gene expression of mMBs and chMBs. As shown in Fig. 1B, undifferentiated mMBs cultured in GM (defined as day 0) were almost mononuclear and negative for myosin heavy chain (MHC), a terminal differentiation marker for myocytes. In DM, nearly 100% of mMBs expressed MHC at day 2, and > 80% of mMBs formed multinuclear myotubes by day 5. Expression of myomaker gene peaked at day 2 and kept higher level at day 4 compared with day 0 as previously reported (Millay *et al.* 2013). Although myogenic differentiation of chMBs were relatively slower than mMBs (Fig. 1C), > 90% of chMBs were MHC^+ and > 70% of chMBs formed myotubes at day 5. Expression pattern of myomaker gene of chMBs was similar to that of mMBs, which is compatible with the previous study (Luo *et al.* 2015). These results indicate that both mMBs and chMBs differentiate into multinuclear myotubes with increasing myomaker expression in DM.

To trace the dynamics of each myoblast during xenogenic fusion, we generated the mMBs having GFP genes within its genome (mMB-GFP) and the chMBs having DsRed genes in the genome (chMB-DsRed) (Fig. 2A). First, undifferentiated mMB-GFP and chMB-DsRed were concomitantly seeded on one dish in the equal mixture of mGM and chGM (Fig. 2Ba). Both mMB-GFP and chMB-DsRed continued to proliferate but did not fuse by day 3 (Fig. 2C).

Next, undifferentiated mMB-GFP and chMB-DsRed were cocultured then induced to differentiate in DM (Fig. 2Bb). At day 1, the multinuclear

myotubes expressing both GFP and DsRed were observed (Fig. 2D), indicating that mMBs and chMBs fuse within 24 h under differentiating condition. These $GFP^+/DsRed^+$ myotubes were able to survive and grew to mature myotubes having more than ten nuclei during day 2-5 (Fig. 2E). However, the ratio of $GFP^+/DsRed^+$ double-positive cells to GFP^+ or $DsRed^+$ single-positive cells were less than 1% and not increased from day 1 onward.

To investigate the impact of differentiation stage of myoblasts on their fusion, undifferentiated chMB-DsRed were seeded on the myotubes derived from mMB-GFP in DM at day 2 (Fig. 2Bc). Unexpectedly, morphological structure of the mMB-GFP-derived myotubes were severely impaired by seeding chMB-DsRed (Fig. 2F). $GFP^+/DsRed^+$ cells were scarcely observed by day 5, demonstrating that chMBs do not fuse to murine myotubes.

Finally, undifferentiated mMB-GFP were seeded on the myotubes derived from chMB-DsRed at day 2. After 24 h of coculture in DM (Fig. 2Bd), $GFP^+/DsRed^+$ myotubes were observed at a maximum rate of 10% (Fig. 2G). However in the mixture of mGM and chGM (Fig. 2Be), undifferentiated mMB-GFP did not fuse to chMB-DsRed-derived myotubes (Fig. 2H). These results indicate that mMBs at an initial differentiation stage but not in undifferentiated situation have a capability to fuse to chick myotubes.

DISCUSSION

In the present study, murine and chick myoblasts spontaneously fused to form hybrid myotubes *in vitro*. This is the first evidence for autonomous xenogenic fusion of mammalian and avian cells, which from distinct animal classes. Previous studies reported the PEG-induced hybrid cell fusion of rat L6 myoblast cell line and chick myoblasts but did not mention spontaneous fusion (Wright 1981; Konieczny & Coleman 1982). As shown in this study, autonomous fusion of mMBs and chMBs under the plain coculture condition were rarely occurred and hard to detect without fluorescent labeling. Visualizing hybrid myotubes intriguingly showed that differentiation stages of the myoblasts are critically important for the efficiency of spontaneous interspecific fusion; that is, mMBs fused to chick myotubes with a relatively high efficiency, while chMBs did not fuse to murine myotubes.

