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Muscle stem cell isolation and *in vitro* culture for meat production: A methodological review

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Abstract

Cultured muscle tissue-based protein products, also known as cultured meat, are produced through *in vitro* myogenesis involving muscle stem cell culture and differentiation, and mature muscle cell processing for flavor and texture. This review focuses on the *in vitro* myogenesis for cultured meat production. The muscle stem cell–based *in vitro* muscle tissue production consists of a sequential process: (1) muscle sampling for stem cell collection, (2) muscle tissue dissociation and muscle stem cell isolation, (3) primary cell culture, (4) upscaled cell culture, (5) muscle differentiation and maturation, and (6) muscle tissue harvest. Although muscle stem cell research is a well-established field, the majority of these steps remain to be underoptimized to enable the *in vitro* creation of edible muscle-derived meat products. The profound understanding of the process would help not only cultured meat production but also business sectors that have been seeking new biomaterials for the food industry. In this review, we discuss comprehensively and in detail each step of cutting-edge methods for cultured meat production. This would be meaningful for both academia and industry to prepare for the new era of cellular agriculture.

KEYWORDS
cultured meat, in vitro culture, method, muscle stem cells

1 | INTRODUCTION

Cellular agriculture is an emerging research field of agribiotechnology that aims to produce agricultural products and by-products using stem cell and tissue engineering without a sacrifice of animals (Post et al., 2020). Currently, various efforts have been conducted to produce agricultural products, such as milk, egg, leather, and meat in a lab (Stephens et al., 2018). Of them, cultured muscle tissue-based protein products, also known as cultured meat, are rising alternatives to conventional meat in the recent food industrial trends (Post, 2012). Cultured meat is artificial meat produced through the culture and differentiation of muscle stem cells *in vitro*. Some researchers and companies have considered the novel food material as a potential protein source for the growing global population along with conventional meat and other analogues including plant meat and edible insects (Lee, Yong, Kim, Choi, & Jo, 2020). However, the ongoing debates on about influences of cultured meat in social and environmental aspects compared with conventional meat and animal farming remain. (The details are well reviewed in Chriki & Hocquette, 2020.) This novel concept for producing a popular food material has drawn significant attention, as it allows to alleviate the above-mentioned problems and promote animal welfare compared with

conventional meat production (Bhat, Kumar, & Fayaz, 2015).

Currently, there are three different available approaches for *in vitro* livestock myogenesis: culture of muscle stem cells (Ding et al., 2017, 2018), directed differentiation of pluripotent stem cells (Bogliotti et al., 2018; Choi et al., 2019), and direct reprogramming using transgenesis (Genovese, Domeier, Telugu, & Roberts, 2017). Since the first report on the production of a cultured muscle-based beef patty, most cultured meat production processes primarily focused on the *in vitro* culture and differentiation of muscle stem cells (Kadim, Mahgoub, Baqir, Faye, & Purchas, 2015). Muscle stem cells (so-called satellite cells) are precursors responsible for the regeneration of the muscle tissue, including quiescent stem cells and their progeny such as proliferating myoblasts (Kuang, Kuroda, Le Grand, & Rudnicki, 2007). The muscle tissue originates from the paraxial mesodermal progenitor cells during fetal development. The paraxial mesoderm differentiates into myoblasts through the sequential developmental process regulated by several growth factors (reviewed in Chal & Pourquie, 2017). The myoblasts generate muscle tissue via cell-to-cell fusion, and a part of them resides beneath the myofiber basal lamina and converts into quiescent satellite cells during the postnatal period. Upon muscle injury, the quiescent satellite cells become activated, differentiate into myoblasts, and contribute to muscle regeneration.

The quiescent satellite cells are defined by the expression of Pax7, but not Myf5 and MyoD (Kuang et al., 2007). Muscle injury triggers myogenic satellite cell commitment through Myf5 and MyoD upregulation and Pax7 downregulation, thereby turning those cells into proliferating myoblasts. Myf5 and MyoD have reciprocal roles in the proliferation and differentiation of myoblasts, respectively (Asakura et al., 2007; Gayraud-Morel et al., 2007). These intrinsic factors could be applied as muscle stem cells markers during the investigation of the cellular states. Muscle stem cells are also defined by surface and cytoskeletal proteins, which interact with the stem cell niche, such as vascular cell adhesion molecule (VCAM), neural cell adhesion molecule (NCAM, also known as CD56), integrin $\alpha 7$ and $\beta 1$ (also known as CD29), CD34, SM/C-2.6, and desmin (Wang, Dumont, & Rudnicki, 2014). In other words, the harmony of extrinsic and intrinsic cues regulates the fate of muscle stem cells. Therefore, to maintain the stemness of muscle stem cells *in vitro*, the supporting physiological features of muscle stem cells should be assured by mimicking the *in vivo*–stem cell niche, including the extracellular matrix (ECM) and paracrine factors. Also, a deep understanding of muscle stem cell physiology would be required for a reliable *in vitro* meat production. Therefore, this review will focus on the basic *in vitro* culture methods including donor animal selection, muscle tissue dissociation, as well as muscle stem cell sorting, cryopreservation, and *in vitro* expansion (Figure 1). This comprehensive review helps both food scientists and industry to understand the details of cultured meat production and to prepare the future cellular agriculture era.

2 | MUSCLE STEM CELL ISOLATION FROM TISSUES

2.1 | Animal age and sex, and the origin of muscles

The muscle stem cells can be isolated through muscle biopsy and from slaughtered animals. As the yield of the isolated muscle stem cell is altered by the conditions of the donor animals, several factors should be considered for a more efficient satellite cell isolation prior to donor animal selection. First, it has been proven in various species that the age of the animals and the location of the muscles affect the number of the obtained muscle stem cells. Satellite cells are reportedly more numerous in the soleus muscle than in the extensor digitorum longus of 1-, 12-, and 24-month-aged mice (Gibson & Schultz, 1983). Another study demonstrated that diaphragm and extraocular muscles have more stem cells than soleus muscles (Keefe et al., 2015). In pigs, the highest number of PAX7-positive muscle stem cells was detected in the psoas major and extensor carpi radialis following the examination of nine different types of muscle tissues including the semimembranosus, semitendinosus, biceps femoris, peroneus tertius, intercostal, longissimus thoracis, psoas major, rhomboideus, and extensor carpi radialis muscles (Ding et al., 2017). Also, satellite cells isolated from the different muscles showed various differentiation potential and proliferation properties. Satellite cells obtained from the diaphragm have a lower proliferation rate and higher myogenic ability than those originated from the semimembranosus muscle in pigs (Redshaw, McOrist, & Loughna, 2010). It is enticing to think that the stem cell niche surrounded by ECM and stromal cells causes distinct satellite cell gene expression in different muscle tissues (Comai & Tajbakhsh, 2014). It has also been proven that a genetic trait, known as "double-muscling" caused by myostatin (*Gdf8*) mutation, can affect the proliferative feature, but not the myogenic potential, of muscle stem cells. The fetal myoblasts of double-muscled cattle maintain their undifferentiated state for longer and have an increased proliferation rate compared with those of wild-type cattle (Quinn, Ong, & Roeder, 1990).

In addition, upon muscle growth during postnatal development, the total number of muscle stem cells increases while their proportion decreases. The absolute

