Investigating the Potential for Myogenic Differentiation in Bovine Embryonic Stem Cells

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To Palestine

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Abstract

In vitro muscle differentiation has had a renewed interest in the context of cultivated meat. One potential cell source for this product is embryonic stem cells (ESCs). Though myogenic differentiation has been studied in mouse and human ESCs, research on livestock species, namely bovine, is still limited. In this study we sought to gain a wider understanding of bovine embryonic myogenesis through genetic analysis of early embryos from gestation day 40 to 65 as well as adult muscle stem cells. With said understanding we gained important context of differentiation in vitro. In that effort, we adapted previous differentiation protocols in human and mice based on WNT and BMP signaling modulation to bovine ESC (bESC) and obtained cells in early paraxial mesodermal state under a serum free myogenic differentiation protocol. This protocol was then improved upon by implementing a more defined cell substrate and a pre-differentiation step. Lastly bESCs were differentiated under spheroid formation identifying that spheroid culture could be used in differentiation but at a restricted capacity. Our findings were limited to expression of the early paraxial mesoderm genes *TBX6* and *PAX3* while other myogenic transcripts remained undetectable. This study provides novel information about the specific myogenic transcripts of bovine myogenic differentiation during the critical period of somitogenesis and beyond. Our in vitro studies highlight the many challenges that still need to be overcome for efficient differentiation of bESCs into muscle in vitro. These findings should aid in future approaches to develop methods for the myogenic differentiation of bovine ESCs.

Chapter 1 Literature Review

Introduction

Muscle is an invaluable tissue that has a highly ordered structure containing multinucleated myotubes aligned into fibers and fascicles. Skeletal muscle allows for structure, movement and function in one's body and myogenic disease, from genetics and aging, can be extremely detrimental to quality of life. Investigating this problem spawned much of the current research on muscle culture and myogenic gene therapy. Beyond clinical settings muscle is one of the main sources of animal protein for consumption, making the culture of muscle cells central for the production of cultivated, or lab-cultured, meat. Recently there has been an increasing effort in developing methods for the production of muscle in vitro exploring the many avenues of cell sourcing and approaches to differentiation and culture.

In pursuing muscle production there are a small collection of cell types one can use. One of the fastest approaches is the utilization of adult muscle stem cells commonly referred to as satellite cells. While these cells are devoted to muscle differentiation, they are limited in their proliferative capacity (Hayflick and Moorhead, 1961) requiring them to be genetically modified for extended use (Stout et al., 2023). Another source of cells for myogenic differentiation is pluripotent stem cells (PSCs) which hold the dynamic potential to differentiate into any cell type in the body. Within clinical settings, induced pluripotent stem cells (iPSCs), produced from a patient's somatic cells, could also be used for regenerative medicine and potentially for animal protein production. However, the previous concern of genetic modifications needed to obtain iPSCs still stands (Jara et al., 2023).

The solution to this problem could be found in embryonic stem cells (ESCs). Unlike satellite cells, ESCs can proliferate indefinitely and create a reliable cell bank for future use. ESCs hold the same pluripotent and proliferative capacity of iPSCs without the use of genetic modification and can be differentiated with growth factors into all cell types found in muscle. Even with these advantages, myogenic differentiation of ESCs, especially in large animal models, remains limited and merits further development. Detailed knowledge about the processes and factors involved in fetal myogenic differentiation in large animals would be extremely helpful to inform the development of this system in vitro. Here we outline the steps of myogenic development in vivo as well as the current state of in vitro differentiation in bovine ESCs (bESC) given the importance of cattle for meat production.

Developmental Biology and Myogenesis

Early Gastrulation Embryo

In recreating myogenesis in vitro the differentiation of ESCs should follow the same developmental steps found in vivo. To properly understand that one must begin at the early embryo. During the first few days of development, the first complex embryonic structure to form is the blastocyst. The blastocyst consists of two cell types being the trophoblast, which go on to form the placenta, and the pluripotent inner cell mass (ICM) which will differentiate into every cell type within the animal (Niakan et al., 2012). The ICM further matures dividing into the hypoblast and the epiblast (Nakamura et al., 2016). The origin of muscle development begins at the next embryonic stage of gastrulation. Gastrulation is brought on by the appearance of the primitive streak, a crease made of cells folding inward in an epithelial to mesenchymal transition,

populating the mesodermal layer and eventually resulting in the three gene layers of ectoderm, mesoderm and endoderm (Kobayashi et al., 2017; Downs, 2009). The streak proceeds along a posterior to anterior path following a gradient of fibroblast growth factor (FGF) mediated expression of the Wnt glycoproteins as seen in mouse (Komiya and Habas, 2008; Ciruna and Rossant, 2001; Naiche et al., 2011) as well as transforming growth factor beta (TGF-Beta) (Gadue et al., 2006). Perpendicular to this posterior-anterior gradient is the bone morphogenic protein (BMP) gradient involved in medial-distal patterning of the mesoderm. BMP signaling at this stage is vital for further mesoderm differentiation as shown in knockout models (Winnier et al., 1995). At the cranial end of the primitive streak, in an indent known as the primitive node, a tube of cells migrates down between the germ layers forming the notochord. This structure is vital for embryo patterning, as reviewed in (Ramesh et al., 2017), and will influence myogenic differentiation later on. Returning to the ectoderm, sections of cells commit to become the neural crest which fold into themselves forming the neural tube. While the notochord is a transient structure the neural tube develops into the spinal cord (Delile et al., 2019). Flanking the neural tube is the developing mesoderm brought about by Wnt3a activation of *Brachyury* (*T*), localized in the notochord (Yamaguchi et al., 1999; Wilkinson et al., 1990; Wehn et al., 2020; Zhu et al., 2016). After its inception, and under the stimulation of the BMP gradient, the mesoderm splits into the 3 sections: the lateral plate mesoderm, the origin of many internal organs, smooth muscle, connective and adipose tissue; the intermediate mesoderm, which will eventually give rise to kidneys and gonads; and most prominently the paraxial mesoderm, which will differentiate into skeletal muscle (James and Schultheiss, 2003).

Somites and Paraxial Mesoderm

When discussing the molecular details and events leading to somite development, most information came from studies using mouse models. Therefore, the mouse model of development will be described here unless specified when information about other species is available. The paraxial mesoderm arises from the nascent mesoderm flanking the notochord and neural tube and matures into clusters of cells called somites. Before the formation of the somites, the pre-somitic mesoderm can be divided into the anterior and posterior sections. While *T* is responsible for the initiation of the mesoderm its expression fades in the posterior pre-somitic mesoderm (pPSM), replaced with another T-box gene, *Tbx6* (Yamaguchi et al., 1999). Activated by Wnt3a (Dunty et al., 2008), *Tbx6*-positive cells inhabit most of the pPSM and are vital for paraxial mesoderm and somite development, with *Tbx6*-knockout models forming neural tubes in place of somites (Chapman and Papaioannou, 1998; Wehn et al., 2020). Along with Wnt3a, *Tbx6* targets the gene *Msgn1* which is responsible for cell migration and aids in paraxial mesoderm commitment, deeming it another vital gene for the pPSM (Nowotschin et al., 2012; Bessho et al., 2003; Manning and Kimelman, 2015; Wittler et al., 2007).

Posterior PSM cells transition to the anterior pre-somitic mesoderm (aPSM) at an intersection of transcription factor gradients known as the *determination front* which consists of the transcriptional cross roads of retinoic acid from the anterior juxtaposed by WNT and FGF8 from the posterior (Dunty et al., 2008; Kennedy et al., 2009; Olivera-Martinez and Storey, 2007). The determination front is maintained from the mutual negative regulation between retinoic acid and FGF8 keeping both concentrations at the determination front low (Cunningham et al., 2015; Del Corral et al., 2003; Kumar and Duester, 2014). In addition to repressing FGF8 to establish the

determination front, retinoic acid also has been shown to be vital in establishing bilateral symmetry of somite formation (Vermot et al., 2005) and is necessary for further myogenic maturation (Hamade et al., 2006). When posterior pre-somitic cells enter this growth factor focal point, they become committed to forming somites and proceed to travel up the now anterior pre-somitic mesoderm before generating the next set of somites in their growing rows. This is done by FGF8 permitting the expression of Wnt3a which begins a signaling cascade, cyclically regulated by *Mesp2* and *Ripply2* (Dunty et al., 2008) as well as *Hes7* and *Lfng* (Bessho et al., 2003). Wnt3a and *Tbx6* unify to activate *Notch* and then *Hairy1/2* which cyclically segments the cells committed to become somites as they migrate to the anterior end (Bessho et al., 2003; Evrard et al., 1998; Jouve et al., 2002; Naiche et al., 2011). *Meox1* also works to help regulate *Notch* signaling as somites form (Mankoo et al., 2003; Reijntjes et al., 2007). As somites populate the anterior end, the determination front is pushed further down the posterior end in tandem with the posterior elongation permitted by the high WNT and FGF gradient as seen in mouse and chicken (Oginuma et al., 2017). These newly formed somites express the principal transcription factor paired box 3 (*Pax3*) which will grant further maturation of the somite into myogenic progenitors (Buckingham and Relaix, 2007). *Pax3* allows for the expression of myogenic factor 5 (*Myf5*), which is in turn positively regulated by *Myf5* itself together with the transcription factors *SIX4* and *TEAD2* to enhance *PAX3* expression (Magli et al., 2019; Lagha et al., 2008; Tapscott, 2005). This expression cascade will also upregulate the paralogue of *Pax3* paired box 7 (*Pax7*) (Magli et al., 2019), which is an important transcription factor for myogenic progenitors and myogenesis, being able to induce myogenesis in vitro (Darabi et al., 2012, 2008). *Pax7* is specifically required for the development of the muscle stem cells called satellite cell (Lagha et

al., 2010). *Pax3* and *Myf5* will go on to activate myogenic differentiation 1 gene (*Myod*) and begin myogenesis (Lagha et al., 2010; Olson et al., 1995).

Somite Maturation, Sclerotome and Dermomyotome

As somites mature, they separate into two distinct sections: the sclerotome and dermomyotome. The former is located closer to the notochord on the ventral side with the latter being closer to the dorsal side. Both sections receive signals from neighboring structures that aid in their development. The sclerotome, responsible for axial skeleton, receives Sonic Hedgehog (*Shh*) signaling from the notochord activating *PAX1/9* and inhibiting expression of *PAX3* as described in mice and avian models (Goulding et al., 1994; Murtaugh et al., 1999). In the dermomyotome, responsible for skeletal muscle, *Pax3* facilitates cellular commitment of myogenesis while *Foxc1/2* positive cells coordinate non-myogenic fates (Buckingham and Rigby, 2014; Lagha et al., 2009) such as brown adipose tissue (Sanchez-Gurmaches and Guertin, 2014; Buckingham and Vincent, 2009). *Pax7* is closely expressed alongside *Pax3* in the dermomyotome sharing similar targets with *Pax3* and assisting in the commitment and survival of myogenic progenitors (Buckingham and Relaix, 2007; Goulding et al., 1994; Lagha et al., 2010, 2008). As shown in zebrafish, *PAX3* and *PAX7* expression are focused in the dermomyotome by the previous gradient of FGF8 and the newer Hedgehog signaling causing their expression to wain as myogenesis proceeds (Hammond et al., 2007). The dermomyotome is also influenced by WNTs and BMPs from the neural tube of the embryo much like its early paraxial mesoderm precursors. Across species, multiple WNTs from the adjacent neural tube shift *PAX3* expression into the medial dorsal and lateral ventral lips of the dermomyotome (Fan et al., 1997; Geetha-Loganathan

et al., 2006; Heilig et al., 2022). BMPs do the same relegating myogenic differentiation to the sides of the dermomyotome as BMP is expressed from the neural tube as well as the lateral plate mesoderm (Marcelle et al., 1997; Patterson et al., 2010).

Myotome

As development continues the cell populations at the dorsal and ventral ends of the dermomyotome begin to migrate medially and ventrally, with the assistance of hepatocyte growth factor (HGF) signaling (Myokai et al., 1995), towards the sclerotome to form a third segment of the mature somite: the myotome. These new myocytes of myotome are the future skeletal muscle cell population. The *MYF5* positive myocytes populating the new myotome arise mainly from *PAX3* precursors in most amniotes (Hutcheson et al., 2009; Lee et al., 2013; Lewandowski et al., 2020; Rupik et al., 2012) but in some cases they may also require *PAX7* like in chicken (Otto et al., 2006). Regardless of precursor expression, the myotome is the sight of an initial, or primary, myogenesis of embryonic muscle. This is achieved by activation of *MYOD* mainly from *MYF5*, and arguably *PAX7*, assisted by the regulatory gene *MEF2* (reviewed in Olson et al., 1995). Other cofactors involved in *MYF5* and *MYOD* activation include *SIX1&4*, which is a regulatory element found at all stages of myogenesis (Maire et al., 2020; Wurmser et al., 2023) and *EYA1&2* (Buckingham and Rigby, 2014; Grifone et al., 2005). As before in the dermomyotome, Wnt proteins exert influence on the myotome and aid in the process of myogenesis supporting *Myf5* and *Myod* (Chen et al., 2005; Hutcheson et al., 2009). During this time Insulin growth factor 1 (IGF-1) signaling also supports myogenesis and metabolic regulation of myoblasts (Chargé and Rudnicki, 2004; van der Velden et al., 2006). As differentiation

continues the myotomic population is also proliferating with assistance from FGF (Itoh et al., 1996).

Terminal differentiation is achieved from *Myod* targeting the gene myogenin (*Myog*) (Kablar et al., 2003). *MYF5* and *MYOD* allow for the initiation of myogenesis changing gene expression, chromatin availability and cell cycle of myoblasts (muscle cell progenitors) but require *MYOG* to fully differentiate to proper muscle (as reviewed in Singh and Dilworth, 2013). In addition to the pervious factors, *Myf6* (also known as *Mrf4*) cannot be discounted for its effect on myogenesis targeting *Myog* and like *Myog* being expressed in terminal differentiation of myotubes (Kassar-Duchossoy et al., 2004). As myoblasts become established *MYOG*, *MYOD* and *MYF6* joined by a myriad of other signals including cell surface interactions and the protein myomerger cause them to fuse together to create multinucleated myotubes (as review in Lehka and Rędowicz, 2020). These embryonic myotubes are unique compared to later fetal and adult myofibers in that they express embryonic myosin heavy chains *MYH3* and slow myosin type 1 *MYH7* and actin *ACTA1* and *ACTC1* (Chal and Pourquié, 2017).

Limb Buds

Depending on their axial position, somites are subjected to different developmental pathways based on Hox genes permitting some somites to populate limb buds (Aulehla and Pourquié, 2010; Ibarra-Soria et al., 2023). *PAX3* is an active player in this morphogenesis being required for limb bud formation (Bober et al., 1994; Buckingham and Rigby, 2014) as well as myocyte migration through upregulation of *cMet* (Epstein et al., 1996; Myokai et al., 1995). As mentioned above myocytes from the developing myotome migrate out and into the

mesenchyme of these limb buds where they will undergo their primary myogenesis (Besse et al., 2020).