One of the key molecules for myoblast fusion is a transmembrane protein, myomaker (Millay *et al.* 2013; Landemaine *et al.* 2014; Luo *et al.* 2015). Expression levels of myomaker are elevated during differentiation both in mMBs and chMBs (Fig. 1B and 1C), suggesting that chick rather than murine myomaker is important for the fusion of mMBs and chMBs. Myoblasts fuse *via* the interaction of myomaker on the cell with its hypothetical ligand on the other cell (Millay *et al.* 2013). It is assumed that chick myomaker is able to interact with murine ligands but murine myomaker can not recognize chick ligands. The latest report showed the critical role of C-terminal tail of myomaker for myoblast fusion (Millay *et al.*

2016). Distinct amino acid sequences in this region may be involved in the asymmetric fusion abilities between mMBs and chMBs. Since amino acid sequences of myomaker are highly conserved among mammals (Fig. 1A), not only murine but also other mammalian myoblasts possibly fuse to chMBs.

Myoblast-based fusogenic technique provide a novel technology to generate hybrid skeletal muscle, which will contribute to the innovative studies of *in vitro* meats, artificial muscles, and chimeric generation of animal organs for transplantation therapy. For basic biology, the xenogenic hybrid myotubes having two distinct cell nuclei may be an evolutional model of intracellular organelles. Interplay of genetic information from different organisms will serve to understand the symbiosis such as with mitochondria and chloroplasts. Establishment and analysis of xenogenic myoblast fusion *in vivo* will contribute to further development of these researches.

Conclusions

Here we showed that murine and chick myoblasts spontaneously fuse to form hybrid myotubes. This is the first evidence for the interspecific fusion of mammalian and avian cells. The myoblast-based technology will provide a novel strategy for autonomous xenogenic cell fusion to create hybrid products.

ACKNOWLEDGMENTS

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Figure legends

Figure 1 (A) Phylogenetic tree for amino acid sequences of myomaker in human (*Homo sapiens*, NP_001073952), cattle (*Bos taurus*, NP_001179975), pig (*Sus scrofa*, XP_003353750), rat (*Rattus norvegicus*, NP_001127989), mouse (*Mus musculus*, NP_079652), chicken (*Gallus gallus*, NP_001305386), quail (*Coturnix japonica*, XP_015734430), gecko (*Gekko japonicus*, XP_015262227), xenopus (*Xenopus laevis*, XP_018089315), and zebrafish (*Danio rerio*, NP_001002088). Numerals indicate the identities of amino acid sequences (%). (B and C) MHC and Hoechst staining images (upper panels), quantifications of MHC^+ cells and fusion indexes (bottom left panels), and qPCR results of myomaker gene expression (bottom right panels) of mMBs (B) and chMBs (C). Scale bars, 100 μm.

Figure 2 (A) mMB-GFP and chMB-DsRed. (B) Schematic representation of culture conditions for myoblast fusion. (C) mMB-GFP and chMB-DsRed at day 3, cultured as in Fig. 2Ba. (D) $GFP^+/DsRed^+$ multinuclear myotube at day 1, cultured as in Fig. 2Bb. (E) $GFP^+/DsRed^+$ mature myotube at day 3, cultured as in Fig. 2Bb. (F) The mMB-GFP-derived myotubes alone (upper panel) and with chMB-DsRed (lower panel) at day 3, cultured as in Fig. 2Bc. (G and H) mMB-GFP seeded on the chMB-DsRed-derived myotubes at day 3, cultured as in Fig. 2Bd (G) and 2Be (H). Arrows indicate $GFP^+/DsRed^+$ myotubes. Scale bars, 50 μm (A and D) or 200 μm (C, E, F, G, and H).