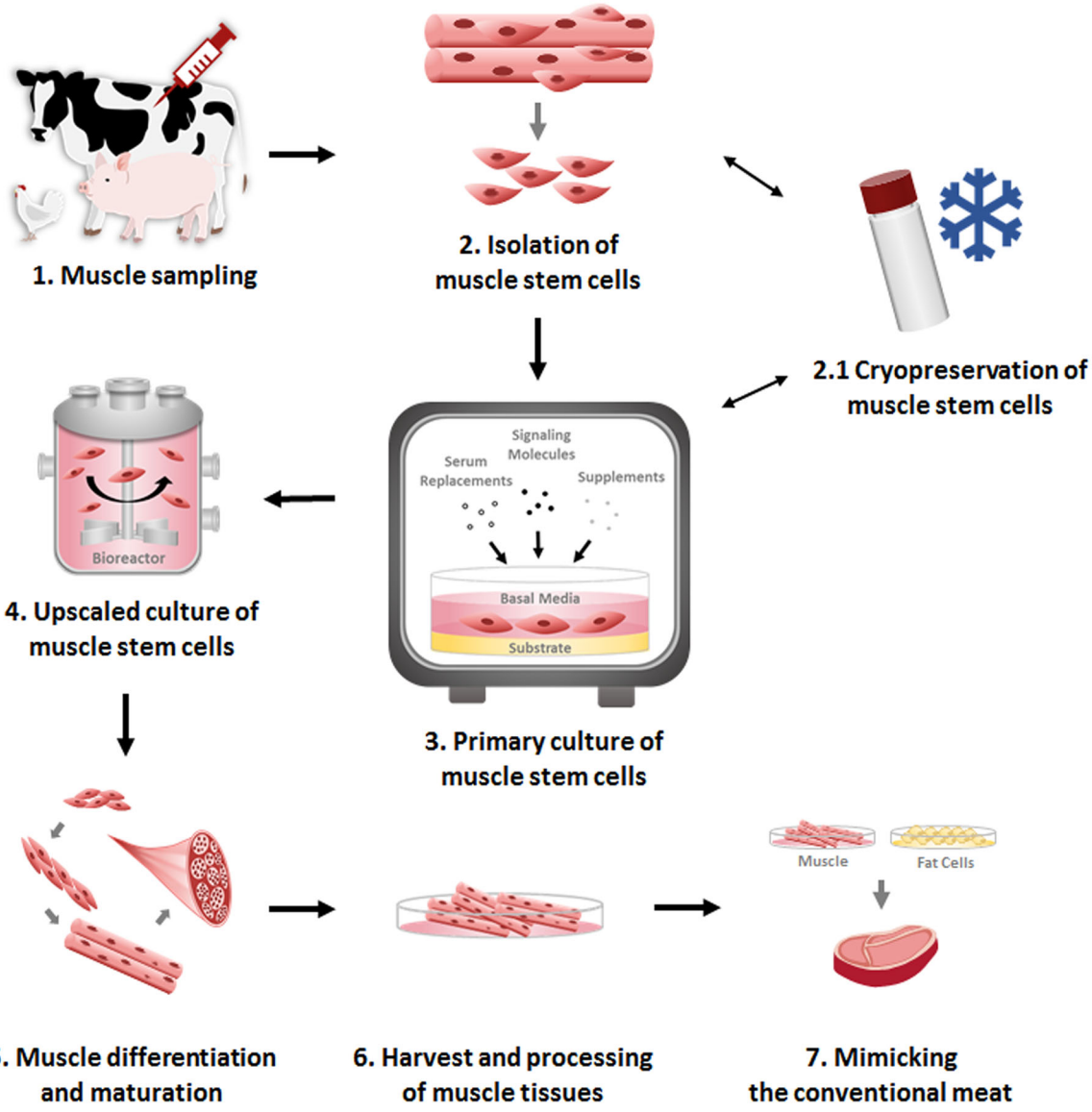

FIGURE 1 Principal process of the cultured meat production The muscle stem cell–based *in vitro* muscle tissue production consists of a sequential process: (1) muscle sampling for stem cell collection, (2) muscle tissue dissociation and muscle stem cell isolation, (3) primary cell culture, (4) upscaled cell culture, (5) muscle differentiation and maturation, and 6) muscle tissue harvest and processing. The muscle tissues can be collected through muscle biopsy and from slaughtered animals. Then, the muscle stem cells are isolated from physically and enzymatically dissociated muscle tissues. *In vitro* muscle stem cell cultures can be achieved by mimicking the *in vivo*–stem cell niches including ECM, signaling molecules (hormones and cytokines), metabolites, and physical environments (temperature, pH, and humidity). The sorted muscle stem cells or dissociated cells from muscle tissues can be stored until use without losing their properties and cultured *in vitro* after thawing. These cells are massively cultured using bioreactor for upscaling muscle production and subsequently differentiated into muscle fiber. Finally, the muscle fibers are harvested and processed to mimic the texture and flavor of conventional meat. In this review, we discuss comprehensively and in detail each step of cutting-edge methods for cultured meat production

number of satellite cells increased throughout the post-hatch growth in the semimembranosus muscle of the Japanese quail, whereas their proportion declined (Campion, Marks, & Richardson, 1982). In mice, the absolute number of satellite cells increased until 12 months and in turn decreased up to 24 months in the soleus muscle, and gradually declined in the extensor digitorum longus with aging (Gibson & Schultz, 1983). Campion et al. showed that the number of satellite cells is increased until 32 weeks, whereas the proportion gradually decreased during post-natal growth in the peroneus longus and sartorius muscles of pigs (Campion, Richardson, Reagan, & Kraeling, 1981). Moreover, along with aging, the proliferative potential of the satellite cells decreased in the gastrocnemius

muscle of rats (Chakravarthy, Davis, & Booth, 2000). The age-induced loss of proliferation ability could be rescued by insulin-like growth factor I (IGF-I) treatment. In the semitendinosus muscle of pigs, the proportion of NCAM-positive cells decreased between 1 and 7 weeks of age, while proliferating cell nuclear antigen (PCNA) immunostainings showed that the percentage of proliferating cells among these NCAM-positive cells declined from 1 to 14 weeks of age (Mesires & Doumit, 2002).

The sex of animals is another factor involved in muscle stem cell growth. Sex hormones such as estrogen and testosterone influence muscle stem cell growth. In the triceps brachii muscle of neonatal boars, the number of satellite cells got significantly reduced upon castration compared with that of noncastrated boars, and this impaired growth was recovered by the administration of testosterone propionate (Mulvaney, Marple, & Merkel, 1988). Mouse studies showed that the males have more satellite cells per myofiber than females and the reduction of satellite cell number per myofiber with age is more dramatic in females than in males (Day, Shefer, Shearer, & Yablonka-Reuveni, 2010). Proliferating satellite cells were more abundant in adult male mice than in females, whereas quiescent satellite cells were present at a similar rate (Neal, Boldrin, & Morgan, 2012). Moreover, sex hormones have a crucial role in establishing the quiescent stem cell population through the activation of the Nodal pathway induced by Mib1 (Kim et al., 2016). In women, the postmenopausal loss of estrogen causes apoptosis and the loss of satellite cells, thereby inducing impaired muscle regeneration in elderly women (Collins et al., 2019).

2.2 | Muscle tissue dissociation

Skeletal muscle is composed of muscle fibers, connective tissues, and stromal cells, as well as various stem cell populations. As muscle stem cells reside on muscle fibers, it is pivotal to efficiently dissociate these cells from the muscle tissue for their upscaled isolation. Generally, proteases such as trypsin, pronase, dispase, and collagenase have been used for purifying muscle stem cells following physical dissociation by scissors and meat mincer. As described in Table 1, several enzymes with multiple combinations could be applied for digesting the muscle tissues. In particular, collagenase and dispase have been widely used for such enzymatic digestion as they reportedly target the ECM including fibronectin and collagen (Stenn, Link, Moellmann, Madri, & Kuklinska, 1989). After the enzymatic dissociation, the fiber fragments, tissue debris, and connective tissues should be separated from the muscle stem cell–containing dissociated cells for an efficient subsequent sorting process. Based on the differences in size and weight, filtering and differential centrifugation are the standard approaches for isolating the mononucleated cell population containing the muscle stem cells (Table 1). Generally, the dissociated tissues are filtrated through a cell strainer or nylon mesh having 20 to 40 μm pores, then centrifuged at a low g-force of about 300 g in order to remove the debris.

2.3 | Muscle stem cell separation from the mononucleated cell population

The resulting supernatant of the differential centrifugation contains various cell types such as somatic cells, blood cells, stromal cells, and muscle stem cells. Therefore, it is essential to perform cell sorting following the dissociation step in order to obtain a highly purified muscle stem cell population. As summarized in Table 1, several methods have been developed for muscle stem cell sorting based on their physical (density gradient centrifugation), biological (preplating and cytochalasin B treatment), and molecular features (flow cytometry and magnetic-activated cell sorting). Since the beginning, density gradient centrifugation and preplating have been widely applied muscle stem cell sorting methods. Density gradient centrifugation is a method for separating cells based on their relative density. The cells can be separated into several subpopulations by centrifugation using a solution that consists of dense substrates such as Percoll and Ficoll. These substrates build up a density gradient through which the sample passes during the centrifugation. In a rat study, the dissociated cells from the hind leg muscles were separated by gradient centrifugation using 70, 50, and 35% Percoll layers (Bischoff, 1997). More than 98% of the cells collected from the interface between the 50 and 70% gradient steps showed the morphology and differentiation rate features of myogenic cells. Gradient centrifugation has also been applied for the isolation of pig muscle stem cells, showing that approximately 95% of the cells expressed the myoblast marker desmin (Mau, Oksbjerg, & Rehfeldt, 2008; Perruchot, Ecolan, Sorensen, Oksbjerg, & Lefaucheur, 2012). These results demonstrated that highly purified muscle stem cells could be sorted by the gradient centrifugation. However, other studies have shown a lower purification efficiency. Yablonka-Reuveni et al. attempted to isolate chicken muscle stem cells using a gradient composed of 60 and 20% Percoll layers (Yablonka-Reuveni & Nameroff, 1987). Only 70% of the resulting cell population was characterized as myogenic cells from the interface between the 60 and 20% gradient layers. Finally, it was reported in a mouse study that 43.1 to 48.6% of the gradient centrifugation-sorted cells were positive for the myoblast marker MyoD (Kastner, Elias, Rivera, & Yablonka-Reuveni, 2000). Therefore, this method is considered to have a wide variety of efficiency and low specificity.

TABLE 1 Summary of dissociation and sorting methods to isolate the muscle stem cells from muscle tissue

Species	Enzymes for muscle dissociation	Sorting methods	References
Rat, rabbit, cattle	Trypsin		Yaffe (1968); Yaffe and Feldman (1965)
Rat, rabbit, cattle	Trypsin	Preplating	Richler and Yaffe (1970)
Mouse	Dispase + collagenase class II	Preplating	Rando and Blau (1994)
Mouse	Dispase + collagenase class D	MACS	Blanco-Bose, Yao, Kramer, and Blau (2001)
Mouse	Type II collagenase	FACS	Fukada et al. (2007)
Mouse	Type I collagenase	FACS	Urbani et al. (2012)
Mouse	Type I collagenase + dispase B		Liu et al. (2012)
Mouse	Type I collagenase		Hitchins et al. (2013)
Mouse	Collagenase A + dispase I + DNase I	FACS	Pannerec, Formicola, Besson, Marazzi, and Sassoon (2013)
Mouse	Type I collagenase		Figeac and Zammit (2015); Sassoli et al. (2014); Wang et al. (2014)
Mouse	Type II collagenase	MACS	Motohashi, Asakura, and Asakura (2014)
Mouse	Collagenase	FACS	Fu et al. (2015)
Mouse	Type II collagenase + dispase	FACS	Liu et al. (2015)
Mouse	Dispase		Suzuki et al. (2015)
Mouse	Collagenase B + dispase I	MACS	Sincennes, Wang, and Rudnicki (2017)
Mouse	Type II collagenase + collagenase D + dispase II	Preplating	Shahini et al. (2018)
Rat	Pronase	Preplating	Allen, McAllister, and Masak (1980); Chakravarthy et al. (2000); Dodson et al. (1985); Machida et al. (2004); Sheehan et al. (2000)
Rat	Pronase	Density gradient centrifugation	Bischoff (1997); Kastner et al. (2000)
Rat	Pronase		Allen et al. (1995); Groux-Muscatelli et al. (1990); Tatsumi et al. (1998)
Rat	Type III collagenase + dispase	Density gradient centrifugation	Dusterhoft, Yablonka-Reuveni, and Pette (1990)
Rat	Type I collagenase + trypsin	Preplating	Dai et al. (2015)
Chicken	Trypsin		Coleman and Coleman (1968); Trotter and Nameroff (1976)
Chicken		Cytochalasin B	Sanger (1974)
Chicken	Trypsin	Density gradient centrifugation	Yablonka-Reuveni and Nameroff (1987)
Pig	Pronase		Choi et al. (2020b); Doumit and Merkel (1992)
Pig	Pronase	Density gradient centrifugation	Mesires and Doumit (2002)
Pig	Trypsin	Density gradient centrifugation	Mau et al. (2008); Miersch, Stange, and Rontgen (2018)
Pig	Type IV collagenase	Preplating	Redshaw and Loughna (2012); Redshaw et al. (2010)
Pig	Trypsin + type II collagenase + DNase	Density gradient centrifugation	Perruchot et al. (2012)
Pig	Protease		Jeong et al. (2013)
Pig	Collagenase D + dispase II	FACS	Ding et al. (2017)
Pig	Pronase	MACS	Choi et al. (2020a)