Secondary Myogenesis

After these embryonic myoblasts mature into myotubes they are joined by a second set of myocytes from the dermomyotome in a secondary wave of myogenesis. This event has twofold purpose: first these new myoblasts will fuse with the primary myotubes creating a more robust and functional myofiber system; and second, a set of descending myoblasts create a precursor satellite cell population (Kassar-Duchossoy et al., 2005). While *Pax7* has mostly been redundant up until this point due to its similarity to *Pax3* (Relaix et al., 2004), its expression is a requirement for secondary myogenesis and establishment of the muscle stem cell population (Seale et al., 2000). During primary myogenesis *PAX7* is shifted to the central dermomyotome (Buckingham and Rigby, 2014); once secondary myogenesis begins *PAX7*-positive cells descends from the central dermomyotome and migrates into the primary myotome following its structure much like a scaffold, as reported in quail-chick chimeras (Gros et al., 2005). *Pax7* activates the fetal myogenic regulatory element *Nfix* (Messina et al., 2010). This results in the addition of new fetal myotubes and the fusion of *Pax7* positive cells into the previous embryonic myotubes beginning to from mature myofibers (White et al., 2010). In addition to maturing the myotome, *PAX7* progenitors also produce the satellite cell population. As seen across species, satellite cells retain *PAX7* expression and exist on the basal lamina of myotubes ready to create or regenerate muscle when damaged (Gros et al., 2005; Kassar-Duchossoy et al., 2005). Satellite cells also express *Pax3* to a certain extent which maintain their function and cell survival (Der Vartanian et al., 2019;

Montarras et al., 2013; Relaix et al., 2005). *Myf6* is also involve in satellite cell function, maintaining epidermal growth factor pathway and keeping satellite cells quiescent (Lazure et al., 2020) alongside the help of *Myf5* (Beauchamp et al., 2000).

Despite shared genetic mechanisms of myogenesis, the timelines of myogenesis between species vary. The gestation period of mice as well as chicken is close to 20 days, bovine is much longer being upwards of 280 days or approximately 9 months. In mice primary myogenesis occurs between embryonic day 10.5 to 12.5 followed by secondary myogenesis between embryonic days 14.5 to 17.5 with satellite cell niche maturing after day 18.5. For chicken, primary occurs between embryonic day 3 to 7 and secondary occurs beyond embryonic day 8 (as reviewed in Chal and Pourquié, 2017). Bovine primary myogenesis occurs between the 1st and 3 months of gestation and secondary between 2 and 7 months (as reviewed in Du et al., 2010). These variations are due to a variety of factors and should be considered when implementing differentiation in vivo.

<u>Embryonic Stem Cells</u>

Embryonic stem cells are derived from the inner cell mass of a blastocyst. With the assistance of microsurgery or other methods, the trophoblast can be detached, enriching the culture with pluripotent ICM cells (Bogliotti et al., 2018; Evans and Kaufman, 1981). Through consecutive passaging in medium designed to maintain pluripotency, the newly-established ESCs can be transferred into a feeder-free culture with the use of substrates such as vitronectin, laminin and/or fibronectin (Kinoshita et al., 2021b; Soto et al., 2021; Xu et al., 2001). ESCs

maintain their pluripotency through a set of genes most significant of which being *POU5F1*, *SOX2*, *KLF4, NANOG* and *c-MYC*.

ESCs can be found at different stages of pluripotency based on the point of their derivation and species. The first ESCs were derived from mouse ICMs and had the unique potential to form chimeric animals when injected into a blastocyst (Evans and Kaufman, 1981). For this higher pluripotency and amenability for chimeric formation mouse ESCs (mESCs) were given the term *naive*. Human ESCs (hESCs) on the other hand, while also being derived from human ICM, more resembled post-implantation mouse epiblast cells (EpiSCs) in both their morphology and molecular state (Tesar et al., 2007; Thomson et al., 1998). This earned them the term *primed* as they resembled mouse epiblast cells which are further developed than the inner cell mass and thus primed for differentiation. In keeping with this understanding some suggest that these cells may be more amenable to differentiation (Bogliotti et al., 2018; Wu et al., 2015). Both types of ESCs express the aforementioned core pluripotency factors *POU5F1* and *Sox2* but other pluripotent genes are more prominent depending on pluripotent state with *Otx2* and *Nanog* being more visible in primed and naive ESCs respectively (as reviewed in Weinberger et al., 2016). Naive and primed pluripotency states have also been established in other model systems including large animal models such as bovine, pig and sheep (Bressan et al., 2020; Jara et al., 2023). It is important to mention that pluripotency is defined as a spectrum rather than a discrete-defined state. ESCs with intermediate characteristics between naive and primed states have been named formative ESCs (Guiltinan et al., 2021; Kinoshita et al., 2021a; Weinberger et al., 2016; Wu and Izpisua Belmonte, 2015; Yu et al., 2021) as well as further extreme ESCs such as post primed state stem cells derived from pre-gastrulation embryonic disk stem cells

(Kinoshita et al., 2021b) and extended potential stem cells that have the ability to create embryonic and extra embryonic lineages (Gao et al., 2019; Yu et al., 2021; Zhao et al., 2021).

The state of pluripotency will dictate the culture conditions of ESCs due to their different signaling requirements to maintain the pluripotent state. A key factor is Wnt signaling, which is vital for naive mESC cultures (Ying et al., 2008) that needs to be repressed for primed ESC maintenance. The primed ESCs models of bovine, sheep and some porcine employ a WNT inhibition via utilization of the tankyrase inhibitor IWR-1 in their maintenance culture conditions (Bogliotti et al., 2018; Choi et al., 2019; Vilarino et al., 2020; Xiao et al., 2021). Along similar lines, the effect of FGFs is depended on pluripotency state. Specifically, FGF2 is considered a requirement for maintenance of primed ESCs but is detrimental to naive mESCs (Greber et al., 2010), enhancing and diminishing the expression of *Sox2* (Jeong et al., 2017; Mossahebi-Mohammadi et al., 2020). In naive mESC, Leukemia inhibitory factor (LIF) is mandatory for stimulation of JAK–STAT3 signaling and maintenance of pluripotency (Kinoshita et al., 2021a; Williams et al., 1988). Although LIF does not seem to be required for the maintenance of primed bESC, it has been used to form primed mESCs when combined with Activin A (Wei et al., 2021). LIF is also utilized in porcine cultures especially during cell line derivation (Choi et al., 2019). Activin A is another factor that enhances pluripotency in primed ESC across species by stimulating TGF-Beta signaling and maintaining expression of *NANOG* (James et al., 2005) often with the assistance of FGF2 (James et al., 2005; Weinberger et al., 2016). This has been shown to be consistent in the bovine and porcine model as well (Soto et al., 2021).

Myogenic Differentiation of ESC

Historically muscle differentiation has been conducted in the human and mouse model to investigate muscular degenerative diseases like muscular dystrophy (Chal et al., 2016, 2015; Shelton et al., 2014). Thus, research on the topic has mainly employed mESCs and human iPSCs to date because hESCs are harder to obtain, and because regenerative medicine would ideally take advantage of hiPSCs to maximize patients' compatibility. Induced PSCs do have similar genetic profiles to ESCs (Marei et al., 2017) and their myogenic differentiation can be very informative to ESC differentiation (Miyagoe-Suzuki and Takeda, 2017; Salani et al., 2012). Large animal models have seen some use in myogenic differentiation but with large animal ESC models still being in its infancy, myogenic differentiation is still very understudied. Regardless of animal model there are a set of signaling molecules whose positive effects on myogenic differentiation are undeniable.

Of the most prominent signals is Wnt. As mentioned previously, Wnt is one of the central signaling molecules of the paraxial mesoderm and has been shown to activate the pre-somitic mesodermal markers *Tbx6* and *Msgn1* as well as the somitic marker *Pax3* and eventually playing a role in *Myf5* and *Myod* expression (Chen et al., 2005; Hutcheson et al., 2009). WNT is activated in vitro using the small molecule CHIR99021 which inhibits the enzyme glycogen synthase kinase-3 (GSK3) allowing for stabilization of beta catenin and activation of WNT target genes (Tan et al., 2013; van der Velden et al., 2006). CHIR99021 has been employed in many myogenic differentiation protocols across species (Chal et al., 2015; Miyagoe-Suzuki and Takeda, 2017; Shelton et al., 2014; Zhu et al., 2023). Sometimes used in conjunction with WNT pathway stimulation is the inhibition of BMP through a small molecule receptor inhibitor LDN193189,

causing the inhibition of the lateral plate mesoderm fate and the encouragement the paraxial mesoderm fate (Chal et al., 2016, 2015; Kinoshita et al., 2021b).

Surprisingly retinoic acid, though acting on the opposite gradient of Wnt, was reported in the mouse model to activate Wnt, *Pax3* and lead to *Myod* expression as well as inhibiting BMP (Kennedy et al., 2009). This differentiation was also supported in hESCs (Ryan et al., 2012). Retinoic acid was detected in mESC culture differentiated to express *Pax3* suggesting that it can produce its own to aid in its differentiation (Chal et al., 2015). While retinoic acid is beneficial in bovine satellite cells (Jin et al., 2024; Kim et al., 2018) its effect in bESCs remains unclear.

TGF-Beta is active in the early mesoderm and can be used for in vitro differentiation (Loh et al., 2016). But when it comes to myogenesis, TGF-Beta has a detrimental effect (Liu et al., 2001; Mahmood et al., 2010). In turn, use of the TGF-Beta inhibitor SB-431542, often alongside BMP inhibition and/or WNT promotion, has been shown to improve myogenic differentiation in human and porcine ESCs (Wu et al., 2018; Zhu et al., 2023). However TGF-Beta also has been shown to improve myoblast fusion (Choi et al., 2016; Gu et al., 2016) but simultaneously regulate it by stopping the process (Melendez et al., 2021). Together this suggests TGF-Beta may be ideal for use in early cultures but detrimental once myogenesis begins.

As stated, growth factors play a significant role in early myogenesis. Debatably the most significant factor, FGF2's effect maintains primed ESCs as well as encourages proliferation and cell survival during in vitro differentiation (Kinoshita et al., 2021b; Shelton et al., 2014). However, FGF2 may also preserve aspects of pluripotency in primed cells and making differentiation difficult. Myogenic protocols often utilize FGF2 at midpoint in the culture after differentiation begins (Borchin et al., 2013; Kinoshita et al., 2021b; Shelton et al., 2014). IGF-1 is another growth

factor used in multiple differentiation protocols for its effect on metabolic regulation and health of muscle and satellite cells (Chakravarthy et al., 2000; Guan et al., 2022). IGF-1 has a hand in proliferation, cell fusion and activation of myogenic regulation factors (Chargé and Rudnicki, 2004; Tahimic et al., 2013). Furthermore, IGF-1 has stimulated myogenic differentiation and myotube formation in C2C12 cells by phosphorylating GSK3 similar to the effect of CHIR99021 (van der Velden et al., 2006). HGF is important for cell migration in the myotome (Brand-Saberi et al., 1996) as well as satellite cell activation and proliferation (Chargé and Rudnicki, 2004; Kolkmann et al., 2022), though higher concentrations of HGF can inhibit myogenesis (Yamada et al., 2010). HGF has been used in terminal differentiation in ESCs across model species in conjunction with IGF-1 and FGF (Caron et al., 2016; Chal et al., 2015; Kinoshita et al., 2021b; Wu et al., 2018; Zhu et al., 2023).

One common bottleneck when it comes to myogenic differentiation is the problem of heterogeneous cell populations. This problem of unwanted cell types could be very problematic for settings of cultivated meat product and even more concerning for regenerative medicine. Genetic and epigenetic analysis of *Pax3*-positive cells showed that, despite the myogenic effect of *Pax3*, these cells still had an assortment of neuro-progenitor and endothelial cells within its population (Khateb et al., 2022). A common solution to this is fluorescence-activated cell sorting (FACS). FACS has been used with great success to isolate satellite cell from mouse limb muscle (Liu et al., 2015). In humans FACS was utilized to sort myoblasts and myoblast precursors differentiated under CHIR99021 and FGF2 treatment (Borchin et al., 2013). Sorting of a *MYF5*/*PAX7* double reporter hESC cell line, allowed the detection of differentiating cells and the identification of the satellite cell surface markers CD10+/CD24– to aid in purer sorting of these

cells (Wu et al., 2018). Other protocols relied heavily on FACS to mediate myogenesis. One protocol in hESCs reported that it was possible to induce differentiation solely through cell sorting cell containing the marker CD73 in simple media containing serum (Barberi et al., 2007). Another protocol used platelet derived growth factor receptor alpha (PDGFRA+) cells which are identified as mesenchymal muscle progenitors (Hwang et al., 2017, 2013; Uezumi et al., 2014). Together this demonstrates FACS can be utilized at different time points for enhancing culture homogeneity based on differentiation protocol.

Another important aspect of culture condition is the use of serum. Original production of muscle, especially using satellite cells, used fetal calf serum and horse serum (Barberi et al., 2007; Caron et al., 2016; Chang et al., 2009, 2009; Hwang et al., 2013). Using a *PAX7* reporter line, serum was shown be close to 100% effective in activating *PAX7* with aid of CHIR in human ESCs (Wu et al., 2018). While serum may be advantageous it is highly variable and leads to batch specific problems as well as being reliant on animal products (Baker, 2016). In the same study knockout serum replacement (KSR) also had high success of *PAX7* activation especially compared to insulin, transferrin and selenium (ITS), but not as strong as serum (Wu et al., 2018). KSR is widely used in many myogenic differentiation protocols creating a more defined procedure (Chal et al., 2015; Zhu et al., 2023) and has only seen some ineffective usage (Caron et al., 2016). A paper by Messmer et al., 2022 approached serum substitution by tailor-making synthetic serum from information gained through RNAseq of serum-containing culture of satellite cells. This highlights that there are many avenues to replace serum and research on the subject continues.

Plate coatings also play a role in myogenic differentiation. Many differentiation protocols use the substrate Matrigel, which is a collection of secretions of EHS mouse sarcoma cell line

including collagen IV, laminin, proteoglycans, and other factors. Matrigel has unique properties of encouraging growth and differentiation and has been shown to be distinctly able to support organoid cultures including somite and skeletal organoids (Budjan et al., 2022; Mavrommatis et al., 2023; Veenvliet et al., 2020). Of note human pluripotent cell culture has a particular affinity to Matrigel (Chal et al., 2016; Hwang et al., 2013). Although its support of differentiation is immense Matrigel suffers from similar problems with serum being largely undefined and variable. However, Matrigel is not a requirement for myogenic differentiation as laminin and fibronectin are useful substitutes in both C2C12 and ESC models (Kinoshita et al., 2021b; Lee et al., 2015). Though it can be used in many cell types, fibronectin is unique in that it also plays a role in myoblast alignment and fusion during tissue repair (Vaz et al., 2012). Laminin also holds great importance to muscle as it is part of the satellite stem cell niche facilitating the preservation of stemness during satellite cell asymmetric division (Rayagiri et al., 2018). Gelatin is another substrate used in myogenic differentiation as it is a form of collagen (Shelton et al., 2014; Zhu et al., 2023). ESCs are also amenable to scaffolds to create large structures and also facilitate in myoblast fusion and myotube growth (Hwang et al., 2017; Zhu et al., 2023).

Spheroid Culture

Three-dimensional cell culture has shown promising leads for benefiting differentiation as it better mimics the in vivo environment. Referred to as spheroids for somatic cells or embryoid bodies (EBs) in ESC models, 3D cell culture enhances cell adhesion through preservation of their endogenous extra cellular matrix and encourage communication improving the growth of the culture (Laschke and Menger, 2017). Research of spheroids and EBs have

shown potential to model the musculoskeletal system (Griffin et al., 2022). Peculiarly the first case of skeletal myogenesis in vitro was in embryoid bodies of a mESC line conducted by Rohwedel et al., 1994. These EBs were cultured for five days, two in hanging drop and three in suspension before being seeded and creating myoblast outgrowths which fused to form myotubes. Amazingly the only growth factor was bFGF highlighting the effect EB formation has on differentiation. However, this differentiation was spontaneous, and the outgrowth population was very heterogeneous and has since been expanded to more sophisticated protocols. The results of this experiment we able to be reproduced with seeding on Matrigel and enhanced with FACS sorting (Chang et al., 2009). In a curious case EB formation and retinoic acid produced myoblast and myotubes without a functional *Pax7* gene. From this study it was discussed that some cell lines have an inclination to differentiate down the myogenic lineage which was constant with Rohwedel's findings (Czerwinska et al., 2016).