マウスおよびニワトリ骨格筋芽細胞の自律的な異種細胞融合

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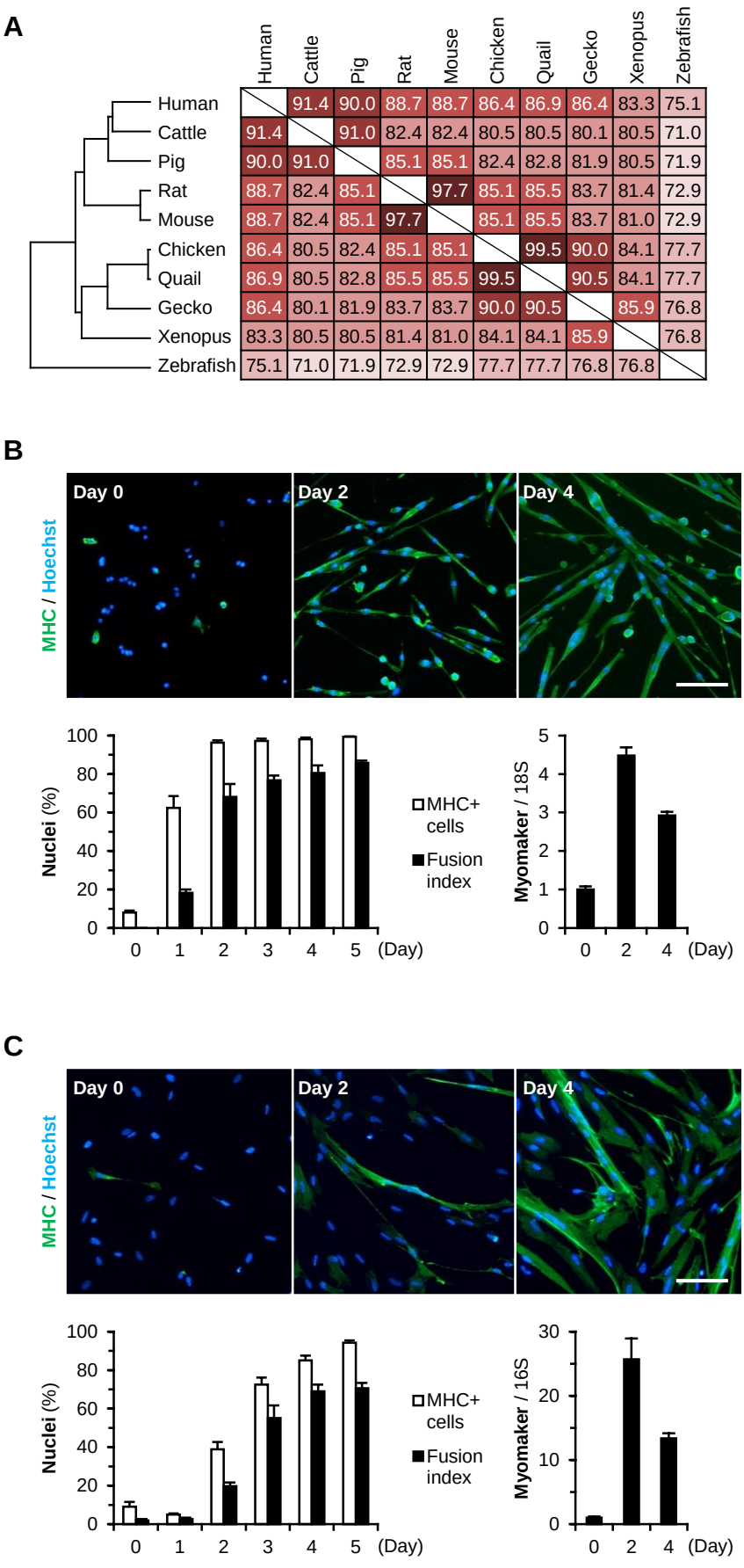
細胞融合は、ハイブリドーマのような有益なハイブリッド細胞の創出に重要な技術である。本研究では、骨格筋芽細胞を用いた自律的な異種細胞融合の新規手法の開発を試みた。筋芽細胞は単核の筋前駆細胞で、筋分化の過程で互いに融合して多核の筋管を形成する。我々は、緑色蛍光タンパク質（GFP）を発現するマウス筋芽細胞（mMB-GFP)、および赤色蛍光タンパク質（DsRed）を発現するニワトリ筋芽細胞（chMB-DsRed）を作出した。共培養した mMB-GFP と chMB-DsRed を分化誘導すると、24 時間後には GFP と DsRed の両方を発現する多核の筋管が観察され、マウスとニワトリの筋芽細胞が異種融合することが示された。この $GFP^+/DsRed^+$ハイブリッド筋管は、多数の細胞核を有する成熟した筋管へと成長することができた。また、未分化な mMB-GFP が、分化した chMB-DsRed 由来筋管に効率よく融合することも明らかになった。本研究は、哺乳類と鳥類の細胞の自律的な異種融合の最初の報告である。筋芽細胞を基盤とした細胞融合技術は、新たなハイブリッド細胞の創出に貢献することが期待される。