(Continues)

TABLE 1 (Continued)

Species	Enzymes for muscle dissociation	Sorting methods	References
Cattle	Trypsin	Preplating or cytochalasin B treatment	Gospodarowicz et al. (1976)
Cattle	Pronase	Preplating	Dodson et al. (1987)
DM cattle[a]	Trypsin		Quinn et al. (1990)
Cattle	Type II collagenase	FACS	Ding et al. (2018)
Human	Trypsin	FACS	Baroffio et al. (1996)
Human		MACS	Brady, Lewis, and Mudera (2008); Martin et al. (2013)
Human	Collagenase + dispase	MACS + FACS	Bareja et al. (2014)
Human	Type II collagenase + dispase	FACS	Charville et al. (2015)

Abbreviations: FACS, fluorescence-activated cell sorting; MACS, magnetic-activated cell sorting.
[a]Double-muscled cattle: they have more muscle mass compared with normal cattle by mutation of the *Gdf8* gene.

The preplating technique has also been a widely used cell separation approach for long. As different cell types take different time for adhering onto the cell culture plate, cell enrichment could also be performed based on these biological features. Among the various cell types of the dissociated muscle tissue, muscle stem cells take longer to adhere onto the cell culture plate than fibroblasts and epithelial cells. After 40 to 60 min from seeding onto the culture plate, a highly purified stem cell population could be obtained by harvesting the supernatant, because most fibroblasts and epithelial cells get already attached onto the culture plate (Richler & Yaffe, 1970). Table 1 shows muscle stem cells sorted by this method in mouse (Contreras, Villarreal, & Brandan, 2018), rat (Dodson, Allen, & Hossner, 1985), cattle (Dodson, Martin, Brannon, Mathison, & McFarland, 1987), and pig (Redshaw & Loughna, 2012). Rando et al. demonstrated that the enriched primary myoblasts, obtained by preplating, could fuse with host muscle cells after transplantation (Rando & Blau, 1994). Various studies have reported that the preplating approach has a very high efficiency, which is beyond 90%. It has also been shown that 95% of the preplated rat muscle cells from the hind limb and back were positive for desmin and MyoD (Sheehan, Tatsumi, Temm-Grove, & Allen, 2000). The purity of mouse myoblasts was increased up to 98% through preplating based on the expression of myogenic markers such as integrin $\alpha 7$, MyoD, and desmin (Shahini et al., 2018). Moreover, Qu-Petersen et al. showed that not only muscle stem cells but also muscle-derived stem cells, a kind of interstitial cells, could be isolated using a 6-step sequential preplating technique (Gharaibeh et al., 2008). However, as this method also has a wide variety of efficiency depending on the experimental subjects (Ding et al., 2017; Gospodarowicz, Weseman, Moran, & Lindstrom, 1976), it is necessary to perform subsequent molecular biological verifications. Another way to isolate myoblasts from fibroblasts is cytochalasin B treatment. Cytochalasin B interferes with the formation of actin filaments, thereby inhibiting cell division and movement. As this compound induces the detachment of myoblasts but not that of fibroblasts, suspended myogenic cells could be sorted through their harvest from the myoblast culture media (Sanger, 1974). This treatment has also been applied for bovine and chicken muscle stem cells, but it reportedly has some cytotoxicity effect on myoblasts (Gospodarowicz et al., 1976; Ojima, Lin, Andrade, Costa, & Mermelstein, 2016).

The cellular gene-expression profile is function- and cell type-dependent. Gene-expression markers are defined as the genes or proteins, which are highly expressed in specific cell types compared with the others. These molecules are specific for certain cell types, which enables cellular identification and specific cell sorting using fluorescence-conjugated antibodies against these marker proteins. The antibody-bound cells could be selected using fluorescence-activated cell sorting (FACS) and magnetic-activated cell sorting (MACS) approaches. FACS is a flow cytometry–based analysis and cell sorting method. Flow cytometry enables cell analysis and isolation based on their physical features such as size and granularity, as well as marker expression through the detection of fluorescent signals. In early studies, the satellite cell population was purified based on cell size (using forward-scattered light, FSC) and complexity (using side-scattered light, SSC) without any antibody staining. Flow cytometry data showed that two different populations could be detected among the human muscle–derived dissociated cells and that 98% of the small-cell population, with lower FSC and SSC levels, were identified as myogenic cells using 5.1.H11 (also known as CD56) immunostaining (Baroffio et al., 1993, 1996). As summarized in Table 2, various fluorescence antibodies have been used for the

TABLE 2 Summary of FACS/MACS methods to isolate the muscle stem cells using cell-surface antigens

Species	Sorting techniques	positive markers	Negative markers	References
Mouse	MACS	Integrin α7		Blanco-Bose et al. (2001)
Mouse	FACS	SM/C-2.6	CD45	Fukada et al. (2007)
Mouse	FACS	SM/C-2.6	CD31, CD45, Sca-1	Urbani et al. (2012)
Mouse	FACS	CD34	CD45, Sca-1, TER119	Pannerec et al. (2013)
Mouse	MACS	Integrin α7	CD31, CD45, Sca-1	Motohashi et al. (2014)
Mouse	FACS	CD34, Integrin α7	CD11, CD31, CD45, Sca-1	Fu et al. (2015)
Mouse	FACS	VCAM	CD31, CD45, Sca-1	Liu et al. (2015)
Mouse	MACS	Integrin α7	CD11b, CD31, CD45, Sca-1	Sincennes et al. (2017)
Human	FACS	Cell size[a]		Baroffio et al. (1993)
Human	MACS	CD56		Brady et al. (2008); Martin et al. (2013)
Human	MACS + FACS[b]	CXCR4, CD56	CD11b, CD31, CD34, CD45	Bareja et al. (2014)
Human	FACS	CD29	CD31, CD34, CD45	Charville et al. (2015)
Pig	FACS	CD29, CD56	CD31, CD45	Ding et al. (2017)
Pig	MACS	CD29		Choi et al. (2020a)
Cattle	FACS	CD29, CD56	CD31, CD45	Ding et al. (2018)

Abbreviations: CD, cluster of differentiation; CXCR4, C–X–C chemokine receptor 4; FACS, fluorescence-activated cell sorting; MACS, magnetic-activated cell sorting; Sca-1, stem cells antigen 1; VCAM, vascular cell adhesion molecule.

[a]The cells were separated based on FSC and SSC, and the smaller population was myogenic cells.

[b]MACS and FACS were used for negative and positive selection, respectively.

isolation of muscle stem cells as well. Generally, positive and negative selections have been simultaneously applied according to species and experimental design (Liu, Cheung, Charville, & Rando, 2015). Negatively selected cells, using antibodies against markers such as CD11, CD31, CD45, and Sca-1, are sorted out, whereas the remaining cells, expressing positive markers such as CD29, CD34, CD56, integrin α7, and SM/C-2.6, are subsequently isolated. These studies have shown a high sorting efficiency beyond 95%. Moreover, MACS is a cell-sorting method based on antigen–antibody interaction, and similar markers can thus be applied during this approach as during FACS (Table 2). Unlike FACS, MACS require the use of magnetic microbead-conjugated antibodies instead of fluorescent antibodies. The microbead-tagged cells could be captured using a strong magnet. As the equipment of MACS is relatively simple and inexpensive compared with that of FACS, including magnets and selection columns, it has widely been used for the enrichment of various cell types.

2.4 | Muscle stem cell cryopreservation

Cryopreservation is a technique that enables cell preservation for extended periods at the extremely low temperatures using deep freezer and liquid nitrogen. As the muscle stem cell self-renewal and myogenic capacities gradually decline in an *in vitro* culture, cryopreservation-based cell banking is required to maintain these properties. The sorted muscle stem cells or dissociated cells from muscle tissues can be stored until use without losing their properties and cultured *in vitro* after thawing. In order to cryopreserve the cells, it is crucial to reduce water crystallization in the media and cytosol during the process of freezing. Several freezing methods have been developed using cryoprotectant-containing media such as dimethyl sulfoxide (DMSO), ethylene glycol, and sucrose. First, the slow-freezing method has widely been applied to the storage of various animal cell types (Freshney, 2015). During slow freezing, the cells in the fetal bovine serum (FBS) and cryoprotectant-containing media are progressively frozen typically at a rate of 1 °C / min, thereby reducing cellular damage through minimizing water crystallization. Vitrification is another rapid freeze-cell preservation method using media with a high concentration of cryoprotectants (Kuleshova, Gianaroli, Magli, Ferraretti, & Trounson, 1999). Of those methods, slow-freezing has generally been applied for the storage of muscle stem cells. Rat and pig satellite cells were frozen using a media containing 20% of FBS and 10% of DMSO (Chakravarthy et al., 2000; Mau et al., 2008). After thawing, no detrimental effects have been observed on cell viability and myogenic potential (Chakravarthy et al., 2000). Moreover, these

freezing methods could also be applied to store muscle tissue. Minced and cryopreserved muscle tissues could be reportedly used for myogenic cell isolation after thawing (Quinn et al., 1990; Witkowski, 1977).