Human embryoid bodies have seen similar success to mice. In comparison of the many approaches utilizing EBs, their use is often to initiate differentiation and then terminally differentiate their outgrowths once seeded (Albini et al., 2013; Czerwinska et al., 2016; Hwang et al., 2013; Zheng et al., 2006). This can be seen in one study where EBs were used to encourage PDGFRA-positive cell populations to be sorted for myogenic differentiation (Hwang et al., 2013). However, these outgrowths may be heterogeneous which is why other protocols dissociate EBs during seeding, in turn reporting higher *PAX7*, *MYF5* and *MYOD* expression and forming engraftable myotubes (Awaya et al., 2012) consistent with C2C12 cells (Jin, 2021). In agreement with 2D culture, EB formation works under CHIR99021 and FGF2 serum-free treatment, granting the culture higher expression of *T*, *TBX6* and *MSGN1* compared to 2D differentiation (Shelton et

al., 2014). Along similar lines retinoic acid has also be used in EB differentiation obtaining *PAX3* expression and increasing myogenic progenitors in culture (Ryan et al., 2012). Other EB differentiation can be facilitated with inducible gene expression like *Pax3* (Darabi et al., 2008) and *MYOD* (Albini et al., 2013).

In addition to ESCs satellite cell differentiation and proliferation also benefit from spheroid formation with an increase in *Myod* and cadherin expression as shown in mice (Jin, 2021; Westerman et al., 2010). Differentiation of C2C12 was recapitulated in bovine satellite cells obtaining proper differentiation and increase outgrowths and fusion compared to dissociated spheroids (Johnson et al., 2024). Whereas in porcine primary muscle spheroids could function in bioreactors but it was also observed that dissociation would encouraged proliferation (Stange et al., 2022). Like other differentiation approaches there is variation between cell type and model but spheroid culture remains unique as it holds the possibility for upscaling production with the use of bioreactor (Stange et al., 2022; Wolf et al., 2022) and be bioprinted for culinary or medical applications (Huang et al., 2013; Johnson et al., 2024; Kang et al., 2021; Wolf et al., 2022).

Conclusions

Like in vivo muscle development, muscle differentiation in vitro is complex. There are a myriad of protocols attempting myogenic differentiation and its approach in just one cell type and model species leaves much to consider. With that being said, there are universal pathways to differentiation, namely WNT, retinoic acid, and BMP inhibition as well as growth factors such as FGF, IGF-1, and HGF. All of these play significant roles in myogenesis in vivo. Furthermore, cell culture in 2D or 3D conditions influence cellular proliferation and differentiation. While there is

a main consensus of gene expression in embryonic and fetal myogenesis there are variations across species (Zhu et al., 2016) and body region (Buckingham and Rigby, 2014) which should be considered in differentiation. Likewise, there is also variation within the ESC model with a range of pluripotency with different “preferences” for myogenic differentiation (Czerwinska et al., 2016). Even with the advancements in myogenic differentiation much of the protocols cited here were developed in human and mouse demonstrating there is still much to be investigated in large animal models. Additionally there is still much work to be done towards removing all animal products from culture media (Pandurangan and Kim, 2015) as well as simplifying culture conditions to upscale production (O’Neill et al., 2021). Despite the current bottlenecks, there is an ever-growing effort to explore in vitro myogenesis. With the knowledge outlined in this chapter the field of ESC myogenic differentiation can be further developed.

Chapter 2: Application of Myogenic Differentiation

Introduction

Generation of muscle cells in vitro has gained a new interest within the pursuit of cultivated meat prompting the investigation of livestock models. Particularly in the case of cultivated meat, exploring cell sources that avoid the need to repeatedly sample animals would be ideal, making pluripotent stem cells a desirable option. There has been a limited number of reports using pluripotent stem cells for myogenic differentiation, with these reports mainly using mouse or human cells. Here we sought out to develop a reliable and high throughput protocol to explore the potential of bovine embryonic stem cells (ESC) as a cell source for cultivated meat applications. A previous report from Chal et al., 2016 is one of the most prominent differentiation protocols and, within the time of this research, was used successfully with adaptations in other models such as bovine embryonic disk stem cells (Kinoshita et al., 2021b) and porcine epiblast stem cells (Zhu et al., 2023). Chal's protocol was originally chosen for its shared consensus with other differentiation protocols noting the stimulation of WNT signaling to initiate mesodermal differentiation while inhibiting BMP signaling to better define a paraxial mesoderm fate. In the pursuit of adapting this protocol the spheroid formation was also incorporated to investigate the potential benefits. However, throughout our trials, thorough myogenic commitment was not obtained. In comparing the muscle development across species, it was apparent that bovine myogenesis both in vivo and in vitro were critically understudied. This led to analyzing early bovine fetuses in conjunction with bovine satellite cells during differentiation to gain a better understanding of bovine myogenesis in vivo, to inform its recreation in vitro. Beyond its use in cultivated meat and possible applications to regenerative medicine, exploring bESC myogenic

differentiation will give grater insight on the nature of ESC and their differentiation in large animal models.

Results

In Vivo Myogenesis

To analyze embryonic and fetal myogenesis in vivo we evaluated the temporal expression of genes important to myogenesis in the hind limbs of early embryos of estimated gestational age 40, 50, 55, and 65 days of development (N=1 for each age). Embryo/fetal ages were estimated via measurement of crown-to-rump (CLR) length (DesCôteaux et al., 2010) (Fig. 1A). Genes of interest included the early paraxial mesoderm genes *TBXT* (aka *T* or *Brachyury*), *TBX6*, *MSGN1*, and *PAX3,* a lateral plate mesoderm marker *FOXF1,* the myogenic differentiation genes *PAX7*, *MYOD*, *MYF5*, *MYOG* and *MYF6*, and the functional myogenic genes *MYH2*, *MYH3*, *NCAM1*, *DES*, and *TNNT1*. We evaluated expression of the pluripotent marker genes *SOX2* and *POU5F1 (OCT3/4)* in parallel for comparison. All genes were normalized to the expression of the housekeeping gene *GAPDH*.

When analyzing early mesoderm genes, we observed an initial spike of expression at day 55 before a drastic drop at day 65 for *TBX6*, *T*, and *PAX3,* but surprisingly not for *MSGN1* which remained low or absent at all time points examined (Fig. 1B). *MEOX1* expression was high and also appeared to have an inverse relation compared to other early mesodermal genes, though not confirmed. *FOXF1*, a marker of the lateral plate mesoderm, had little to no expression. The pluripotency markers *SOX2* and *POU5F1* were detected with *SOX2* having two peaks of

expression at day 40 and 65 of development compared to *POU5F1* which remained low and continued to fall as differentiation continued (Fig. 1F).

The myogenic genes *PAX7*, *MYOD* and *MYF5* had a gradual increase between days 40 and 50 and peaked at day 55 alongside the early paraxial mesoderm markers described above (Fig. 1C). As expected, these myogenic genes had a higher expression than the mesodermal genes as the embryo developed; *MYF5* had considerably higher expression compared to *PAX7* and *MYOD*. Consistent with the early mesoderm genes, expression of myogenic genes dropped at day 65. Mature myogenic markers had similar expression patterns with a gradual increase from day 40 to 50 followed by a sharp increase at day 55 before decreasing at day 65. (Fig. 1C & E). The transcription factor *MYOG* was present at all timepoints evaluated, with a small increase at day 50 and 55 compared to day 40, returning to lower levels at day 65. *MYF6,* another transcription factor present in mature muscle together with *MYOG*, was barely detected in fetal muscle. This expression profile of high *MYOG* and high *MYF5*, but little *MYF6*, depicts a stage of early embryonic/fetal myogenesis. Specifically, within the functional components of muscle, there was a great increase from day 50 to day 55 in the muscle cytoplasmic element *DES* as well as the slow skeletal muscle troponin *TNNT1*. Myogenic cell surface marker *CD56* (*NCAM1*) also followed this expression pattern. *DES* and *TNNT1* genes were the most highly expressed of all that were examined (Fig. 1D). Regarding myosin genes, we detected expression of the embryonic myosin heavy chain *MYH3* while the adult *MYH2* had only dismal expression (Fig. 1E). Though each timepoint was N=1, these data give us a unique temporal landscape of myogenesis in vivo between days 40 and 65 of development.

Satellite cells Differentiation

To compare fetal and mature myogenic differentiation, muscle satellite cells (MuSCs) were cultured in a serum starvation differentiation medium based on the protocol defined by Hu et al., 2021. In addition to nondifferentiated satellite cells (day 0), samples were harvested on each day of differentiation between days 1 and 7, at which point confluent myofibers were observed. These samples were processed for RT-qPCR. Expression of *PAX3*, *MYOD*, *MYOG*, and *MYF5* gradually increased at day 1 before spiking at day 2 (Fig. 2A & B). As expected, *TBX6* had lower expression level compared to the above markers. After its initial increase at day 1 and spike at day 2 of differentiation, *MYOG* continued to be expressed consistently with the expression of *MYF5* and *MYOD* at the same timepoints. *MYOD* however, began to fade after day 5. *PAX3* seemed to also follow this pattern but it was much more sporadic, spiking at day 2, and then again to a lower extent at days 4 and 7 (Fig. 2A). Different from this pattern was *MYF6* which had expression that gradually increased after day 1 as differentiation continued (Fig 2B). The early mesoderm genes *TXB6* and *MSGN1* were present with *TBX6* having relatively low expression but increasing alongside *PAX3,* whereas *MSGN1* was barely detected (Fig 2A). The functional muscle genes appeared to oscillate after day 3 (Fig. 2C & D). Expression of *DES* and *TNNT1* increased and decreased consistent with each other, as did *MYH3*. Although satellite cells are adult, somatic stem cells, the embryonic myosin *MYH3* was more abundant than *MYH2*, consistent with the expression patterns observed in embryos. The surface marker *NCAM1* had somewhat of an oscillating expression but different from that of *DES*, *TNNT1* and *MYH3* (Fig. 2E). Though having only one replicate, these results serve as an important reference to compare in vivo myogenesis as well as a baseline of our in vitro differentiation experiments.

Myogenic Differentiation

Our first attempt at myogenic differentiation using bESC followed the protocol described by Chal et al., 2016. Bovine ESCs were plated onto Matrigel coated wells in NBFR for a pre-differentiation step. After 2 days they were passaged to a Matrigel coated plate for direct differentiation. As a control one Matrigel coated plate was kept in NBFR for the duration of the trial. Initially, culture morphology was a combination of colony like formations and cell monolayers. In the control condition colony like formations increased in size forming dome like structures after 6 days. Although we were able to maintain cells for 40+ days using this protocol, the cells did not differentiate towards the myogenic fate nor were they confluent. In this and three subsequent trials, we also observed pronounced cell death stating at day 8 of differentiation. This appeared as holes in the cell monolayers with some cells surviving and remaining attached to one another via ECM resulting in a spiderweb like appearance (Fig. 3A). Culture conditions also created varying cell densities.

Matrigel substitution

Next, we investigated if Matrigel could be replaced by a more defined substrate. In ours and other labs, bovine ESC are routinely grown and maintained in a pluripotency state on vitronectin coated plates (Soto et al., 2021); therefore, we tested whether bESC would respond better to the differentiation protocol when cultured on vitronectin as compared to Matrigel. On vitronectin, morphological changes were observed as the rounded bESCs adopted a sharper

morphology at the first 3 days of differentiation with media containing WNT promotor CHIR99021 and BMP inhibitor LDN193189. Furthermore, genetic analysis showed an upregulation of *TBX6* and *PAX3* on vitronectin as compared to Matrigel. Compared with expression levels in bESC, *TBX6* was upregulated of 2.4-fold on vitronectin compared to 1.3 on Matrigel at day 3. PAX3 had a 2.8-fold increase on vitronectin compared to 4.3 on Matrigel also on day 3. This experiment suggested that bESC were able to begin paraxial mesoderm differentiation, and that vitronectin could be a suitable replacement for Matrigel. However, high level of cell death was still observed during the first week of differentiation. In the original attempt using Matrigel cell death was seen in the first 3 days, as reported from the original protocol. In the original attempt culture success was variable but when cultures did fail it was consistently after day 8. However, when using vitronectin cell death in the first 3 days constantly persisted throughout trials with many cultures failing within the first 3 days, and of the cultures that did survive, none could reach past day 8.

Manipulation of FGF2 concentration in differentiation medium

With vitronectin having the advantage of less variability but alone not ideal for culture conditions we set out to increase cell viability. The original differentiation protocol described by Chal et al., 2016 removed FGF2 during the first 3 days of differentiation. Given that bESC require FGF2 as opposed to mouse ESC, we hypothesized that maintaining FGF2 during early differentiation would promote cell survival. To test this, we included 10 ng/ml FGF2 during the first 3 days of direct differentiation (Fig. 3B). Supplementation with FGF2 had a positive effect on the culture as seen by increased confluence of cells possessing the sharp morphology previously described, and progression into a cell monolayer. Although there was still cell death by day 8,

overall the culture was much more viable with less variable cell densities and less susceptibility for cell sheets to peel off the culture plate. In addition, differentiation was not inhibited by the addition of 10 ng/ml of FGF2 as observed by expression of *TBX6* and *PAX3* being similar to culture without FGF2, though not directly compared (Fig. 3C).

Additional improvements to differentiation protocol

Although the matrix and media adaptations improved culture conditions and cell survival during the early differentiation protocol, we still observed a considerable rate of cell death and as a result, inconsistent success of cell differentiation with upregulation of *TBX6* and *PAX3* expression but not of markers of further maturation such as *MYOD.* We therefore continued testing conditions for protocol improvement following results reported by Kinoshita et al., 2021b who successfully adapted the original mouse ESC protocol for the differentiation of bovine embryonic disk stem cells. The main changes in the protocol were a shorter time for the initial differentiation from 3 days to 1 day (Fig. 4A & B). Reducing this period during which FGF2 is omitted greatly reduced cell death in our cultures. Another important difference was the use of 10 µg/ml laminin and 16.7 µg/ml fibronectin as cell culture substrate. These culture conditions resulted in *TBX6* and *PAX3* expression consistently at day 8 and 12 as well as higher expression of these markers (Fig 4D). Initial results utilizing the above adaptations seemed very promising for survivability with the culture reaching day 30 of culture. Though a much longer culture, it still contained cell death, with high levels of cell debris (Fig. 4B).

To improve cell proliferation and survival later in culture, we included 10 ng/ml of FGF2 for the two days of DK-I media (days 7 to 9 of culture) while keeping all other differentiation

media the same (Fig. 4C). Both culture conditions were able to reach longer periods (15 days) of differentiation, but confluence was varied between technical replicates both with and without FGF2. At the cellular level, there were no morphological differences between the two conditions. In addition, cell fusion into myotubes remained absent in both culture conditions.