Figure 1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Day 0
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Nuclei (%)
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Myomaker / 18S
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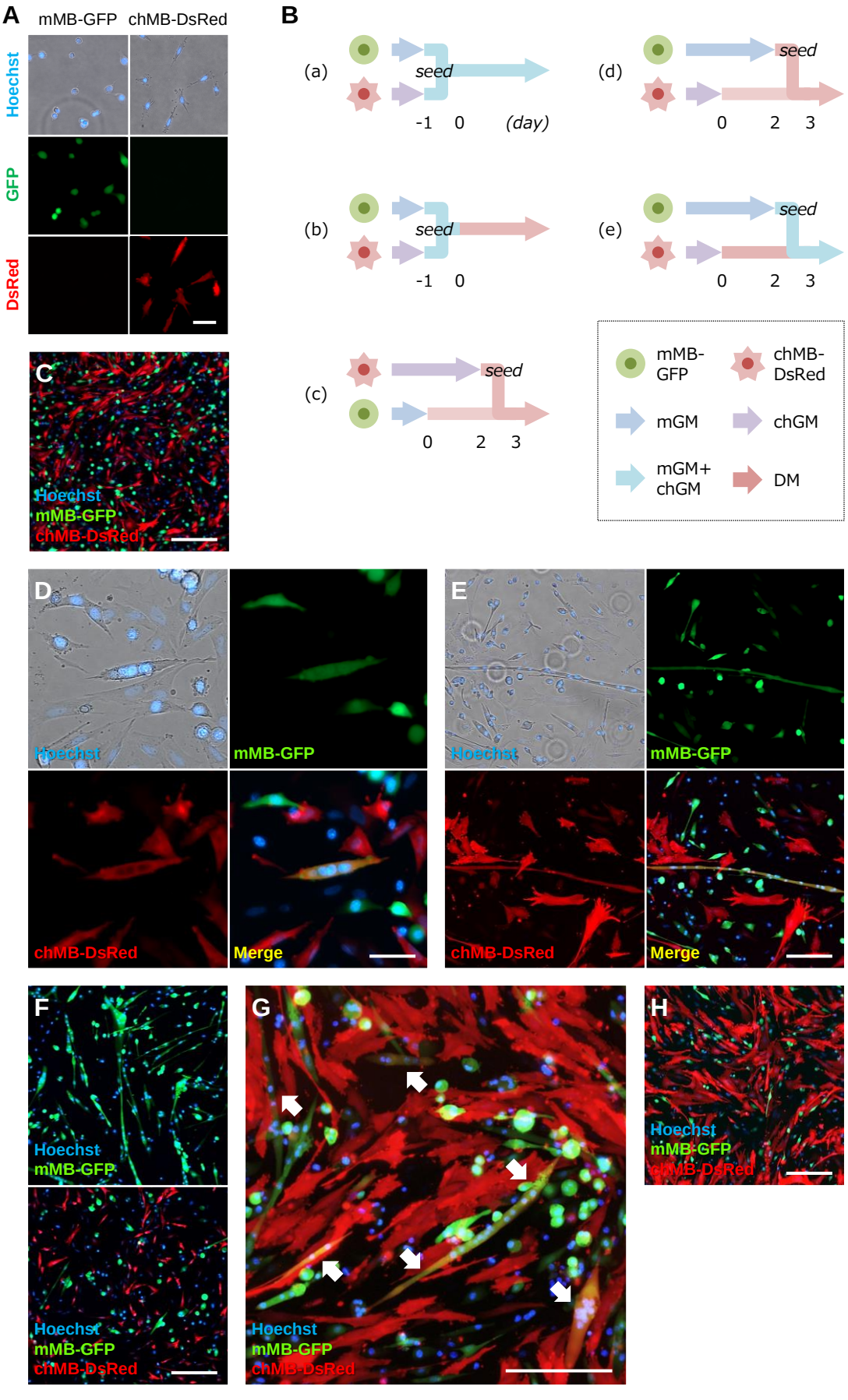
Myomaker / 16S

Figure 2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