3 | *IN VITRO* CULTURE OF MUSCLE STEM CELLS

In vitro muscle stem cell cultures can be obtained by mimicking the *in vivo*–stem cell niches including ECM, signaling molecules (hormones and cytokines), metabolites, and physical environments (temperature, pH, and humidity). These components surrounding muscle stem cells *in vivo* have been recapitulated *in vitro* using synthetic chemicals and artificial devices (Table 3). The *in vitro* culture environment includes growth media, cell substrates, and incubators. The role of each component will be described in the succeeding sections.

3.1 | Cell substrates

The basic role of cell substrates is providing an adherent surface for the cells, which influences the cadherin and integrin receptor–mediated proliferation, viability, and cellular aging in stem cells (Guilak et al., 2009). For the muscle stem cells, several ECM proteins such as fibronectin, laminin, and collagen provide the *in vivo* physical environment, which have also been applied for *in vitro* cell cultures (Yin, Price, & Rudnicki, 2013). *In vivo* studies showed that fibronectin overexpression stimulates satellite cell expansion, whereas its downregulation induces the impaired repopulation of the satellite cell niche (Bentzinger et al., 2013). It has been verified that the fibronectin activates the Wnt7a signaling through the receptor complex composed of Syndecan-4 (Sdc4) and Frizzled-7 (Fzd7), thereby regulating the proliferation ability of satellite cells. Moreover, it has been shown that the fibronectin expression gradually reduces with aging in the skeletal muscle, leading to a loss of muscle stem cell through the inactivation of the p38-mediated integrin signaling pathway (Lukjanenko et al., 2016). These defects of the aged muscle stem cell regenerative abilities could be rescued by intramuscular fibronectin injection in old mice. Collagen also plays a crucial role in the muscle stem cell self-renewal and differentiation *in vivo*. Type IV collagen knockout reportedly induces a reduction of satellite cell regeneration capacity upon muscle injury in mice (Urciuolo et al., 2013). The laminin family is also crucially involved in muscle tissue regeneration. Muscle injury induces an expression increase of laminin family members in the muscle, leading to the promotion of satellite cell self-renewal (Rayagiri et al., 2018).

The treatment of ECM proteins resulted in similar effects on muscle stem cells both *in vitro* and *in vivo*. The muscle stem cells cultured on ECM protein–coated plates reportedly exhibit improved proliferation and differentiation abilities. Moreover, as the cells show species-specific ECM preferences, a comparative analysis has been conducted to find among the ECM proteins to identify the most suitable candidates for muscle stem cell *in vitro* cultures (Table 3). Doumit et al. demonstrated that using four substrates including Primaria (Becton Dickinson), fibronectin, type III collagen, and gelatin, the pig satellite cells cultured on gelatin-coated plates have significantly higher proliferation and differentiation rate compared with those cultured on the other three substrates (Doumit & Merkel, 1992). On the other hand, it has been shown that mouse myoblasts cultured with type I collagen and laminin showed rapid doubling compared with those cultured with type IV collagen and fibronectin (Rando & Blau, 1994). In addition to single ECM molecules, a mixture of ECM components, known as Matrigel, secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells, has been widely applied for *in vitro* muscle stem cell expansion. Matrigel imitates an *in vivo* niche due to its specific components such as laminin, type IV collagen, entactin, and trace growth factors (Vukicevic et al., 1992). Moreover, the stemness of mouse myogenic progenitor cells could also be maintained for an extended period on Matrigel-coated dishes (Wang et al., 2014). When compared with other substrates, Matrigel facilitated the expression of integrin and Wnt signaling pathways and enhanced the proliferation and differentiation capacities in porcine muscle stem cells (Wilschut, Haagsman, & Roelen, 2010). In a recent study using immersion rotary jet spinning-produced microfibrous gelatin, researchers recapitulated myogenesis, including its structural and mechanical aspects as well (MacQueen et al., 2019).

3.2 | Basal media

Cell growth media generally contain basal media, serum or serum replacements, and cell signaling molecules. Basal media, composed of basic elements such as nutrients (amino acids, carbohydrates, and lipids), vitamins, inorganic salts, and trace minerals, provide a soluble microenvironment for the cells *in vitro*. The basal media, just like body fluids *in vivo*, take part in buffering pH and osmotic pressure, as well as nourishing cells *in vitro*. As different metabolites and components are required for maintaining different cell types, various basal media have been developed for these specific purposes such as GMEM for BHK-21 cells (Stoker & Macpherson, 1961), F10/F12 nutrient medium for CHO cells (Ham, 1965), or

TABLE 3 Summary of culture conditions to maintain the muscle stem cells *in vitro*

Species	Substrates	Basal media	Serums	Others	Antibiotics	Signaling molecules	Incubator conditions	References
Rat, rabbit, cattle		Media 199 + MEM[a] for primary culture; media 199 for cell line culture	10% HS for primary culture; 10% HS or FBS[b] for cell line culture	CEE for primary culture; CEE + bovine serum albumin for cell line culture				Yaffe (1968)
Rat, rabbit, cattle		Media 199 + DMEM	10% HS	CEE			37 °C, 10% CO_2	Richler and Yaffe (1970)
Mouse	Collagen	F10	20% FBS			FGF2[c]	37 °C,5% CO_2	Rando and Blau (1994); Sincennes et al. (2017)
Mouse	Laminin	F10	20% FBS		P + S	FGF2	37 °C,5% CO_2	Blanco-Bose et al. (2001)
Mouse	Matrigel	DMEM	20% FBS		P + S	FGF2	37 °C,5% CO_2	(Fukada et al. (2007)
Mouse		DMEM	20% FBS + 10% HS	CEE	P + S		37 °C, 5% CO_2,2% O_2	Urbani et al. (2012)
Mouse	Collagen	F10	15% FBS		P + S	FGF2	37 °C, 5% CO_2,1% O_2	Liu et al. (2012)
Mouse		DMEM	10% FBS	CEE		WNT1 and WNT6	37 °C,5% CO_2	Hitchins et al. (2013)
Mouse	Gelatin	DMEM	20% FBS + 10% HS	L-glutamine, sodium pyruvate	P + S	FGF2		Pannerec et al. (2013)
Mouse	Matrigel	DMEM	20% FBS + 10% HS	L-glutamine, CEE	P + S	Sphingosine 1-phosphate and VEGF	37 °C,5% CO_2	Sassoli et al. (2014)

(Continues)

TABLE 3 (Continued)

Species	Substrates	Basal media	Serums	Others	Antibiotics	Signaling molecules	Incubator conditions	References
Mouse	Matrigel	F10	20% FBS			FGF2		Motohashi et al. (2014)
Mouse		DMEM	10% FBS	L-glutamine	P + S		37 °C,5% CO_2	Horbelt et al. (2015)
Mouse	Matrigel	DMEM	20% FBS + 10% HS	CEE	AA (Thermo)		37 °C,5% CO_2	Wang et al. (2014)
Mouse	Collagen	F10	10% FBS			FGF2, IL-1α, IL-13, IFN-γ and TNF-α	37 °C,5% CO_2	Fu et al. (2015)
Mouse	Matrigel	DMEM	20% FBS + 10% HS	Glutamax (Thermo), CEE	P + S	FGF2	37 °C,5% CO_2	Figeac and Zammit (2015)
Mouse	Matrigel	F10	10% HS		P + S	FGF2	37 °C,5% CO_2	Liu et al. (2015)
Mouse	HPLAAT	DMEM	10% FBS		P + S		37°C,5% CO_2	Xie et al. (2015)
Mouse	PLGA + GO-PLGA-Col	DMEM	10% FBS		AA		37 °C,5% CO_2	Shin et al. (2015)
Mouse		DMEM	20% FBS				37 °C, 5% CO_2,5% O_2	Majmundar et al. (2015)
Mouse		DMEM	10% FBS	L-glutamine, sodium pyruvate, nonessential amino acids (Thermo)	P + S	IWR-1 (negative effects)	37 °C,5% CO_2	Suzuki et al. (2015)
Mouse	PEG-fibrinogen	DMEM	10% FBS		P + S		37 °C,5% CO_2	Costantini et al. (2017)
Mouse	Matrigel	DMEM	20% FBS + 10% HS	CEE	AA + G + plasmocin prophylactic	FGF2	37 °C, 10% CO_2	Shahini et al. (2018)

(Continues)

TABLE 3 (Continued)

Species	Substrates	Basal media	Serums	Others	Antibiotics	Signaling molecules	Incubator conditions	References
Mouse	Nanofiber yarn/hydrogel core shell scaffolds	DMEM	10% FBS		P + S		37 °C,5% CO_2	Wang, Wu, Guo, and Ma (2015)
Mouse, rat		DMEM	20% FBS	Glutamine	P + S		37 °C,5% CO_2	Di Carlo et al. (2004)
Rat		DMEM	10% FBS			Insulin	37 °C,5% CO_2	Mandel and Pearson (1974)
Rat		Parker media 199 + MEM (1/3 v/v)	10% HS	CEE				Delain and Wahrmann (1975)
Rat		DMEM	5% HS	CEE	AA (Thermo)	IGF-2		Florini et al. (1984)
Rat		75% DMEM, 25% MCDB 104 medium	10% HS			Somatomedin[d], MSA[e], insulin and dexa-mathasone		Dodson et al. (1985)
Rat	Gelatin	DMEM	10% FBS + 10% HS			FGF1	37 °C,5% CO_2	Groux-Muscatelli et al. (1990)
Rat	Gelatin	DMEM	2% FBS + 10% HS	CEE			8% CO_2	Dusterhoft et al. (1990)
Rat	Fibronectin	DMEM	10% HS		Antibiotics mixture + G	Heparin and HGF	37 °C,5% CO_2	Allen et al. (1995)
Rat	Gelatin	MEM	10% HS	CEE	AA	TGF-β and HGF (chemotactic effect)	37 °C,5% CO_2	Bischoff (1997)