Pre-differentiation

With cultures being successful in reaching day 12 and beyond with adequate cell survival, successful myogenic differentiation was the next goal of our research. The absence of myogenic commitment alluded to the possibility that there was a lack of homogeneity within the culture and possibly other cell populations present. Furthermore, cell death remained a challenge in later steps of culture. In efforts to address these problems we elected to add a pre-differentiation step to our protocol (Fig. 5A). For this we used a mesodermal differentiation medium previously developed in our laboratory to successfully induce *T* and *TBX6* expression in bESCs (data not shown). It was hypothesized that two days of pre-differentiation before direct differentiation would benefit the culture by increasing commitment to the mesoderm fate and therefore *TBX6* expression, thus allowing for better paraxial mesoderm differentiation. In addition, this pre-differentiation step was hypothesized to improve culture viability by reducing the strain of differentiation and reducing cell death.

Bovine ESC responded well to the pre-differentiation step, and as a result there was a decrease in cell death (Fig. 5B). The culture reached day 12 consistently and developed a sharp and widen morphology earlier than in previous conditions. However, adding the pre-differentiation step improved cell survival but did not induce the expression of myogenic

commitment markers. The early paraxial mesoderm makers *TBX6* and *PAX3* were detected as before (Fig. 5C & D). Also as reported before, pre-differentiation upregulated *TBX6* which was seen in RT-qPCR data with TBX6 peaking at day 8, while always remaining higher than non-pre-differentiation cultures.

Spheroids

With pre-differentiation being a suitable addition for health of long-term cultures, further myogenic differentiation and commitment in addition to increasing cell confluence in later cultures still had yet to be achieved. Previously mentioned were the benefits of spheroid formation in differentiation and proliferation. We therefore decided to test spheroids as a method to induce myogenic differentiation beyond the early paraxial mesoderm stage (Fig. 6A - C). Bovine ESCs underwent pre-differentiation in 2D before being aggregated into spheroids for direct differentiation. The duration of spheroid culture was compared between 4 and 6 days before seeding into a 2D culture system. Cultures were terminated at day 12. Controls for this experiment included 2D and 3D conditions for the 12 days of cell culture.

Spheroid size during the experiment was variable regardless of the condition tested (Fig. 6D & E). Overall control spheroids demonstrated a gradual decrease in size. Though measuring theses spheroids became increasingly hard due to high cell debris blocking the edge of the spheroids. Also, when retrieving control spheroids after day 12 it was shown that they had lost their defined border and uniform circular shape (Fig. 6C). Test spheroids remained mostly consistent but also deteriorated after day 6, sharing similar morphology to that of the control spheroids lacking their defined border and circular shape (Fig. 6C).

Consistently we found that spheroids plated after 6 days of direct differentiation did not adhere to the plate and ultimately died. In contrast, spheroids plated after 4 days of direct differentiation consistently created outgrowths. Outgrowths held a wide and sharp morphology as seen in the 2D control and created outgrowths from the point of seeding (Fig. 6C). Gene expression analysis at day 12 revealed expression of *PAX3* and *TBX6* comparable to 2D pre-differentiation. However, other myogenic genes were not detected. Comparison to the 3D control was not possible due to the deterioration mentioned before (Fig. 6F & G).

Discussion

Here we report for the first time the transcriptional landscape of myogenic differentiation during an early window of early limb formation in the bovine embryo and also during the differentiation of satellite cells in vitro. This information was used to inform the initial development of myogenic differentiation of bESCs. Evaluation of the temporal expression of myogenic factors provided an accurate and novel look at bovine embryonic and fetal myogenesis. These data have a special significance in the context of ESC differentiation. Pluripotency genes remained expressed at a low level except for two spikes in *SOX2* expression at day 40 and again day 65 of development. This could be due to differences in sample collection where when removing the limb there was more of the spinal cord included, where *Sox2* would be expressed in addition to in the neural cells innervating skeletal muscle (Javali et al., 2017). The early mesoderm marker *T* was detected which while very low may also have been a sign of residual notochord expression (de Bree et al., 2018). Of the early paraxial mesoderm markers, *TBX6* was present, but not *MSGN1*. However, the absence of *MSGN1* in the presence of its activator *TBX6* could be due to timepoints perhaps being too early in development. We also observed that *PAX3*

was still present in the embryo after somite formation which speaks to *PAX3's* continued role in myogenesis and limb bud formation. *MEOX1* was expressed at a higher level compared to early mesoderm genes. While past the point of somitogenesis the function of *Meox1* is also to regulate vertebrae formation pointing to a more sclerotome characteristic which could explain the inverse relationship it has with early mesoderm genes but not with dermomyotome genes as *MEOX1* mediates *PAX3* and *PAX7* (Mankoo et al., 2003). Embryos also had high expression of *MYOG* and *MYF5* but not *MYF6* which together indicates primary stage of myogenesis in these embryos. This is also displayed by the presence of embryonic myosin heavy chain 3 (*MYH3*) and not adult myosin heavy chain 2 (*MYH2*). Our findings were consistent with primary myogenesis in bovine beginning around 60 days of gestation (Du et al., 2010).

When it came to satellite cell differentiation there were important distinctions in myogenesis. While *MYOD* and *MYOG* shared a pattern of consistency between satellite cells and embryos, *MYF6* became more expressed alongside *MYF5* but notably both at a lower expression as compared to other myogenic genes within satellite cells. We also observed unexpected oscillations of *DES, TNNT1* and *MYH3* expression, which could be due to variations in mRNA isolation quality. In addition, we found a somewhat erratic expression of *NCAM1*. This marker is also expressed in other cell types but, while possible that the MuSC is not a perfectly homogenous culture, this variation seen in MuSC was not identified in the embryos we examined.

Curiously, *PAX7* expression was exceptionally low to the point of no detection in satellite cells. *PAX7* is a hallmark of satellite cells and its absence was concerning. However it was confirmed in the Smith Lab that these MuSCs were positive for *PAX7* in immunostaining. After

consulting with the Smith lab it was suspected that these MuSCs were at a stage seen where *Pax7* is extremely low especially as *Myog* increases all the while *Myod* is sustained as shown in mouse (Halevy et al., 2004). Smith lab reported that their MuSC cultures were at significantly lower passage around passage 0-1. The MuSC we cultured were at passage 4 which may have been at a point where the myogenic differentiation potential had strongly diminished. However, others have reported MuSC cultures which lack *Pax7* but were still able to go through myogenesis (Czerwinska et al., 2016; Helinska et al., 2017). These variations outline the challenges of using satellite cells for muscle production without genetic engineering to preserve their potency.

Our study illuminated many challenges to direct myogenic differentiation. Predominantly, challenges with culture survival hindered much of our progress. Initially, culture failure occurred mostly at the beginning of differentiation around days 3 and 4. The initial protocol developed for mouse ESC included a FGF2 withdrawal step of 3 days duration, which was detrimental to our cells given their primed state and therefore, dependence on FGF2 (Weinberger et al., 2016). We found that either supplementing the medium with lower levels of FGF2, or later shortening the FGF2 withdrawal period from 3 to 1 day as described by Kinoshita et al., 2021b helped improve cell survival. Similarly, adding a mesoderm pre-differentiation step was beneficial for cell survival during differentiation. All these modifications increased cell survival without impairing initial commitment into the paraxial mesoderm lineage as seen by increased expression of *TBX6* and *PAX3* across experiments. Similarly, the replacement of Matrigel with vitronectin and later fibronectin/laminin as culture substrates allowed for a more defined system for cell differentiation. Both vitronectin and fibronectin/laminin have been reported as suitable

substrates for the culture and differentiation of bESCs (Kinoshita et al., 2021b; Soto et al., 2021) while Matrigel has not been widely reported.

Aside from reducing cell debris and improving overall cell health another effect of adding the pre-differentiation step was a more sustained expression *TBX6*. The effect of prolonged *TBX6* has yet to be fully examined in this model; in human iPSCs and mouse ESCs models it has been shown to inhibit cardiac muscle development as well as promoting chondrocyte and skeletal muscle development (Sadahiro et al., 2018). Notably this sustained *TBX6* could induce a more chondrocyte population, highlighting the need to include analysis of chondrogenic markers in future studies.

Although we obtained consistent expression of *TBX6* and *PAX3* with all experimental approaches, further differentiation and myogenic commitment did not occur during the time points tested. One aspect that should be mentioned is the genetic profile of the ESCs used. The original Chal et al., 2016 protocol was effective to accomplish myogenic differentiation in vitro using hiPSCs and hESCs (Rao et al., 2018). Accordingly, others have found success using bovine embryonic disk stem cells (Kinoshita et al., 2021b) and porcine epiblast stem cells (Zhu et al., 2023) through adapted protocols. Human PSC reflect a primed state and thus a further developed state with a lesser degree of pluripotency compared to mouse naïve ESCs. The ESCs of large animal models such as bovine share this primed state of pluripotency. However, of the examples that adapted Chal's protocol (Kinoshita et al., 2021b; Zhu et al., 2022), some cell lines have a bias toward different differentiation pathways; this is also confirmed by Rohwedel et al., 1994. In the first reported attempt to differentiate bESC into the germline, Murakami et al., 2023 highlighted

the heterogeneity between ESC lines that resulted in high variability in differentiation success. One important limitation of our studies was that the protocols were only tested in one bES cell line. Further studies should incorporate additional lines for a more comprehensive evaluation of the individual ability of bESC lines to commit to myogenesis. Bovine ESCs used in this study which were derived from the ICM of a full blastocyst stage embryo which could be considered more of a baseline primed state. While there are variations to each differentiation approach, pluripotency profile is an extremely important consideration when beginning differentiation.

Beyond variability in between cell lines, it has also been documentation of different DNA methylation patterns in between clones of mESCs (Humpherys et al., 2001). In addition to this, passage number effects the state of ESCs as outlined in Zhu et al., 2023 which showed the difference between high and low passages of porcine EpiSCs still had pluripotent characteristics but different gene expression. There have been epigenetic investigations of myogenic differentiation in mice (Khateb et al., 2022) but to our knowledge not in bovine. Understanding the epigenomic differences between cell lines as well as passage number could be vital for defining a reliable myogenic protocol.

Another bottleneck of our approach is the lack of validated surface markers of early bovine differentiation that would allow for cell sorting and therefore, purification of differentiating cells. The gene expression profiles conducted on the cultures were limited in their scope and while there was some investigation into lateral plate markers such as *FOXF1*, there was a lesser degree of investigation into neural markers. Performing flow cytometry analysis of *TBX6*- and *PAX3*-positive cells would be useful to evaluate the proportion of cells actually

committing to myogenesis within our population; however, this would also require full validation of TBX6 and PAX3 antibodies in bovine cells for reliable results. In fact, transcriptomics analysis of differentiating mESC in the report by Chal et al., 2016 revealed a notably heterogeneous population (Khateb et al., 2022).

The appropriate substrate for bovine cell differentiation may still be a factor that requires more investigation. Proper integrin function is very influential in cell function and differentiation with loss of some integrins, like Beta1, delay myogenic differentiation as seen in mice (Rohwedel et al., 1998). Furthermore, the elasticity of a substrate can influence satellite cell self-renewal (Higuchi et al., 2013) so other substrate factors could influence the development into the satellite population. Laminin remains a key element in muscle which was present in our protocol. However, the influence of other factors found in Matrigel used in the original protocol are still unknown for our model. Furthermore, Chal's protocol pre-differentiated cells on Matrigel before direct myogenic differentiation making the genetic landscape of the culture at time of differentiation unknown. Following the approach of Kinoshita et al., 2021b, it was hypothesized that the substrates of laminin and fibronectin would be beneficial to culture conditions compared to Matrigel or vitronectin due to laminin being is present in satellite stem cell niches and fibronectin being important for integrin activation. While the individual effect of these substrates was not determined in our studies, when used in combination they were a suitable substitution for Matrigel and vitronectin and ultimately beneficial to the culture of bESC.

Throughout the culture conditions tested the expression of *PAX3* was consistent which was a positive sign due to its importance in somitogenesis and myogenesis. However, *PAX3* also

triggers other effects that can be detrimental to myogenesis. As reported in mice, high and prolonged *PAX3* expression can activate myogenic inhibitors (Lagha et al., 2010). One *PAX3* target is *Sprouty1* that, from regulation of FGF, can act as a hold on myogenesis regulating the population of myogenic progenitors in the dermomyotome similar to how it acts to preserve quiescence in satellite cells (Lagha et al., 2008). Similar to *Sprouty1*, *Mbnl3* is also a target of *Pax3* that can inhibit premature myogenesis by antagonizing *Myod* (Lee et al., 2008). In addition, *Pax3* also shares a binding site with *Pax7* on the gene Id3 which is used to maintain quiescence of satellite cells. Curiously *Pax3* can also target *SFRP3* which can inhibit Wnt (Lagha et al., 2010). With *PAX3* expression persisting in culture conditions there is a possibility that these inhibitory effects could arise. Compared to our results, Kinoshita et al., 2021b reported much lower expression of *PAX3* at day 12.

Genetic expression and epigenetic profile aside, there is the possibility that our differentiation protocol lacked certain components that could be required for bovine myogenesis. Of the growth factors present in media, each one could be subject to a change to find their optimal concentration. HGF while active in satellite cell activation and cell migration and proliferation can also inhibit myogenic differentiation via the ERK pathway (Wang et al., 2008). At concentrations above 20 ng/ml, HGF can preserve quiescence and prevent differentiation of primary rat cells (Chazaud, 2010). IGF-1 is very beneficial to muscle proliferation and differentiation; however, the concentration of IGF-1 could be a factor in its effectiveness with Chal et al., 2016 only using 2 ng/ml when other concentrations are used (eg. 10 ng/ml in porcine EpiSC) (Zhu et al., 2023) and a wide range from 10-100 ng/ml in bovine satellite cells (Jara et al., 2023) as well as upwards of 80 ng/ml in C2C12 cells (Yang et al., 2007). The same variability

can also be seen in FGF (Jara et al., 2023). Taken together, there is still much work needed to find optimal concentrations of growth factors for the bovine model.

One reagent that appears in other protocols but not the one used in these studies are inhibitors of TGF-Beta signaling. As stated before, TGF-Beta is present in the early mesoderm and can aid in pre-somitic mesoderm differentiation (Loh et al., 2016) but can hinder muscle differentiation. Inhibition of TGF-Beta in early stages of the differentiation protocol was shown to have a positive effect in porcine culture (Zhu et al., 2023). With our maintenance media as well as the pre-differentiation media using Activin A to stimulate the TGF-Beta pathway this could have resulted in well-defined pre-somitic differentiation as our data suggests, but could have detrimental effects of myogenic differentiation proper. Investigating TGF-Beta inhibition remains a possible next step for testing our model.

Another reagent that was not used in our protocol was retinoic acid. Being one of the major morphogens during somitogenesis, retinoic acid has been used in a variety of papers including zebrafish, mouse and human (Chal et al., 2015; Patterson et al., 2010; Ryan et al., 2012). Previously mentioned, retinoic acid was shown to be endogenous for *PAX3* positive cultures in mice and thus not a requirement for culture conditions (Chal et al., 2015). However, also in mice, the use of retinoic acid displayed upregulation of *Pax3* and *Myod* with retinoic acid has a wide-ranging effect potentially substituting the use of Wnt and also having an inhibitory effect on Bmp signaling (Kennedy et al., 2009; Ryan et al., 2012). This would mean that there would be a large overhaul of culture conditions with potential benefit of having less reagents to make a more efficient and economically beneficial production especially in the terms of upscale production.

Retinoic acid has also been used in the context of generating embryoid bodies (Czerwinska et al., 2016). Though studies have highlighted retinoic acid's focus on producing muscle progenitors (Ryan et al., 2012), retinoic acid has also been used in neural differentiation in mice (Rohwedel et al., 1998), and adipogenesis in human (Taura et al., 2009), signaling that there may be a heterogeneous culture population if retinoic acid is used, however that is problem found in many approaches and can be solved through cell sorting by FACS.