(Continues)

TABLE 3 (Continued)

Species	Substrates	Basal media	Serums	Others	Antibiotics	Signaling molecules	Incubator conditions	References
Rat	Polylysine + fibronectin	DMEM	10% HS		AA + G		37 °C,5% CO_2	Tatsumi et al. (1998)
Rat	Collagen	F10	20% FBS	L-glutamine, CEE	P + S	IGF-1	37 °C,5% CO_2	Chakravarthy et al. (2000); Machida et al. (2004)
Rat	Gelatin	DMEM	25% FBS + 10% HS	CEE	P + S			Kastner et al. (2000)
Rat	Polylysine + fibronectin	DMEM	10% HS		AA + G		37 °C,5% CO_2	Sheehan et al. (2000)
Rat	Collagen	DMEM/F12	20% FBS + 10% HS	Leucine		FGF2		Dai et al. (2015)
Rat	Laminin	DMEM/F12	25% FBS		AA	FGF2 and dexamethasone		Syverud et al. (2016)
Rat, Chicken	Type I collagen and fibronectin	DMEM for rat, MEM for chicken	20% FBS for rat or 10% FBS+5% chicken serum for chicken			TGF-β		Massague et al. (1986)
Chicken	Collagen		15% HS	CEE			36.5 °C,5% CO_2	Coleman and Coleman (1968)
Chicken		MEM	10% HS	CEE	P + S + F		37 °C,5% CO_2	Trotter and Nameroff (1976)
Chicken	Gelatin	MEM	10% HS	CEE	F + G		37.5 °C,5% CO_2	Yablonka-Reuveni and Nameroff (1987)

(Continues)

TABLE 3 (Continued)

Species	Substrates	Basal media	Serums	Others	Antibiotics	Signaling molecules	Incubator conditions	References
Pig	Gelatin	MEM	FBS		P + S + G + F		37 °C,5% CO_2	Doumit and Merkel (1992)
Pig	Gelatin	MEM	10% FBS + 10% HS	L-glutamine	P + S + F		37 °C,5% CO_2	Mau et al. (2008)
Pig	Type I collagen	αMEM	20% FBS		P + S + AB		37 °C,5% CO_2	Redshaw et al. (2010)
Pig	Type I collagen	αMEM	20% FBS	L-glutamine	P + S + AB		37°C, 5% CO_2,5% O_2	Redshaw and Loughna (2012)
Pig	Matrigel	DMEM	10% FBS + 10% HS		G + AB		37 °C,5% CO_2	Perruchot et al. (2012)
Pig	Matrigel	MEM	10% FBS		P + S		37 °C,5% CO_2	Jeong et al. (2013)
Pig		F10	15% FBS		P + S	FGF2		Ding et al. (2017)
Pig	Type I collagen	αMEM	20% FBS		P + S + AB + G		37 °C,5% CO_2	Miersch et al. (2018)
Pig	Matrigel	SkGM-2 (Lonza)	10% FBS	Glutamax	AA	EGF, dexamethasone, and SB203580	37 °C,5% CO_2	Choi et al. (2020a, 2020b)
Cattle	Gelatin	MEM + DMEM	10% FBS	CEE		FGF	37 °C,5% CO_2	Gospodarowicz et al. (1976)
Cattle	Fibronectin						37 °C,5% CO_2	Dodson et al. (1987)
DM cattle[f]	Gelatin	MEM	10% FBS	Conditioned media from fibroblasts	P + S + G + F		37 °C,5% CO_2	Quinn et al. (1990)
Cattle	Type I collagen	F10	20% FBS		P + S	FGF2 and SB203580		Ding et al. (2018)

(Continues)

TABLE 3 (Continued)

Species	Substrates	Basal media	Serums	Others	Antibiotics	Signaling molecules	Incubator conditions	References
Human		F10	15% FBS					Baroffio et al. (1996)
Human	Gelatin and type I collagen for 3D culture	DMEM	20% FBS		P + S		37 °C,5% CO_2	Brady et al. (2008)
Human	Gelatin and fibrin gel	DMEM	20% FBS		P + S		37 °C,5% CO_2	Martin et al. (2013)
Human	Type I collagen + laminin	F10	20% HS		P + S	FGF2		Bareja et al. (2014)
Human	ECM	DMEM/MCDB	20% FBS	Insulin-transferrin-selenium	P + S	SB203580		Charville et al. (2015)
						Heparan sulfate and FGF2		Rapraeger et al. (1991)
	Collagen	F10	20% FBS		P + S	FGF2 and IL-6	37 °C,5% CO_2	Serrano et al. (2008)

Abbreviations: AA, antibiotic-antimycotic; AB, amphotericin B; CEE, chick embryo extract; DMEM, Dulbecco's Modified Eagle Medium; F, fungizone; F10, Ham's F-10 nutrient mixture; F12, Ham's F-12 nutrient mixture; FBS, fetal bovine serum; FGF, fibroblast growth factor; G, gentamicin; GO-PLGA-Col, collagen impregnated with graphen oxide; HGF, hepatocyte growth factor; HPLAAT, hyperbranched PLA copolymers; HS, horse serum; IFN-γ, interferon gamma; IGF-1, insulin-like growth factor 1; IL, interleukin; IWR-1, Wnt/beta-catenin signaling inhibitor; MCDB, Molecular cellular development biology medium; P + S, penicillin and streptomycin; PEG, photocurable semisynthetic biopolymer; PLGA, poly(lactic-coglycolic acid); TGF-β, transforming growth factor beta; TNF-α, tumor necrosis factor alpha; VEGF, vascular endothelial growth factor; WNT1, wingless-type MMTV integration site family; αMEM, alpha MEM.

[a]Also known as Eagle's media, MEM and Eagle's MEM (EMEM).

[b]Also known as fetal calf serum.

[c]Also known as a basic fibroblast growth factor (bFGF).

[d]Also known as insulin-like growth factor 1 (IGF-1).

[e]Also known as IGF-2.

[f]Double-muscled cattle: they have more muscle mass compared with normal cattle by mutation of the *Gdf8* gene.

IMDM for hematopoietic cells (Guilbert & Iscove, 1976). Therefore, several studies have been conducted to find a suitable basal medium for muscle stem cell cultures. These studies showed that the type of the medium influences stem cell proliferation and differentiation properties. Pig satellite cells showed the highest proliferation rate when cultured with five media including McCoy's 5A, Ham's F12, DMEM, minimum essential media (MEM), and DMEM/F12, whereas they exhibited the worst myogenic potential in MEM and McCoy's 5A media (Doumit & Merkel, 1992). Another study reported that Ham's F10 nutrient medium selectively facilitated mouse myoblast growth, whereas fibroblasts continued to overgrow in DMEM, Waymouth's medium, and M199 medium (Rando & Blau, 1994). Moreover, myosin heavy chain expression studies showed that the F10 nutrient medium suppressed myoblast myogenic differentiation compared with other media mentioned above. A similar effect was observed in cultured rat satellite cells (Machida, Spangenburg, & Booth, 2004).

3.3 | Serum and serum replacements

The serum is the fluid component of the blood that remains after coagulation and contains various protein sources, nutrients, and growth factors. Therefore, it has been widely used as a cell culture medium supplement. In muscle stem cell cultures, FBS and horse serum (HS) have been widely applied. It has been proved that muscle stem cell growth was facilitated, and spontaneous myogenic differentiation was suppressed when cultured at a high serum concentration. Pig satellite cells exhibited the highest proliferation and myogenic potentials in FBS-containing media when cultured with four different sera, including FBS, HS, porcine serum, and lamb serum (Doumit & Merkel, 1992). The stemness of the cells significantly increased in a dose-dependent manner under these conditions. Furthermore, high serum-containing media with 20% FBS and 10% HS exclusively promote mouse muscle stem cell proliferation compared with that of nonmyogenic cells, especially fibroblasts (Shahini et al., 2018; Wang et al., 2014). Currently, due to the donor animal-based wide variations in their composition, serum replacements, such as knockout serum replacement (KSR), and N2/B27 supplements are used to obtain consistent experimental results. However, serum-free media still have lower effects on myoblast *in vitro* expansion compared with those of serum-containing media, indicating that muscle stem cell culture serum replacement remains to be resolved (Kolkmann, Post, Rutjens, van Essen, & Moutsatsou, 2020).

3.4 | Incubators and their specificities (temperature and CO_2 and O_2 concentration)

It is an essential criterion to create a physical environment similar to the body for *in vitro* cell cultures. Incubators have been generally used in order to maintain a constant temperature, humidity, pH for the cellular homeostasis. Carbon dioxide (CO_2) concentration adjustment in the incubator enables the maintenance of the culture medium pH at equilibrium through the presence of sodium bicarbonate buffer in the cell culture medium. In order to culture muscle stem cells, the general incubator settings are 36.5 to 37.5 °C, similar to the body temperature, and 5 to 10% carbon dioxide concentration for pH control (see Table 3). Interestingly, it has been recently reported that changes in cellular respiration and mitochondrial activity due to the differences between the physiological (1 to 6%) and the atmospheric oxygen concentrations (~20%) affect cellular characteristics such as differentiation and proliferation (Abdollahi et al., 2011). Moreover, several recent studies have shown that hypoxia influences muscle stem cell stemness. Urbani et al. demonstrated that the proliferation of mouse satellite cells cultured under hypoxic conditions (2% O_2) increased by two-fold compared with that under atmospheric oxygen concentrations (Urbani, Piccoli, Franzin, Pozzobon, & De Coppi, 2012). Upon their transplantation into cardiotoxin-injured muscle, the cells expanded at hypoxia formed a higher number of new muscle fibers than those expanded at normoxia. The myogenic potential of pig satellite cells was also significantly enhanced at a low oxygen level (Redshaw & Loughna, 2012). Hypoxia-inducible factors (HIFs), known as oxygen sensors, are reportedly involved in regulating muscle stem cell stemness under hypoxia. Hypoxia-induced HIF1α expression hampered muscle regeneration, whereas it did not affect embryonic and fetal myogenesis (Majmundar et al., 2015). It was also revealed that hypoxia-upregulated HIF1α suppressed the canonical WNT pathway, thereby impeding myogenic differentiation *in vivo*. Albeit the genetic ablation of HIFs accelerates muscle regeneration (Majmundar et al., 2015), HIF downregulation also induces the reduction of myoblast number through activating the Notch signaling pathway (Yang, Yang, Wang, & Kuang, 2017). These results indicate that hypoxia has exclusive effects on muscle stem cell proliferation and differentiation. Accordingly, physical environments, including temperature and CO_2 and O_2 concentration, should be carefully manipulated to promote the proliferation and differentiation ability of muscle stem cells.

3.5 | Cell signaling molecules: Cytokines, hormones, and signaling inhibitors

Hormones and growth factors, as well as nutrients, are essential for cell growth and maintenance. They are secreted from endocrine organs and surrounding stromal cells and bind to receptors in the cell membrane or cytoplasm to activate signaling pathways involved in cell growth and differentiation. Therefore, it is possible to regulate the cell physiological features of *in vitro* cultures by using the aforementioned components in various combinations. It was reported that *in vitro*–cultured muscle stem cells gradually lose their stemness in the lack of signaling molecules (Machida et al., 2004). It was also demonstrated that the abundance of PAX7-positive rat satellite cells was reduced from 90 to 55% upon subculturing. Therefore, various signaling molecules have been examined to maintain the undifferentiated muscle stem cell state *in vitro*, which will be presented in the following paragraphs.

3.5.1 | Fibroblast growth factors 2 (FGF2 or basic FGF)

As shown in Table 3, FGF2 has been most widely used for *in vitro*–cultured muscle stem cells. FGF2 is reportedly produced by stromal cells, such as fibroblasts, in a paracrine manner, rather than by proliferating satellite cells in an autocrine manner, having a trophic effect on myoblasts *in vivo* (Groux-Muscatelli, Bassaglia, Barritault, Caruelle, & Gautron, 1990). Heparan sulfate proteoglycans in the cell membrane are required for the binding of FGF2 to their receptors, and heparan sulfate blocking reportedly causes myoblast differentiation (Rapraeger, Krufka, & Olwin, 1991). Out of different culture conditions with growth hormone, insulin, testosterone, or FGF supplementation, FGF treatment increases bovine myoblast growth rate through inhibiting myogenic differentiation, whereas the other factors induce no significant effect (Gospodarowicz et al., 1976). Another comparative study showed that among various growth factors (FGF1, FGF2, PDGF-AA, PDGF-BB, IGF1, IGF2, EGF, and LIF), FGF2 treatment stimulated the growth of mouse myoblasts by more than 25% (Rando & Blau, 1994). Once withdrawn from *in vitro* cultures, the spontaneous differentiation of myoblasts significantly increases (Shahini et al., 2018).

3.5.2 | P38 signaling inhibitors

The P38 signaling pathway has a crucial role in the differentiation and proliferation of muscle stem cells *in vivo*. Upon muscle injury, p38 pathway activation induces satellite cell self-renewal and differentiation through exiting quiescence, whereas a subset of satellite cells remain quiescent by suppressing the p38 pathway (Troy et al., 2012). The genetic ablation of p38 in satellite cells led to growth defects due to a delayed satellite cell differentiation during postnatal development (Brien, Pugazhendhi, Woodhouse, Oxley, & Pell, 2013). Similar to *in vivo* results, treating *in vitro*-cultured bovine satellite cells with the p38 inhibitor SB203580 prevents PAX7 downregulation and enables extended maintenance of their differentiation capacity (Ding et al., 2018). When transplanted, *in vitro*–cultured SB203580-treated satellite cells show a higher frequency of chimeric muscle generation and engraftment in the sublaminar niche compared with untreated cells (Charville et al., 2015). Furthermore, it has been proved that p38 signaling is involved in aging-related muscle tissue degeneration. Upon aging, the p38 pathway gets upregulated in mice, thereby decreasing the self-renewal capacity of satellite cells, whereas cell signaling inhibition with small molecules such as SB203580 and BIRB 796 recovers satellite cells from the age-related defects (Bernet et al., 2014). Repression of the p38 pathway also improves the aged satellite cell engraftment through restoring their myogenic potential when transplanted (Cosgrove et al., 2014).

3.5.3 | Insulin and IGFs

Insulin is a hormone commonly used for *in vitro* stem cell cultures, which promotes stem cell survival and self-renewal (Godoy-Parejo, Deng, Liu, & Chen, 2019). IGFs, which belong to the insulin superfamily, also have roles in stemness maintenance *in vitro* (Teng, Jeng, & Shyu, 2018). It has been observed that increased IGF-1 levels in satellite cells lead to their rapid expansion during skeletal muscle regeneration, indicating their trophic role in muscle stem cells *in vivo* (Jennische, Skottner, & Hansson, 1987). Likewise, IGF-2 increases cultured myoblast proliferation and reduces their differentiation (Florini et al., 1984). Supplementation of insulin instead of serum maintains the differentiation ability of myoblasts *in vitro*, although they lose their myogenic potential in the absence of FBS (Mandel & Pearson, 1974). Dodson et al. demonstrated that the treatment of IGF-1 (known as somatomedin), IGF-2 (known as MSA, multiplication-stimulation activity), and insulin enhances satellite cell growth, and insulin supplementation, along with IGF-2 but not with IGF-1, has an additive effect on their self-renewal potential (Dodson et al., 1985). Similar to the p38 signaling inhibition, IGF-1 also restores the decreased proliferative potential of the aged satellite cells isolated from 30-month-old mice (Chakravarthy et al., 2000).

3.5.4 | Wnt signaling pathway

The Wnt signaling pathway has a crucial role in stem cell proliferation and tissue regeneration. Grand et al. found that the quiescent satellite cells highly express Wnt7a and its receptor Fzd7 during muscle regeneration (Le Grand, Jones, Seale, Scime, & Rudnicki, 2009). Wnt7a increases the number of satellite cells by promoting symmetric cell division but does not affect myoblast differentiation and proliferation. In addition, Wnt signaling can be activated by ECM protein binding, such as fibronectin, or HIF upregulation, thereby suppressing satellite cell differentiation (Bentzinger et al., 2013; Majmundar et al., 2015). The Wnt signaling pathway reportedly comprises of canonical and noncanonical pathways (Rao & Kuhl, 2010). In muscle stem cells, both pathways are coordinately involved in the regulation of proliferation and differentiation. The treatment of the canonical Wnt pathway inhibitor IWR1-endo causes a decreased myoblast proliferation activity and inhibits myoblast fusion during myogenic differentiation (Suzuki, Pelikan, & Iwata, 2015). Fieac and Zammit found that these Wnt pathways are finely regulated by Axin1 and Axin2, known suppressors of the canonical pathway (Figeac & Zammit, 2015). Axin1 and Axin2 are expressed in proliferating satellite cells and differentiating cells, respectively, and the lack of Axin 1 suppresses proliferation and induces myogenic differentiation. Furthermore, Sulf1a, a known enhancer of the canonical pathway, is reportedly expressed in myoblasts, promoting cell growth through the regulation of the noncanonical, Wnt6-activated pathway (Hitchins, Fletcher, Allen, & Dhoot, 2013).

3.5.5 | Dexamethasone

Dexamethasone is a synthetic glucocorticoid, playing a role in anti-inflammatory and immunosuppressive processes. Glucocorticoids reportedly influence the proliferation of various cell types through catabolism regulation. In muscle stem cells *in vitro*, dexamethasone decreases myoblast doubling time, leading to their increased proliferative ability (Guerriero & Florini, 1980). Dexamethasone also amplifies the effect of IGF-1 and IGF-2 on satellite cell growth (Dodson et al., 1985). Moreover, dexamethasone treatment also improves the myogenic differentiation of satellite cells characterized by advanced sarcomere formation and enhanced contraction in the resulting myotubes (Syverud, VanDusen, & Larkin, 2016).

3.5.6 | Transforming growth factor-beta family

The transforming growth factor-beta (TGF-β) family includes various growth factors such as TGF-β, growth and differentiation factors (GDFs), and bone morphogenetic proteins (BMPs), which play pivotal roles in tissue development and immune response. The TGF-β treatment prevents the myogenic differentiation of *in vitro*–cultured myoblasts, whereas their suppressed differentiation ability could be recovered upon the removal of TGF-β (Massague, Cheifetz, Endo, & Nadal-Ginard, 1986). GDF8, known as myostatin, represses myogenic differentiation, whereas its inhibitors, dorsomorphin and LDN-193189, restore myogenesis in GDF8-treated myoblasts (Horbelt et al., 2015).