Finally, we also tested the use of spheroid culture which to our knowledge has not yet been attempted in bESC. As stated earlier, spheroids hold potential benefits for improving cellular communication and ultimately growth and differentiation. Based on our experiments, we concluded 4 days of spheroid formation is better for bESCs differentiation within our protocol. There was variability in spheroid size between wells which is likely due to technique of dispersing cells into microwells as well as different growth rates. Spheroids that were seeded after 6 days did not seed well and the cultures ultimately disappeared. Spheroids seeded after 4 days were very reliable and should be further investigated to for necrotic core through live/dead staining on a confocal microscope to enhance efficiency (Johnson et al., 2024). Once seeded, outgrowths were present but not highly proliferative. This was limited also from the low seeding density employed in our study. It is possible that increasing the number of spheroids seeded/well we could reach a confluence that would be better for growth and differentiation. Though with considerations, spheroids remain a possible avenue for myogenic differentiation especially with the potential of converting culture to a suspension culture model.

Conclusion

Though the variety of approaches employed in our study did not achieve full myogenic differentiation, evidence of early paraxial mesoderm differentiation was observed. Our data speak to the challenges of reproducibility in myogenic differentiation protocols across species and cell types. Considerations must be made of differences between pluripotency states which range from differences in derivation methods and species, as well as differentiation protocols. Like all models, there will need to be constant optimization to reach the production goals envisioned for this technology. Outlined in this study are a myriad of other approaches that can be added to our current understanding of in vitro muscle generation. With our finding we hope to take the first steps towards a reliable bovine model in myogenic differentiation.

Methods

All reagents were purchased from Thermo Fisher Scientific unless otherwise noted.

ART EMBRYO PROCESSING

Embryos received from Applied Reproductive Technology LLC, Madison WI, were measured to obtain their CLR to approximate their age. The hind limbs were dissected and RNA was isolated using TRIzol (ThermoFisher) and treated with DNAse I (ThermoFisher). RNA was then processed to cDNA using with Thermo Scientific RevertAid First Strand cDNA Synthesis Kit as before.

SATELLITE CELL CULTURE AND DIFFERENTIATION

Bovine satellite cells (CD56+/CD34-) were isolated from brisket muscle biopsies from an Angus-Hereford cross steer and provided by the UC Davis meat laboratory. Satellite cell sorting was performed by the Smith Lab. Satellite cells were isolated using Miltenyi Satellite cell Isolation Kit

and validated through PAX7 immunofluorescence. Satellite cell culture was conducted using previously validated protocols including MuSC media containing F-10 basal media, 20% FBS, 1% Penicillin and Streptomycin, 5 ng/ml FGF2, 50 ug/ml Gentamicin, and 1 µg/ml Amphotericin (Hu et al., 2021).

Satellite cell differentiation was conducted when culture reached confluency. Differentiation media consisted of DMEM high glucose basal media supplemented with 2% FBS and 1% Penicillin and Streptomycin. Media was changed every 3 days.

bESC derivation and culture

Bovine embryonic stem cells were derived from a full blastocyst stage embryo (day 7 of development) generated from in vitro fertilization and culture. Blastocysts were seeded onto MEF feeder line (A34180 Gibco) and outgrowths were cultured at 37 °C and 5% CO_2 as described in Bogliotti et al., 2018. Bovine ESC maintenance medium NBFR was changed daily. Bovine ESCs were passaged every 3-4 days using TrypLE. BovineESCs were transitioned to feeder free culture on vitronectin-N coated plates through consecutive passaging. All lines were karyotyped to confirm chromosome stability over time.

Myogenic differentiation

Myogenic differentiation was first conducted from the protocol outlined by Chal et al., 2016. The protocol consisted of bESC culture in basal medium DMEM/F12 with 3 µM CHIR99021 and 500 nM LDN193189 for 3 days following the same media but with 20 ng/ml FGF2. Then the media was changed to one supplemented with 15% KSR, 10 ng/ml HGF, 2 ng/ml IGF, 20 ng/ml FGF2 and 500 nM LDN193189 for 2 days then changed to media containing 15% KSR and 2 ng/ml IGF for 4 days before culturing the terminal media of 15% KSR, 10 ng/ml HGF, 2 ng/ml IGF.

Second application of myogenic differentiation was from the protocol of Kinoshita et al. (34874452). Kinoshita et al. uses the same approach as Chal et al. but with 1 day of DMEM/F12 with 3 µM CHIR99021 and 500 nM LDN193189 and 2 days of media containing 15% KSR and 2 ng/ml IGF. Cells were grown on 10 µg/ml laminin (L2020 Sigma) and 16.7 µg/ml fibronectin (F1141 Sigma) coated plates.

Pre-differentiation media was produced and used for 2 days of culture before starting myogenic differentiation via Kinoshita et al.

SPHEROID FORMATION AND CULTURE

Spheroids were formed using 1.45 x 10^5 cells in 29 microwell of a 1.5% agarose mold spun at 100 Gs for 2 minutes with Rho kinase (ROCK) inhibitor Y-27632 (10 µM). Spheroids were collected whole and seeded on laminin and fibronectin plates.

RT-QPCR

RNA was collected using the Qiagen RNeasy mini kit. RNA amount was normalized before conversion to cDNA using Thermo Scientific RevertAid First Strand cDNA Synthesis Kit. Quantitative PCR conducted using BioRad SYBR® Green master mix and processed in CFX96 Touch Real-Time PCR Detection System using Bio-Rad CFX Maestro 1.0 software.

Primers were designed on NCBI primer BLAST and validated via sanger sequencing in bESCs and MuSCs. Other primers were taken from Dohmen et al., 2022 ordered by the Smith Lab including *TNNT1*, *DES*, *NCAM1* and *PAX7*.

IMAGE PROCESSING

Cell culture images taken on Echo Revolve microscope. Image processing done via Fiji Image J software.

Statistics and Graphs

Statistical analysis was conducted using SAS: Analytics, Artificial Intelligence and Data Management on the ΔCts were calculated from the RT-qPCR data using 1-way ANOVA statistical method. Relative abundance of mRNA was calculated using $2^{-\Delta Ct}$ and graphed using Graphpad Prism software.

Tables

Table 1: Media

Media formulation for differentiation and maintenance of bESC and satellite cells. MuSC media are Bovine Cell GM and Bovine Myogenic media. All other media are used in bESCs.

Bovine Cell GM (bogrow)		
Reagents	**Volume**	**Final**
F-10	39ml	base
FBS	10ml	20 %
P/S	0.5ml	1 %
FGF2	100µl	5 ng/mL
Gentamicin	0.25ml	50 µg/mL
Amphotericin	0.2ml	1 µg/mL

Bovine Myogenic Differentiation Media (BoDM)		
Reagents	**Volume**	**Final**
DMEM	48.5ml	base
FBS	1ml	2%
P/S	0.5ml	1%

bESC maintenance media NBFR		
Reagents	**Volume (100ml)**	**Final Concentrations**
BSA Stock (100 mg/mL)	10ml	10% (vol/vol)
DMEM/F12	40ml	40% (vol/vol)
Neurobasal	50ml	50% (vol/vol)
N2 Supplement	500µl	0.5% (vol/vol)
B27 Supplement	1ml	1% (vol/vol)
MEM Non-essential Amino Acid Solution	1ml	1% (vol/vol)
Penicillin-Streptomycin	1ml	1% (vol/vol)
2-Mercaptoethanol	10µl	0.1 mM
bFGF	200µl	20 ng/mL
IWR-1	50µl	2.5 µM
Activin A	20µl	20 ng/mL

Pre Differentiation Media		
Reagents	**Volume (50ml)**	**Final Concentrations**
GMEM	40.4ml	

KSR	7.5ml	15% (vol/vol)
Nonessential Amino Acids	500µl	0.1mM
Glutamax	500µl	1% (vol/vol)
Sodium Pyruvate	500µl	1% (vol/vol)
BME	5µl	0.1mM
Penicillin/streptomycin	500µl	1% (vol/vol)
Activin A	35µl	70 ng/mL
CHIR99021	28µl	3 µM (0.00139ug/ul)

DiCL (Dmem-ITS-Chir-Ldn)		
Reagents	**Volume (250ml)**	**Final Concentrations**
DMEM/12	244.5ml	
ITS	2.5ml	1% (vol/vol)
Nonessential Amino Acids	2.5ml	1% (vol/vol)
Penicillin/streptomycin	0.5ml	0.2% (vol/vol) (20 IU + 0.02 mg)/ml
CHIR99021	75µl	3µM
LDN193189	125µl	0.5µM

DiCLF (Dmem-ITS-Chir-Ldn-Fgf)		
Reagents	**Volume (250ml)**	**Final Concentrations**
DMEM/12	244.5ml	
ITS	2.5ml	1% (vol/vol)
Nonessential Amino Acids	2.5ml	1% (vol/vol)
Penicillin/streptomycin	0.5ml	0.2% (vol/vol) (20 IU + 0.02mg)/ml
CHIR99021	75µl	3 µM
LDN193189	125µl	0.5 µM
Recombinate FGF-2	500µl	20 ng/ml

DK-HIFL (Dmem-KSR-Hgf-Igf-Fgf-Ldn)		
Reagents	**Volume (250ml)**	**Final Concentrations**
DMEM/12	209.5ml	
KSR	37.5ml	15% (vol/vol)
Nonessential Amino Acids	2.5ml	1% (vol/vol)
Penicillin/streptomycin	0.5ml	0.2% (vol/vol) (20 IU + 0.02 mg)/ml
2-Mercaptoethanol	454µl	0.1 mM
Recombinate Hgf	25µl	10 ng/ml
Recombinate Igf-1	50µl	2 ng/ml
Recombinate Fgf-2	500µl	20 ng/ml

LDN193189	125µl	0.5 µM
DK-I		
Reagents	**Volume (250ml)**	**Final Concentrations**
DMEM/12	209.5ml	
KSR	37.5ml	15% (vol/vol)
Nonessential Amino Acids	2.5ml	1% (vol/vol)
Penicillin/streptomycin	0.5ml	0.2% (vol/vol)
2-Mercaptoethanol	454µl	0.1 mM
Recombinate Igf-1	50µl	2 ng/ml
DK-HI		
Reagents	**Volume (250ml)**	**Final Concentrations**
DMEM/12	209.5ml	
KSR	37.5ml	15% (vol/vol)
Nonessential Amino Acids	2.5ml	1% (vol/vol)
Penicillin/streptomycin	0.5ml	0.2% (vol/vol)
2-Mercaptoethanol	454µl	0.1 mM
Recombinate Hgf	25µl	10 ng/ml
Recombinate Igf-1	50µl	2 ng/ml

Table 2: Primers

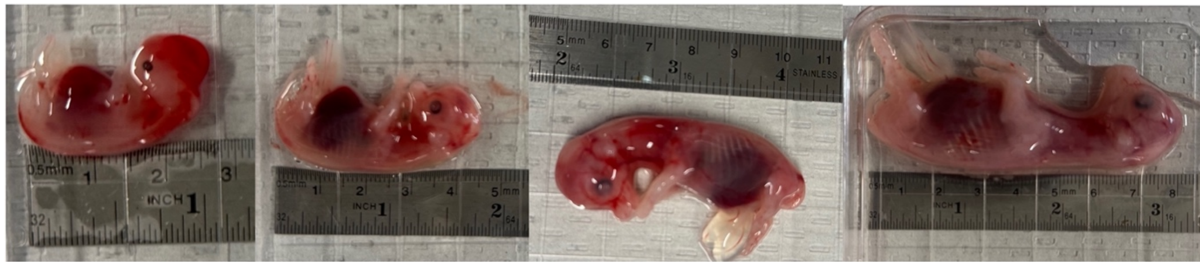
Primers used for RT-qPCR

Gene	Sequence ID		Sequence
GAPDH	NM_001034034.2	FWD:	5' - CGCATCCCTGAGACAAGATGG
		REV:	5' - CTTGCCGTGGGTGGAATCAT
SOX2	NM_001105463.2	FWD:	5' - CATTAACGGCACACTGCCCC
		REV:	5' - TGAAAATGTCTCCCCCGCCC
OCT4 (POU5F1)	NM_174580.3	FWD:	5' - AACGAGAATCTGCAGGAGATATG
		REV:	5' - TCTCACTCGGTTCTCGATACT
TBXT (T)	NM_001192985.1	FWD:	5' - GTTCTCCAACCTATTCCGACAG
		REV:	5' - TGTGGGCAGACATTCCAAG
TBX6	NM_001192630.1	FWD:	5' - TTTCTTCTGGACGTGGTCCC
		REV:	5' - TGTAAACACGGTCAGGCAGG

MEOX1	NM_001035376.2	FWD:	5' - GGAGAATTCAGACAACCAGGAG
		REV:	5' - TGAGCAAACTCAGCTTCGAG
MSGN1	XM_002691521.6	FWD:	5' - GAGGCCTGAGTCAGCTCAAA
		REV:	5' - ACAACCTGCGAGAGACCTTC
FOXF1	XM_002694754.7	FWD:	5' - GCCTCCTACATCAAGCAGCA
		REV:	5' - GTTCTGGTGCAGATACGGCT
PAX3	NM_001206818.2	FWD:	5' - GCACCAGGCATGGATTTTCC
		REV:	5' - GGTCAGAAGTCCCATTACCTGA
MYF5	NM_174116.1	FWD:	5' - ACCAGCCCCACCTCAAGTTG
		REV:	5' - GCAATCCAAGCTGGATAAGG
MYF6 (MRF4)	NM_181811.2	FWD:	5' - GCGAAAGGAGGAGGCTAAAGAAAATCAACG
		REV:	5' - TGGAATGATCGGAAACACTTGGCCACTG
MYOD	NM_001040478.2	FWD:	5' - CAGGGAAGTGCGAGTGTTCC
		REV:	5' - GCAACAGCGGACGACTTCTA
PAX7	XM_015460690.2	FWD:	5' - CTCCCTCTGAAGCGTAAGCA
		REV:	5' - GGGTAGTGGGTCCTCTCGAA
MYOG	NM_001111325.1	FWD:	5' - GGCCTTCCCAGATGAAACCA
		REV:	5' - TGAGGAGGGGATAGTCTGGC
MYH2	NM_001166227.1	FWD:	5' - AGAGCAGCAAGTGGATGACCTTGA
		REV:	5' - TGGACTCTTGGGCCAACTTGAGAT
MYH3	NM_001101835.1	FWD:	5' - AGATGAGGGGGACACTGGAA
		REV:	5' - GTTGTCGTTCCTCACGGTCT
TNNT1	XM_005219671.5	FWD:	5’- CCTCTGATCCCGCCAAAGAT
		REV:	5’- GGTCCTTTTCCATGCGCTTC
DES	NM_001081575.1	FWD:	5’ - GGAAGCCGAGGAATGGTACA
		REV:	5’ - TCGATCTCGCAGGTGTAGGA
NCAM1 (CD56)	XM_005215806.5	FWD:	5’ - CCGAGAAGGGTCCCGTAGA
		REV:	5’ - ATTTGTGTGGCATCGTTGGG

Figures

A



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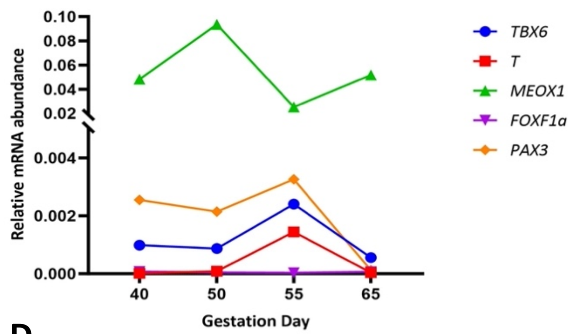