3.5.7 | Interleukins and inflammatory cytokines

Upon muscle injury, infiltrated immune cells increased the secretion of proinflammatory cytokines such as interleukins (ILs), tumor necrosis factors (TNFs), and interferons (IFNs) in the damaged area. Based on this, Fu et al. attempted the long-term *in vitro* muscle stem cell culturing through an inflammatory cytokine treatment using T-cell–secreted IL-1α, IL-13, TNF-α, and IFN-γ (Fu et al., 2015). The combination of these four cytokines allowed the *in vitro* maintenance of muscle stem cells in an undifferentiated state over 20 passages. Moreover, when engrafted, these cells were capable of repairing injured muscles *in vivo*. IL-1β expression showed a 20-fold increase 5 days after the injury, and the IL-1β treatment facilitated the proliferation of *in vitro*–cultured myoblasts through the upregulation of the NF-κB pathway (Otis et al., 2014). IL-6 also plays a pivotal role in muscular hypertrophy. It has been proved that IL-6 promotes myoblast proliferation and migration through the Stat3 signaling pathway and that a growth defect could occur in the absence of IL-6 (Serrano, Baeza-Raja, Perdiguero, Jardi, & Munoz-Canoves, 2008).

3.5.8 | Other factors

The effect of paracrine factors, secreted by mesenchymal cells, on muscle stem cells has also been investigated as mesenchymal cells are reportedly involved in muscle repair. Hepatocyte growth factor (HGF) is one of the crucial factors for the activation of quiescent satellite cells *in vivo* upon muscle injury (Miller, Thaloor, Matteson, &

Pavlath, 2000). They are released from the damaged muscle and stimulate satellite cell proliferation in the early stage of muscle regeneration (Tatsumi, Anderson, Nevoret, Halevy, & Allen, 1998). HGF induces quiescent satellite cell transition toward the proliferation state and facilitates *in vitro* proliferation in a dose-dependent manner (Allen, Sheehan, Taylor, Kendall, & Rice, 1995). Moreover, *in vitro*–cultured satellite cells express HGF in an autocrine manner (Sheehan et al., 2000). It was proved that sphingosine 1-phosphate (S1P), a mesenchymal cell–secreted lipid, promotes myoblast proliferation and that mesenchymal cells secrete vascular endothelial growth factor (VEGF), stimulating S1P release from myoblasts themselves (Sassoli et al., 2014). The supplementation of chicken embryo extract, as a mixture of growth factors, triggers the quiescent satellite cells to enter the cell cycle and has a mitogenic effect on muscle stem cell cultures (Bischoff, 1986). In addition, it was found that myoglobin, one of the heme proteins exclusively expressed in muscle, activated the proliferation and metabolism of *in vitro*–cultured satellite cells (Simsa et al., 2019).

Taken together, various cell signaling molecules including cytokines, hormones, and signaling inhibitors are crucially involved in the stemness and aging of muscle stem cells. It would be pivotal to find the signaling molecule combination for improving the yield of cultured meat production.

3.6 | Additional nutrients and supplements

In addition to the above-mentioned media components, various nutrients and supplements such as nonessential and essential amino acids, L-glutamine, antioxidants (e.g., 2-mercaptoethanol, and glutathione), and buffering agents have also been widely applied for the *in vitro* cultures of various stem cells. In order to further optimize the *in vitro* culturing of muscle stem cells, the use of the aforementioned components should also be carefully investigated (Table 3).

3.7 | Antibiotics and antimycotics

During *in vitro* animal cell culturing, contamination by bacteria, fungi, and yeast occurs frequently. To date, several antibiotic and antimycotic compounds, including penicillin, streptomycin, amphotericin b, and gentamicin, have been generally used to prevent such contamination (Table 3). However, the antibiotics mentioned above should be avoided during cultured meat production due to their side effects upon inhalation. Reportedly, the long-term exposure of antibiotic residues in foods, even if they are trace amounts, could cause health problems including hypersensitivity reaction and reproductive disorder, and increase emerging antibiotic-resistant pathogenic microorganisms in humans (Ngangom, Tamunjoh, & Boyom, 2019). To produce safe food products, contamination should be carefully monitored in order to obtain clean muscle samples through the use of Mycoplasma detection kits and the discovery of natural antibiotics.

4 | UPSCALED MUSCLE STEM CELL CULTURING

As muscle stem cells, including satellite cells or myoblasts, are adherent cells that require a so-called ECM as an adherent surface for their survival and proliferation *in vivo*, they have been usually cultured on flat culture dishes coated with a hydrogel *in vitro*. However, it has been proven that this planar cell culture has limitations such as a low surface-to-volume ratio, or difficulties in controlling pH, gas, and metabolic concentrations (Bodiou, Moutsatsou, & Post, 2020). Therefore, these obstacles hampered the culture capacity scale-up using the 2-dimensional (2D) culture method (Rowley, Abraham, Campbell, Brandwein, & Oh, 2012). For an upscaled muscle stem cell production, a more efficient culture system is required, providing a more space-, time-, resource-, and labor-efficient solution. Bioreactors have been suggested to solve these problems, as they are commonly employed for mass culture and enable the maintenance of high-density cell cultures using microcarriers and cell aggregates (Kempf, Andree, & Zweigerdt, 2016; Moritz, Verbruggen, & Post, 2015; Panchalingam, Jung, Rosenberg, & Behie, 2015; Zhang et al., 2020). Although the cell aggregate method, known as a myosphere culture, enables the production of higher-density cell cultures and the recapitulation of *in vivo* cellular niches, it reportedly has a more crucial role in supporting the myogenic potential rather than improving the muscle stem cell yield (Aguanno et al., 2019; Bodiou et al., 2020; Hosoyama, Meyer, Krakora, & Suzuki, 2013; Wei et al., 2011; Westerman, Penvose, Yang, Allen, & Vacanti, 2010). Therefore, to date, the microcarrier-based *in vitro* expansion seems to be more suitable for upscaled muscle stem cell cultures.

Microcarriers, such as ECM protein–coated small polystyrene beads (100 to 200 μm in diameter), provide an attachment surface and growth platform for adherent cells (Moritz et al., 2015; Panchalingam et al., 2015). Generally, such adherent cells grown in bioreactors, attached to floating microcarriers. Various microcarriers are commercially available for the culture of different stem cell types (Rafiq, Coopman, & Hewitt, 2013). In order

to select the microcarriers for the culture of muscle stem cells, it is important to consider several issues, including their edibility and the nature of the coated substrates. As mentioned above, substrates influence muscle stem cell physiology, the materials used for microcarrier coating are thus also important. Gelatin, collagen, fibrin, and Matrigel have been generally used for adherent cell cultures, as well as edible materials, such as proteins, polysaccharides, native ECM, and decellularized plants, which could all be used for microcarrier coating (Ben-Arye & Levenberg, 2019; Campuzano & Pelling, 2019). Moreover, the use of edible and biodegradable materials would save the effort of separating muscle stem cells from microcarriers prior to myogenic differentiation. For this reason, cross-linked pectins, such as a pectin-thiopropionylamide (PTP), nonmammalian biopolymers, and RGD-containing polypeptides, such as a tiolated cardosin A, could be used as a microcarrier source (Enrione et al., 2017; Zhang et al., 2020). Indeed, it remains an ongoing endeavor to optimize upscaled muscle stem cell production using microcarriers. When cultured using various microcarriers, including DE-53 cylinders, glass bead, and Cytodex3 in a stationary and stirred bioreactor, C2C12 myoblasts on Cytodex3 showed significantly higher growth and myotube formation compared with the other solutions (Bardouille, Lehmann, Heimann, & Jockusch, 2001). Bovine myoblast could be expanded using Syntehmax, Cellbind, and Cytodex1 in spinner flasks (Verbruggen, Luining, van Essen, & Post, 2018).

The bioreactor is a device to culture microcarrier-attached cells or cell aggregates maintaining the optimized conditions through regulating the conditions of their *in vitro* environment, such as pH, temperature, gas, and metabolite or nutrition concentrations. Various factors including cell density, bioreactor volume, inoculation efficiency, media requirements, cleaning scaffold source, passaging, inoculation, and cell removal methods should be considered for the mass production of muscle stem cells using bioreactors (Allan, De Bank, & Ellis, 2019). Several studies have employed the bioreactors for an upscaled muscle stem cell production as described in Table 4. Spinner flasks (Stirred tank bioreactors) are the most commonly applied for muscle stem cell cultures. Although these agitated reactors provide a homogeneous environment, their impeller system also gives rise to a cell shear stress, which is one of the major drawbacks for upscaled muscle stem cell production (Moritz et al., 2015). Indeed, although C2C12 myoblasts have similar physiological features, including the surface adherence, differentiation ability, and marker gene expression, on Cytodex3 beads in stationary and stirred flask compared with the conventional 2D culture, other cell lines such as M12 and M2.7-MDX show poor differentiation ability, potentially caused by shear stress (Bardouille et al., 2001). When rat myoblasts are cultured on Matrigel-coated Cytodex3 in High-Aspect-Ratio-Vessel (HARV) bioreactors, although they reportedly exert lower shear stress on cells than other bioreactors, cell proliferation is also reduced compared with 120-hr 2D flask cultures (Molnar, Schroedl, Gonda, & Hartzell, 1997). To date, for reducing the side effects, various types of bioreactors such as the fluidized bed bioreactor, hollow-fiber bioreactors, and packed bed bioreactors have been developed and could also potentially be applied for muscle stem cell cultures (Allan et al., 2019). Accordingly, finding and developing an optimal bioreactor and scaffolds for upscaled muscle stem cell production are required to industrialize the cultured meat.