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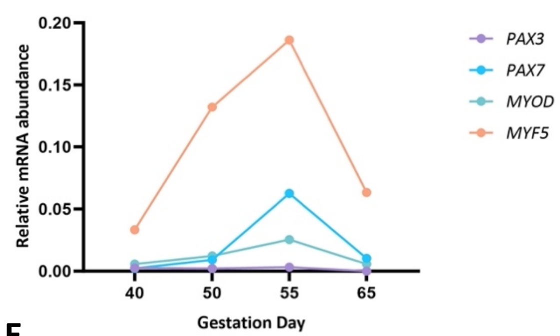

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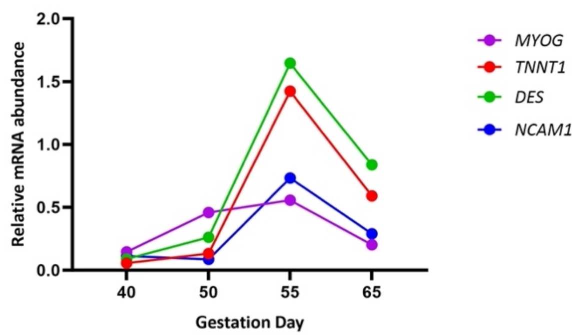

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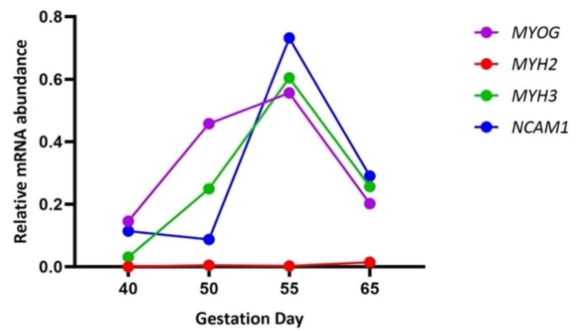

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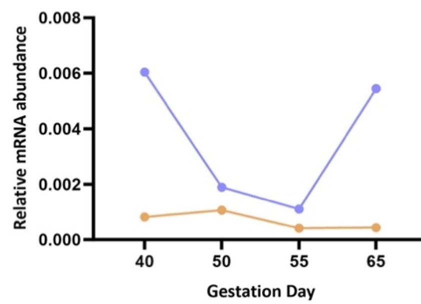


Figure 1: Genetic Analysis of ART Embryos (A) Pictures of embryos from estimated gestation day 40 to 65. (B-F) Relative mRNA abundance of (B) early paraxial mesoderm markers, (C) myogenic markers, (D) actin elements and (E) myosin elements (F) pluripotency markers in muscle obtained from embryonic/fetal hind limbs. Each time point N=1.

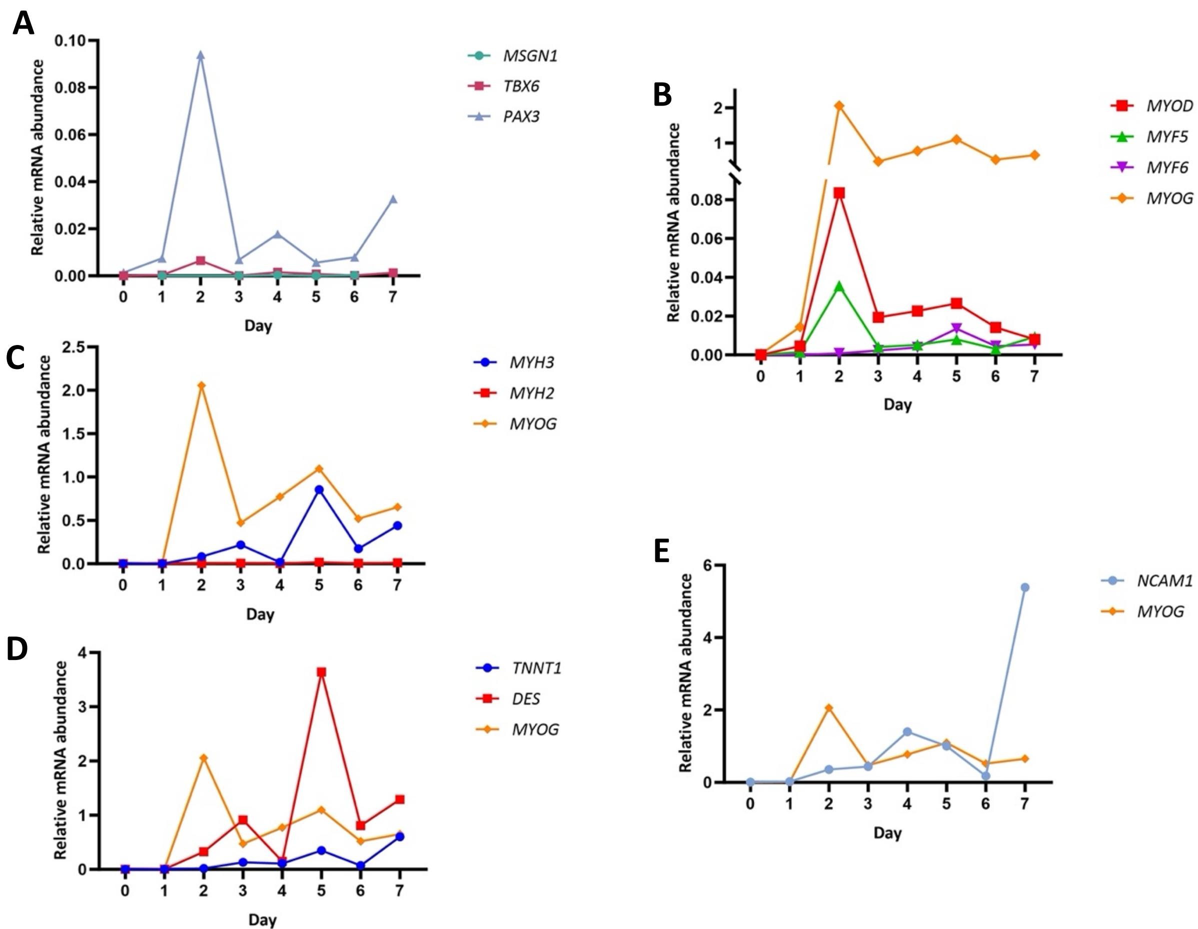
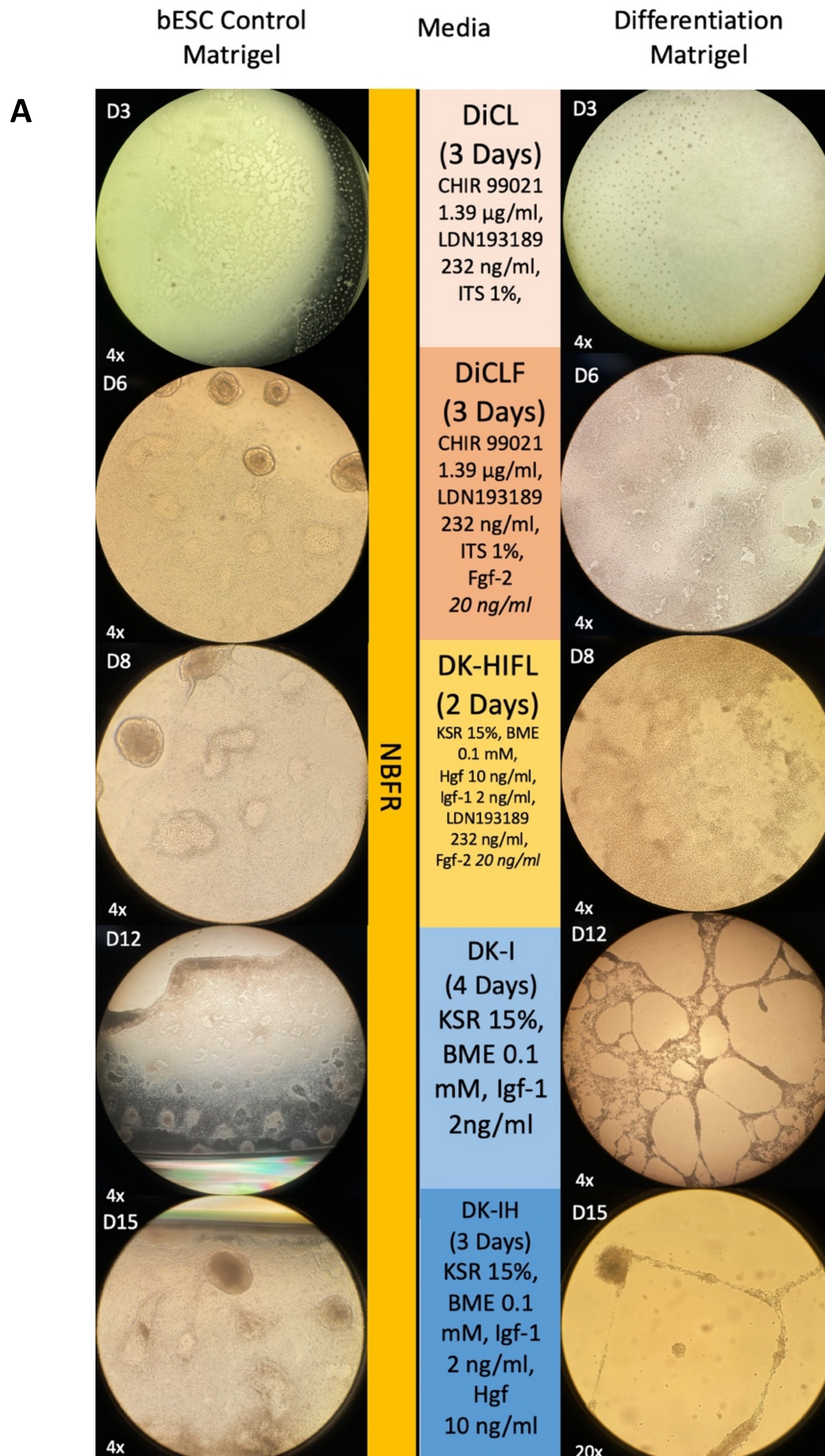
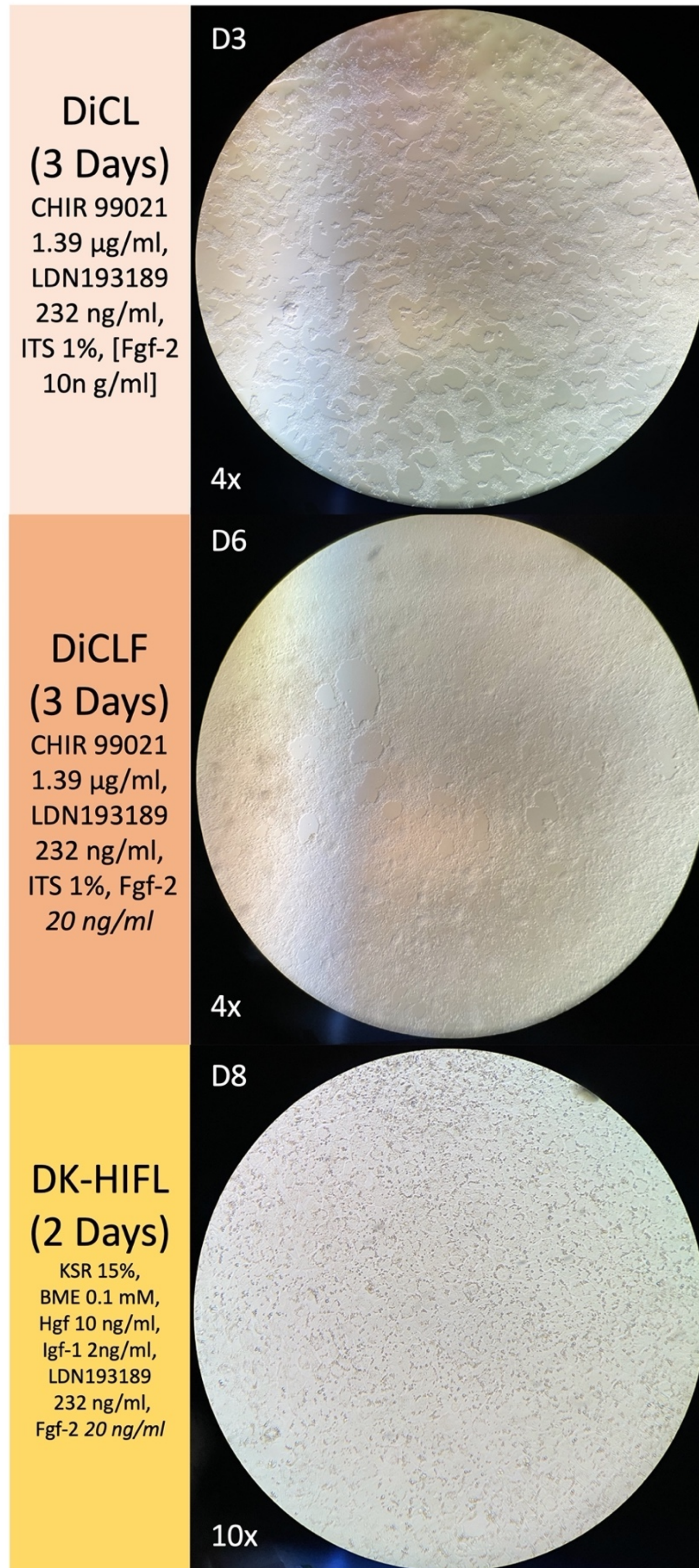


Figure 2: Genetic Analysis of Satellite Cells. Relative mRNA expression compared to housekeeping gene *GAPDH* of bovine satellite cells in maintenance media (Day 0) and during differentiation (day 1 – 7). (A) Early paraxial mesoderm markers. (B) Myogenic differentiation markers. (C)

Myosin markers. (D) Actin markers. (E) Satellite cell surface marker. MYOG was used as a reference gene on graphs (C-E).




B
Media
Vitronectin
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(3 Days)
CHIR 99021
1.39 µg/ml,
LDN193189
232 ng/ml,
ITS 1%, [Fgf-2
10n g/ml]
D3
4x
DiCLF
(3 Days)
CHIR 99021
1.39 µg/ml,
LDN193189
232 ng/ml,
ITS 1%, Fgf-2
20 ng/ml
D6
4x
DK-HIFL
(2 Days)
KSR 15%,
BME 0.1 mM,
Hgf 10 ng/ml,
Igf-1 2ng/ml,
LDN193189
232 ng/ml,
Fgf-2 20 ng/ml
D8
10x

C

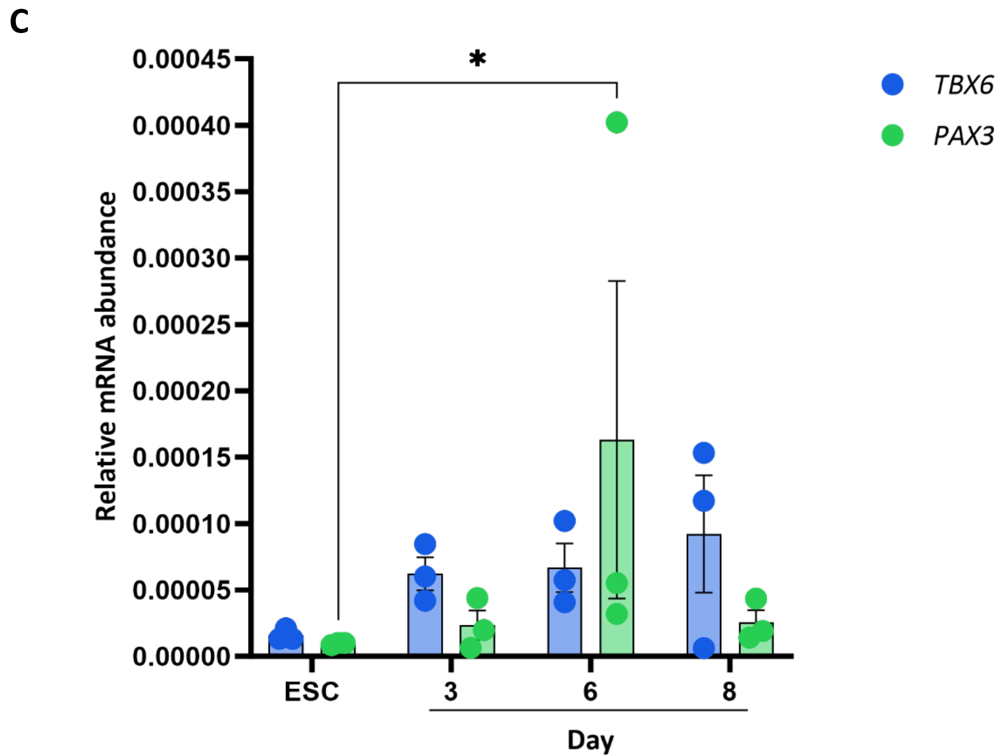
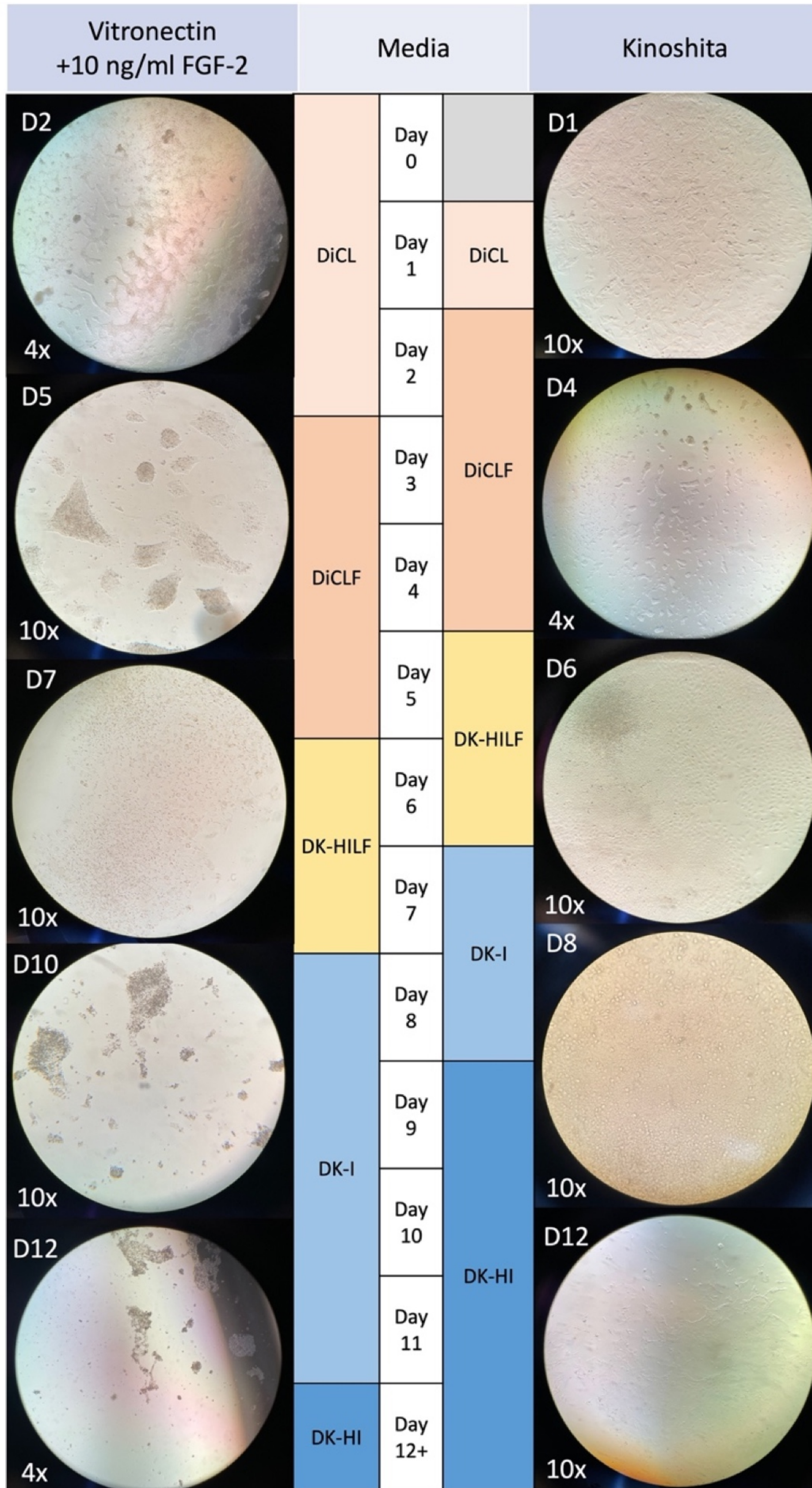


Figure 3: bESC Differentiation. bESC differentiation using adaptations of the differentiation protocol described in Chal et al., 2016. (A) Morphology of original protocol compared (right) to control of bESC maintenance media control (left). (B) Morphology of original protocol on vitronectin and 10 ng/ml of FGF-2 in the first 3 days. (A-B) Timepoint in days depicted in upper left corner for each photo. (C) Relative mRNA abundance of *TBX6* and *PAX3* compared to housekeeping gene *GAPDH* during differentiation on vitronectin and 10 ng/ml of FGF-2 in the first 3 days compared to bESCs. N=3.

A

	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12+
Original Kinoshita Protocol		DiCL CHIR 99021 1.39 µg/ml, LDN193189 232 ng/ml, ITS 1%	DiCLF CHIR 99021 1.39ug/ml, LDN193189 232ng/ml, ITS 1%, Fgf-2 *20ng/ml*			DK-HILF KSR 15%, BME 0.1 mM, Hgf 10 ng/ml, Igf-1 2 ng/ml, LDN193189 232 ng/ml, Fgf-2 *20 ng/ml*		DK-I KSR 15%, BME 0.1 mM, Igf-1 2 ng/ml		DK-IH KSR 15%, BME 0.1 mM, Igf-1 2 ng/ml, Hgf 10 ng/ml			
Original Chal Protocol	DiCL CHIR 99021 1.39 µg/ml, LDN193189 232 ng/ml, ITS 1%			DiCLF CHIR 99021 1.39 µg/ml, LDN193189 232 ng/ml, ITS 1%, Fgf-2 *20 ng/ml*			DK-HILF KSR 15%, BME 0.1 mM, Hgf 10 ng/ml, Igf-1 2 ng/ml, LDN193189 232 ng/ml, Fgf-2 *20 ng/ml*		DK-I KSR 15%, BME 0.1 mM, Igf-1 2 ng/ml				DK-IH KSR 15%, BME 0.1 mM, Igf-1 2 ng/ml, Hgf 10 ng/ml

B



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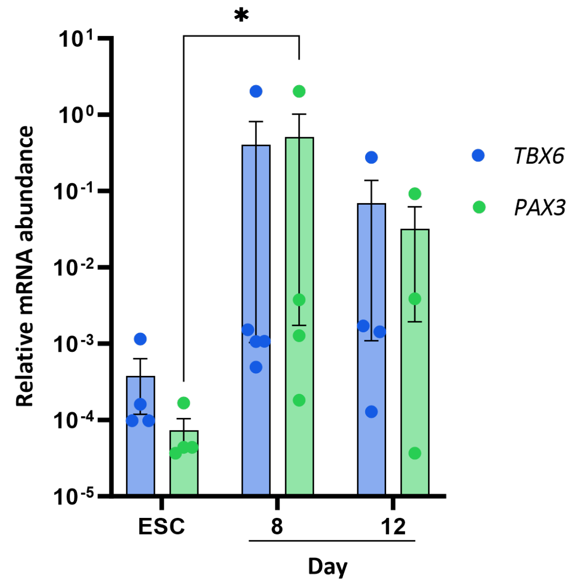

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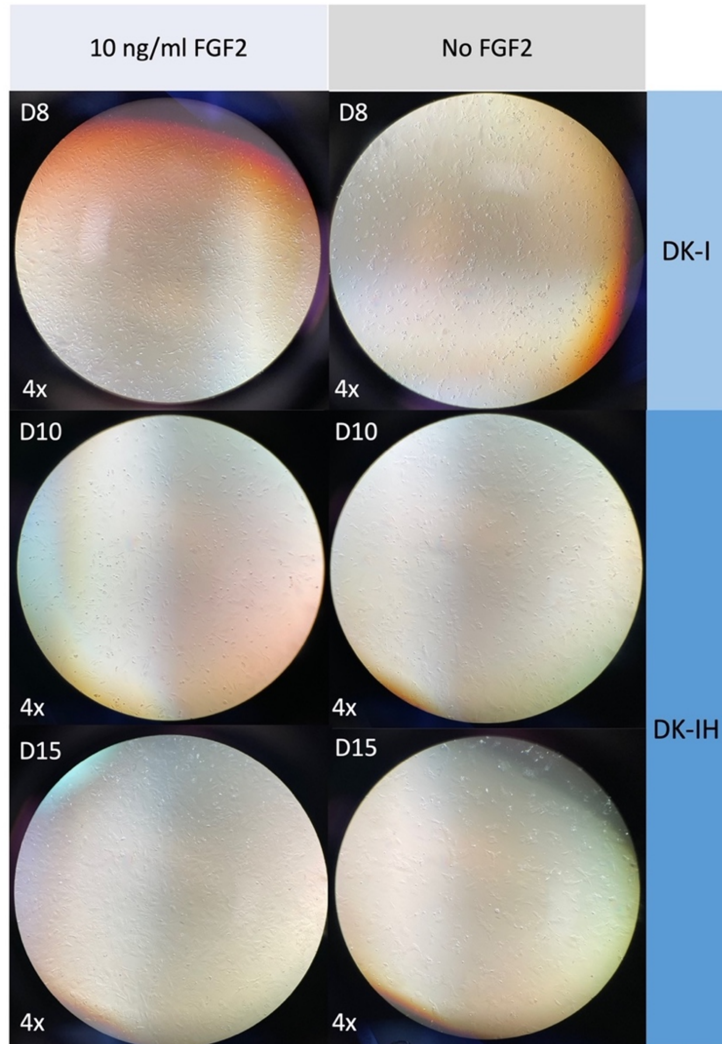

Figure 4: Alternative bESC differentiation. bESC differentiation using adaptations of the differentiation protocol described in Kinoshita et al. (A) Media components of new protocol compared to control (media described in Chal et al., 2016). (B) Illustrative cell morphology of original protocol (vitronectin and 10 ng/ml of FGF2) in the first 3 days compared to Kinoshita protocol. (C) Relative mRNA abundance of *TBX6* and *PAX3* compared to housekeeping gene *GAPDH* during differentiation compared to bESCs. Number were transformed using Log 10 scale. N=5. (D) Alternative protocol with the addition of 10 ng/ml of FGF2 added at day 7 in DK-I media compared to no FGF2. (B&D) Timepoint in days depicted in upper left corner for each photo. Magnification depicted in lower left corner of each photo. N=1.

A

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KSR 15%
CHIR 1.39 µg/ml
Activin A 70 ng/ml
BME 0.1 mM
Glutamax 1%
Sodium Pyruvate 1%</td>
<td>DiCL
CHIR 99021
1.39 µg/ml,
LDN193189
232 ng/ml,
ITS 1%</td>
<td colspan="3">DiCLF
CHIR 99021 1.39 µg/ml,
LDN193189 232 ng/ml,
ITS 1%, Fgf-2 20 ng/ml</td>
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KSR 15%, BME 0.1 mM,
Hgf 10 ng/ml, Igf-1
2 ng/ml, LDN193189
232 ng/ml,
Fgf-2 20 ng/ml</td>
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</tr>
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<td>Day -2</td><td>Day -1</td><td>Day 1</td><td>Day 2</td><td>Day 3</td><td>Day 4</td><td>Day 5</td><td>Day 6</td><td>Day 7</td><td>Day 8</td><td>Day 9</td><td>Day 10+</td>
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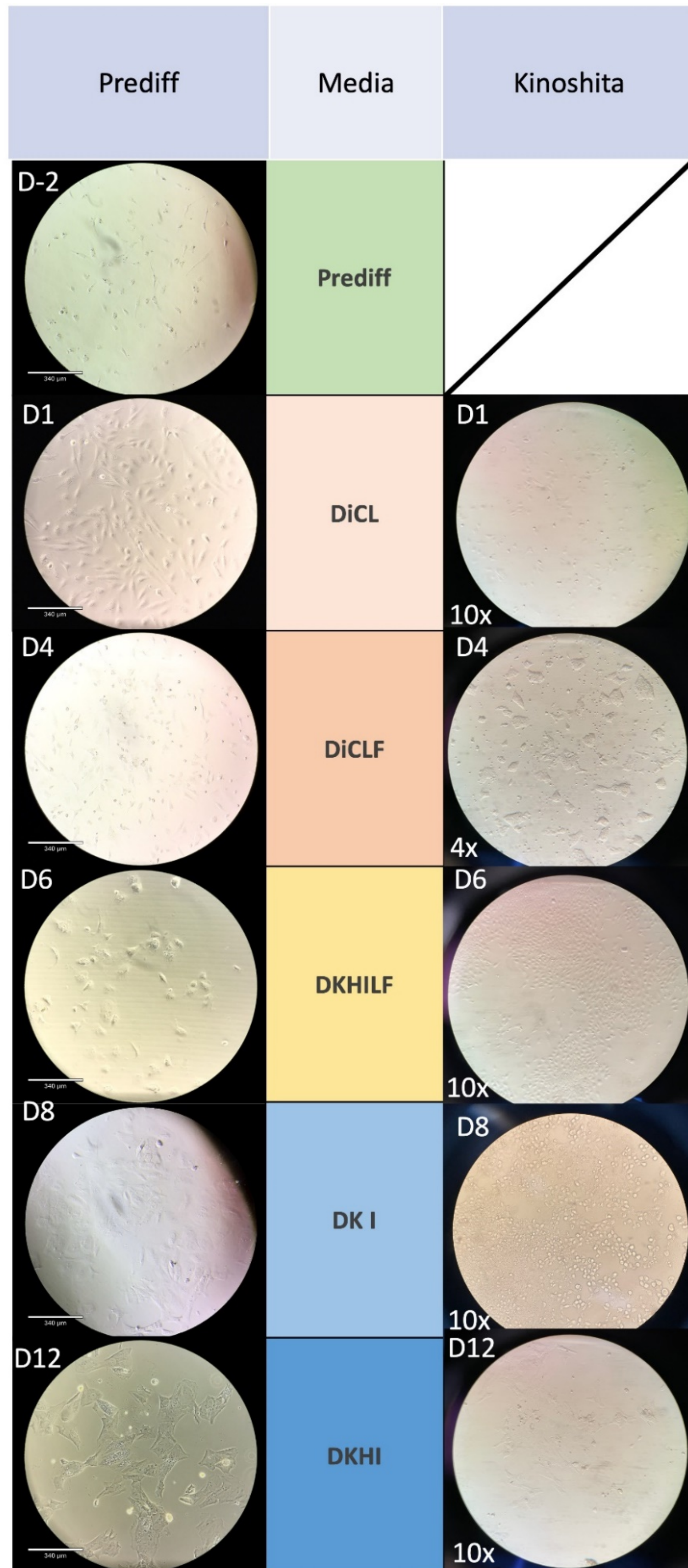
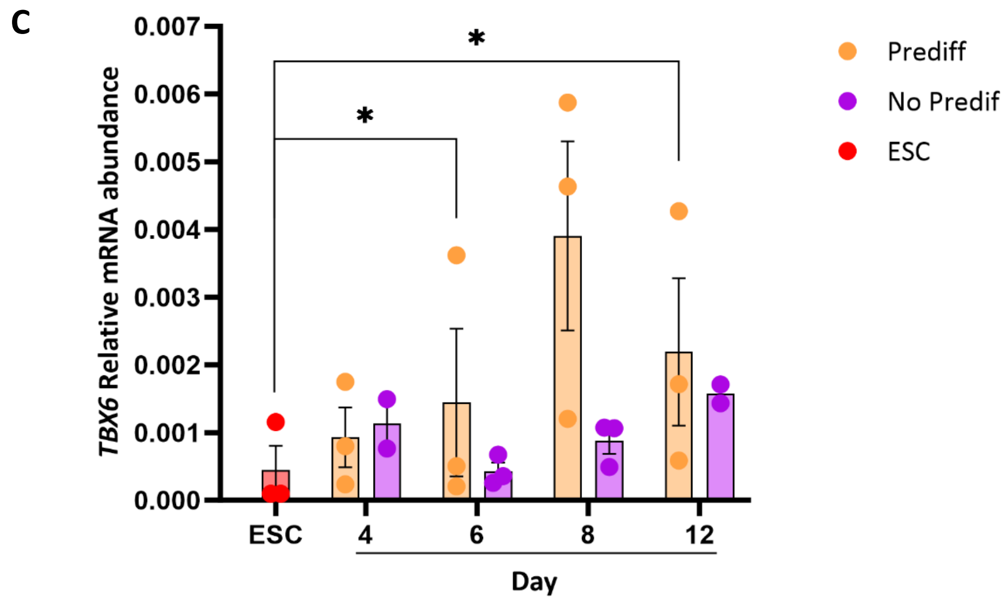
B