5 | MUSCLE DIFFERENTIATION AND MATURATION

The muscle differentiation begins with the activation of quiescent satellite cells *in vivo*. Muscle injury causes the transition of satellite cells from a quiescent to a proliferating, so-called myoblast, state. Through proliferation, a sufficient number of myoblasts could be obtained for muscle regeneration, and a part of the myoblasts returns into quiescent satellite cell state to reserve the satellite cell population. Then, the myoblasts exit a cell cycle and undergo cell-to-cell fusion with each other, and in turn, develop into myotubes (Schmidt, Schuler, Huttner, von Eyss, & von Maltzahn, 2019). In the case of *in vitro* cultures, as the proliferation and differentiation are mutually exclusive events, the muscle stem cell–derived muscle differentiation generally occurs through the removal of growth factors or treatment by differentiation-stimulatory factors. Most of all, the starvation of serum supplements has been generally applied for the myogenic differentiation of muscle stem cells *in vitro* (Ostrovidov et al., 2014). Although the serum containing various growth factors supports proliferation, its withdrawal leads to the cell-cycle arrest, thereby inducing myogenic differentiation in the muscle stem cells *in vitro* (Doumit & Merkel, 1992; Zhang et al., 1999). Otherwise, the supplement of stimulatory factors such as nitric oxide (NO) and arachidonic acid results in the differentiation of muscle stem cells. It has been reported that NO treatment stimulates the production of follistatin through cGMP signaling, causing myoblast fusion both *in vitro* and *in vivo* (Pisconti et al., 2006). The fused nascent myotubes are maturated through growing and obtaining their contractile function, which is achieved by several anabolic growth factors including IGFs and androgen. The growth and maturation of muscles are one of the most important anabolic events in the body (McCarthy & Esser, 2010). IGF-1 plays a pivotal role in the growth of muscle as well as

TABLE 4 Summary of bioreactors and scaffold for the upscaled muscle stem cell culture

Species/cell lines	Bioreactor	Scaffold	Scale	References
Rat	HARV bioreactor	Microcarriers (Cytodex 3 and Biosilon) coated with Matrigel	10 mL	Molnar et al. (1997)
C2C12	Spinner flask	Microcarriers (SoloHill Labs glass coated polymer)	1350/1600 mL	Breese and Admassu (1999)
C2C12	Spinner and SuperSpinner flask	DEAE cellulose (DE-53), cellulose (Asahi microcarrier), glass (Sigma), plastic (Sigma), polyester (Fibra-Cel), collagen-coated microcarrier beads (Cytodex 3).	200, 600 mL for the spinner flask and SuperSpinner flask, respectively	Bardouille et al. (2001)
C2C12	Hollow-fiber spinning bioreactor	PLLA hollow fiber	Not specified	Bettahalli, Steg, Wessling, and Stamatialis (2011)
C2C12	Perfusion bioreactor	Polypropylene hollow-fiber, polysulfone hollow fiber, and polyethersulfone hollow fiber	Not specified	Bettahalli et al. (2011)
C2C12	Hollow-fiber bioreactor	Cellulose triacetate hollow fibers Scaffold=collagen	1 mL	Yamamoto et al. (2012)
C2C12	Hollow-fiber bioreactor	Polysulfone Hollow fibers with C2C12 spheroid of diameter 300 μm	Not specified	Baba and Sankai (2017)
C2C12	Spinner flask	CultiSpher G microcarrer and Cellnest microcarrier	20 mL	Confalonieri, La Marca, van Dongen, Walles, and Ehlicke (2017)
Cattle	Spinner flask	Microcarriers (Cytodex 1, Synthemax II and CellBIND)	95/250 mL	Verbruggen et al. (2018)
Human	Spinner flask	Cytodex 1 and Cytodex 3	40/100 mL and 500 mL	Bardouille et al. (2001)
Human	Spinner flask	Cytodex 3	100 mL	Rozwadowska et al. (2016)

Abbreviations: HARV, high-aspect-ratio vessel; PLLA, poly(lactic acid).

the proliferation of muscle stem cells. IGF-1 is transiently expressed following the myogenic differentiation and promotes the myofiber hypertrophy in an autocrine fashion (Tollefsen, Lajara, McCusker, Clemmons, & Rotwein, 1989; Vandenburgh, Karlisch, Shansky, & Feldstein, 1991). Recently, it has been revealed that the androgens including testosterone and 5-α-dihydrotestosterone increase the muscle mass via the IGF-1 and follistatin signaling pathways (Seo, Kim, & Kong, 2019). Also, the treatment of arachidonic acid as a precursor of prostaglandins, such as PGE_2, $PGF_{2\alpha}$, and PGI2, stimulates the proliferation of myoblasts and the *in vitro* growth of myotubes (Leng & Jiang, 2019).

However, it remains challenging to recapitulate the matured muscle tissues using the muscle stem cells *in vitro*. Indeed, the therapeutic muscle regeneration technique could be one of the potential approaches to produce muscle bundles *in vitro* (Specht, Welch, Clayton, & Lagally, 2018). The *in vitro* muscle regeneration techniques include the applications of biomaterials, mechanical and electrical stimulations, and coculturing with the interstitial cells. ECM proteins also play a pivotal role in the growth and survival of muscle fibers as well as in maintaining the stemness of muscle stem cells (Hinds, Bian, Dennis, & Bursac, 2011). The natural ECM proteins, including collagen, fibrin, gelatin, hyaluronic acid, and chitosan, as well as various synthetic polymers have been applied (the details are well reviewed in Qazi, Mooney, Pumberger, Geissler, & Duda, 2015). The surface patterning and topography of the substrates improve the myogenic fusion through supporting the parallel alignment of myoblasts by the spatial restriction (Lam, Huang, Birla, & Takayama, 2009). Besides, the structural properties of 2D and 3D scaffolds influence the organization of muscle fibers (the details are well reviewed in Ostrovidov et al., 2014). Recent reports have also shown that bioartificial muscle (BAM) could be fabricated with hydrogels using three-dimensional (3D) culture or bioprinting. Upon physical stimulation, the fibrin hydrogel–embedded C2C12 mouse myoblast cell line could form mature myotubes in terms of diameter and length (Heher et al., 2015). Moreover, in contrast to 2D cultures, myoblast-derived human myotubes developed sarcomeres and were capable of spontaneous contractions when cultured in a scaffold made of collagen and Matrigel (Shima, Morimoto, Sweeney, & Takeuchi, 2018). When bioprinting a mixture of C2C12 and PEG-fibrinogen into a fiber-like structure, it shows enhanced myotube formation, having a unidirectional alignment (Costantini et al., 2017). In addition, multicellular patterning could be achieved if scaffold construction would be performed through the simultaneous use of two different cell types. Furthermore, instead of natural compounds, synthetic materials have also been applied to creating artificial muscle tissues. The attractive part of this approach is that we can design ourselves the physical features of the materials such as conductivity, degradability, and ductility, according to the experimental purposes (Riboldi, Sampaolesi, Neuenschwander, Cossu, & Mantero, 2005). Xie et al. attempted to culture the myogenic precursors with a hyperbranched ductile polylactide copolymerized with aniline tetramer (HPLAAT), which was electroactive, ductile, and degradable (Xie et al., 2015). On the HPLAAT substrate, C2C12 cells could be stably maintained, and their myogenic potential was significantly improved as demonstrated by their muscle maturation index.

In the body, the mechanical and electrical stimulations by the exercise and motor neurons play a crucial role in the growth and maturation of muscle fibers (Michael, 2000; Ross, Duxson, & Harris, 1987). For muscle stem cells *in vitro*, the mechanical stretching enhances the myogenic differentiation and maturation along with the increase of diameter (Powell, Smiley, Mills, & Vandenburgh, 2002), and the improvement of contractile functions (Moon du, Christ, Stitzel, Atala, & Yoo, 2008) in the differentiated myofiber. Likewise, electrical stimulation reportedly improves the myogenic differentiation of muscle stem cells and the contractile force thereof (Ito et al., 2014; Langelaan et al., 2011). However, it is proven hard to optimize the electrical conditions such as amplitude and frequency for applying to the tissue engineering (Balint, Cassidy, & Cartmell, 2013). Finally, the coculture with the interstitial cells, including fibroblasts, endothelial cells, and neurons, regulates the *in vitro* myogenesis of muscle stem cells. *In vivo* skeletal muscle composes of connective tissue, vascular tissue, and nervous tissue. They are involved in the growth and maturation of muscle fibers via the cell-to-cell interactions and affect the flavor of the meat as well. When cocultured with fibroblasts and neurons, the differentiated muscle fibers from myoblasts showed higher maturity and contractility compared with the control group (Cooper et al., 2004). Moreover, the coculture of myoblasts and HUVEC by layers resulted in the capillary-like structures (Sasagawa et al., 2010). Accordingly, because the muscle differentiation and maturation are crucial steps in realizing muscle structure from cells, further studies are required to optimize the efficient production process and more desirable texture of cultured meat.

6 | CONCLUSION AND FUTURE PERSPECTIVES

Cultured meat, as a field of cellular agriculture, is considered as a novel alternative protein source. Also, the

material could be applied as a functional food via customizing nutrients and bioactive compounds (Young et al., 2013). Along with conventional meat and meat analogues, including edible insects and plant-based products, it will become a useful material of alternative protein source in the future (Lee et al., 2020). However, several hurdles remain to be solved for reaching this goal. First of all, to create animal-free protein sources, animal-sourced culture components such as serum and ECMs have to be replaced with animal-free components. No or significantly less animal sacrifice should be made to produce cultured meat. Furthermore, several previously used culture supplements may not be suitable in muscle stem cell cultures for cultured meat production due to their toxicity upon ingestion. The deep understanding of muscle stem cell physiology should help the replacement of such components with sustainable and edible compounds. Creating flavor and texture for the cultured muscle tissue–derived artificial meat, mimicking conventional meat in composition is another ongoing endeavor for tissue engineering. Finally, it is essential to lower the price of production, through the optimization of the production process such as mass culture and media recycling, so that consumers can easily access cultured meat. Besides the technical issues, the social and ethical issues including consumer acceptance and national food safety regulations and international harmonization are important for this novel food material. The constant endeavors of scientists and relevant organizations are required to pave the way for the era of cellular agriculture. If accompanied with ongoing endeavors to achieve this goal, they will be served on the center of the plate along with conventional meat in the near future.

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CONFLICTS OF INTEREST

The authors declare no potential conflict of interest relevant to this article.

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