C



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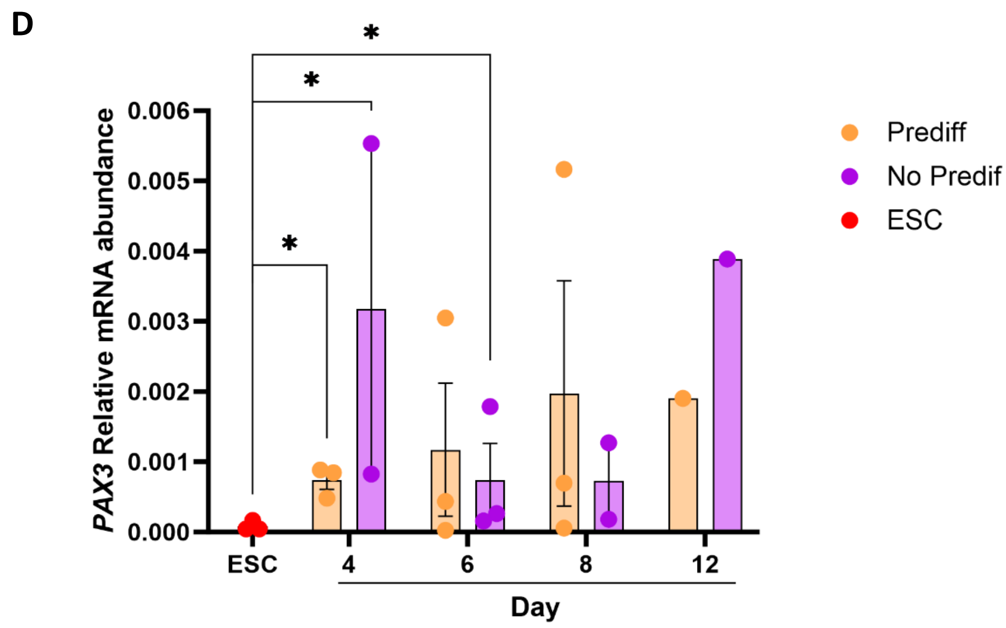
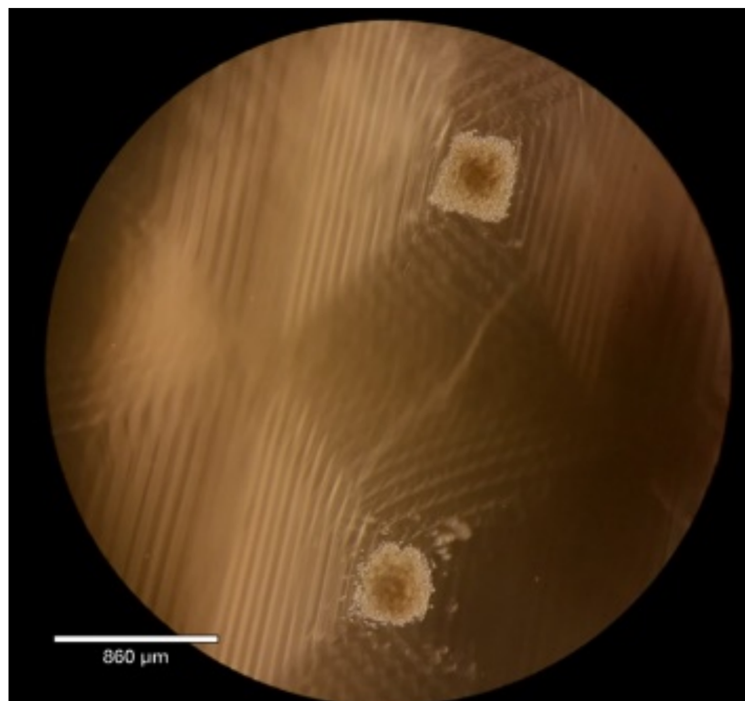


Figure 5: Pre-differentiation. (A) Media components of alternative protocol with the addition of a pre-differentiation step. Pre-differentiation steps identified as day –1 and –2. (B) Morphology of alternative protocol vs alternative protocol with a pre-differentiation step. Timepoint in days depicted in upper left corner for each photo. Magnification depicted in lower left corner of each photo. Scale bar 340µm. (C) Relative mRNA expression compared to housekeeping gene *GAPDH* between Pre-differentiation and no Pre-differentiation of *TBX6* (C) and *PAX3* (D) compared to bESCs. N=3.

A

	2 Days	1 Day	3 Days	2 Days	2 Days	4 Days
	Prediff	**DiCL**	**DiCLF**	**DKHILF**	**DK I**	**DKHI**
2D control	2D	2D	2D	2D	2D	2D
4 Day Spheroid	2D	Spheroid	Spheroid	2D	2D	2D
6 Day Spheroid	2D	Spheroid	Spheroid	Spheroid	2D	2D
3D Control	Spheroid	Spheroid	Spheroid	Spheroid	Spheroid	Spheroid

B



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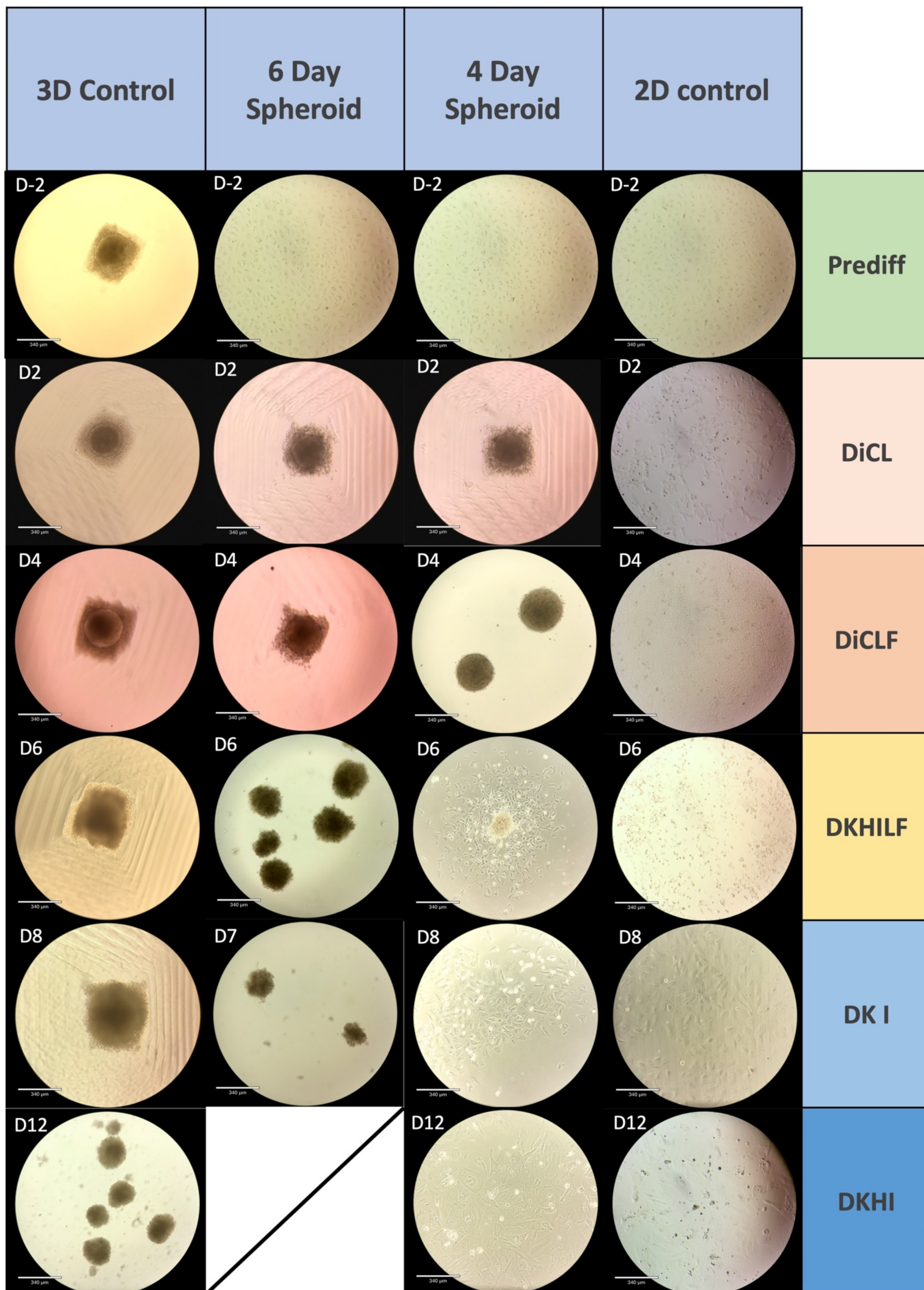

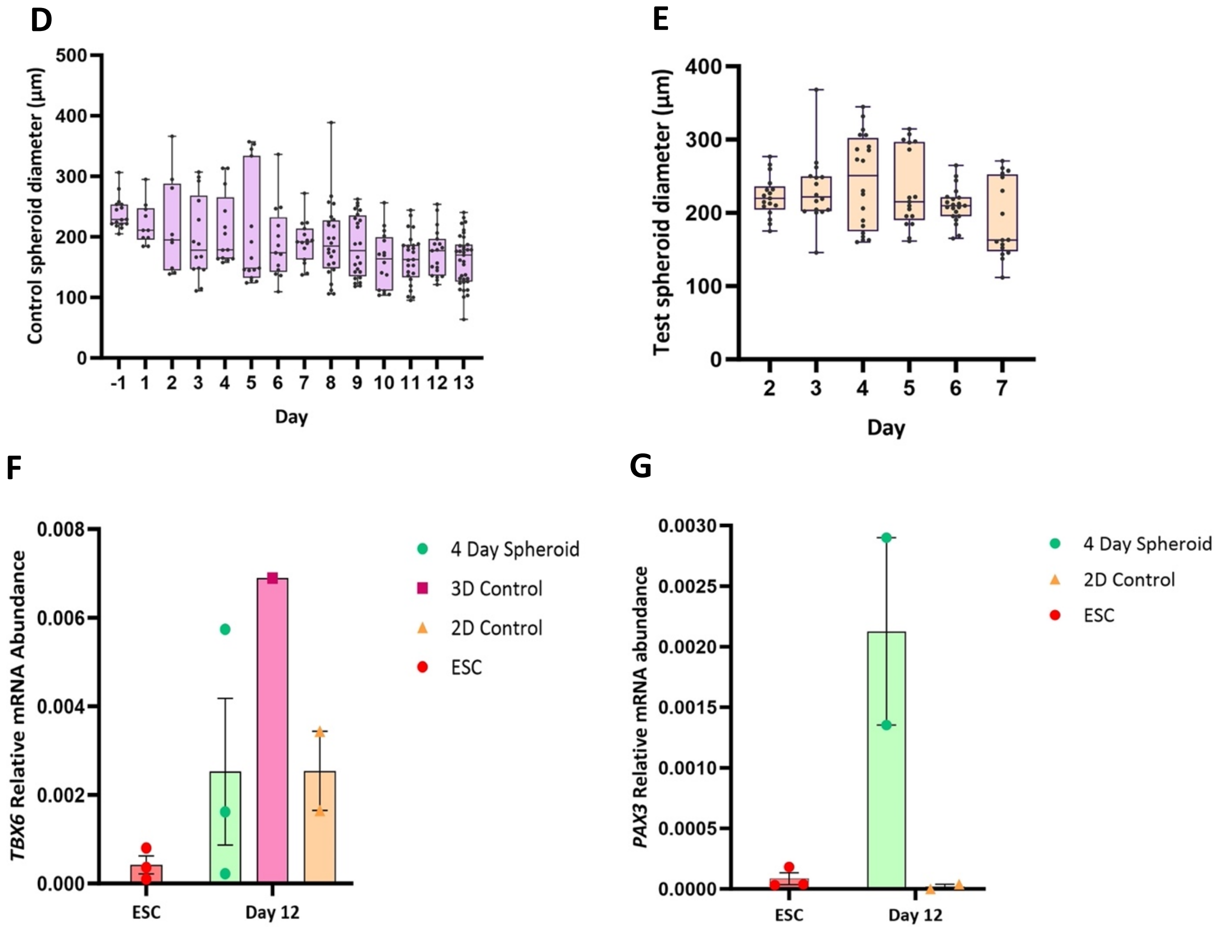

Figure 6: Spheroid Differentiation. (A) Spheroid media chart depicting the time culture would be in spheroid formation compared to 2D culture. (B) Representative images of spheroids successfully formed and maintained in agarose microwells. Scale bar = 860 µm. (C) Morphology of spheroid culture and seeding with a 2D and 3D control. Scale bar 340 µm. Culture failure for 6 Day Spheroids shown as dead spheroids at day 7 and slash signifying no day 12. (D) Average spheroid diameter of 3D control (culture that remained in spheroid formation until day 12). Negative days represent pre-differentiation period. (E) Average spheroid diameter of test condition containing both 4 Day Spheroids (day 1 – 4, seeded after day 4) and 6 Day Spheroids (day 1 – 6, seeded after day 6). Both conditions up to day 4 were identical. First day of spheroid formation for each condition was not measured to let spheroids form. Spheroids measured on

day 7 as they seed. N = 3 replicates. (F) Relative mRNA expression of *TBX6* compared to housekeeping gene *GAPDH* in spheroid culture (G) Relative mRNA expression of *PAX3* in spheroid culture.

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